Pesticide residues in food – 2013

Joint FAO/WHO Meeting on Pesticide Residues

EVALUATIONS 2013

Part II – Toxicological

Pesticide residues in food – 2013

Toxicological evaluations

Sponsored jointly by FAO and WHO

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

Geneva, Switzerland, 17–26 September 2013

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* First full evaluation

** Evaluated within the periodic review programme of the Codex Committee on Pesticide Residues

2013 Joint Meeting of the FAO Panel of Experts on
Pesticide Residues in Food and the Environment
and the WHO Core Assessment Group on Pesticide Residues

Geneva, 17–26 September 2013

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Abbreviations used

β-NF	beta-naphthoflavone
ACTH	adrenocorticotrophic hormone
ADI	acceptable daily intake
ADME	absorption, distribution, metabolism, excretion
ALAT	alanine aminotransferase
ALT	alanine transaminase
ANCOVA	analysis of covariance
ANOVA	analysis of variance
AP	alkaline phosphatase
APTT	activated partial thromboplastin time
ARfD	acute reference dose
ASAT	aspartate aminotransferase
AST	aspartate transaminase
AUC	area under the plasma concentration–time curve
BMD	benchmark dose
$BMDL_{10}$	lower 95% confidence limit on the benchmark dose for a 10% response
$BMDL_{SD}$	95% lower confidence limit of the dose corresponding to a change equivalent to 1 standard deviation in the response for unexposed animals
BMDS	Benchmark Dose Software
BP	benzo(*a*)pyrene
BrdU	5-bromo-2′-deoxyuridine
BROD	benzyloxyresorufin *O*-debenzylase; benzyloxyresorufin *O*-debenzylation
bw	body weight
$C_{24\ h}$	concentration after 24 hours
CAS	Chemical Abstracts Service
CHL	Chinese hamster lung
CHO	Chinese hamster ovary
CI	confidence interval
CLA	clofibric acid
C_{max}	maximum concentration
CMC	carboxymethyl cellulose
CPA	cyclophosphamide
CPK	creatine phosphokinase
CYP	cytochrome P450
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
7-EC	7-ethoxycoumarin *O*-deethylase
EH	epoxide hydrolase
eq	equivalents
EROD	ethoxyresorufin *O*-deethylase; ethoxyresorufin *O*-deethylation
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
GC	gas chromatography
GD	gestation day
GGT	gamma-glutamyltransferase
GST	glutathione *S*-transferase
HDW	haemoglobin distribution width
^{1}H-NMR	hydrogen nuclear magnetic resonance
HPAA	4-hydroxyphenyl acetate
HPLA	4-hydroxyphenyl lactate
HPLC	high-performance liquid chromatography
HPPD	4-hydroxyphenylpyruvate dioxygenase
HPRT/Hprt	hypoxanthine–guanine phosphoribosyltransferase
IC_{50}	concentration that caused a 50% reduction in enzyme activity
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
ip	intraperitoneal
IPCS	International Programme on Chemical Safety (IPCS)
ISO	International Organization of Standardization
IU	International Units
IUPAC	International Union of Pure and Applied Chemistry
iv	intravenous
JMPR	Joint FAO/WHO Meeting on Pesticide Residues
LC	liquid chromatography
LC_{50}	median lethal concentration
LD	lactation day
LD_{50}	median lethal dose
LDH	lactate dehydrogenase
LI	hepatocyte labelling index
LLNA	local lymph node assay
LOAEL	lowest-observed-adverse-effect level
LOD	limit of detection
LOQ	limit of quantification
LRD	limit of reliable determination
M	male
3-MC	3-methylcholanthrene
MCH	mean corpuscular haemoglobin; mean cell haemoglobin
MCHC	mean corpuscular haemoglobin concentration; mean cell haemoglobin concentration
MCV	mean cell volume
MMAD	mass median aerodynamic diameter
MPP	1-methyl-4-phenylpyridine
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	messenger ribonucleic acid
MROD	methoxyresorufin *O*-demethylase
MRT	mean residence time

MS	mass spectrometry
NA	not analysed
NC	not calculated
NCE	normochromatic erythrocyte
ND	not determined; not detected
NE	not evaluated
NK	natural killer
NOAEC	no-observed-adverse-effect concentration
NOAEL	no-observed-adverse-effect level
NQO	4-nitroquinoline 1-oxide
NTBC	2-(2-nitro-4-trifluoromethylbenzoyl)-cyclohexane-1,3-dione
NZW	New Zealand White
OECD	Organisation for Economic Co-operation and Development
P	parental generation
P	probability
PAS	Periodic-Acid Schiff
PB	phenobarbital; phenobarbital sodium
PCE	polychromatic erythrocyte
PCNA	proliferating cell nuclear antigen
PCV	packed cell volume
PDC	2,3-pyridine dicarboxylic acid
PEG	polyethylene glycol
PNAS	*p*-nitroanisole *O*-demethylase
PND	postnatal day
po	per os (by mouth)
ppm	parts per million
PROD	pentoxyresorufin *O*-depentylase; pentoxyresorufin *O*-depentylation
PT	prothrombin time
PTU	propylthiouracil
QA	quality assurance
RITA	Registry of Industrial Toxicology Animal-data
RT	retention time
rT_3	reverse triiodothyronine
S9	$9000 \times g$ supernatant fraction from rat liver homogenate
SD	Sprague-Dawley
SDH	sorbitol dehydrogenase
SEM	standard error of the mean
sRBC	sheep red blood cell
$t_{½}$	half-life
T_3	triiodothyronine
T_4	thyroxine
TK	thymidine kinase
TLC	thin-layer chromatography
T_{max}	time to reach C_{max}
TOPPS	1,2,3,4-tetrahydro-1-oxopyrido-(1,2-a)-5-pyrazinium salt
TRR	total radioactive residue
TSH	thyroid stimulating hormone

U	uniformly labelled; units
UDP	uridine diphosphate
UDPGT	uridine diphosphate–glucuronosyltransferase
UDS	unscheduled DNA synthesis
USA	United States of America
USEPA	United States Environmental Protection Agency
v/v	volume per volume
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 17–26 September 2013.

Eleven of the substances evaluated by the WHO Core Assessment Group (benzovindiflupyr, bixafen, cyantraniliprole, fenamidone, fluensulfone, imazapic, imazapyr, isoxaflutole, tolfenpyrad, triflumizole and trinexapac-ethyl) were evaluated for the first time. One compound (diquat) was re-evaluated within the periodic review programme of the Codex Committee on Pesticide Residues (CCPR). Information provided to the Meeting on two other compounds (chlorfenapyr and dithianon) permitted a further evaluation of their metabolites. 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 219*. That report contains comments on the compounds considered, acceptable daily intakes 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 2013, 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 2013 Joint Meeting. A special acknowledgement is made to those experts and to the Members of the Joint Meeting who reviewed early drafts of these working papers.

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

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

BENZOVINDIFLUPYR

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Explanation

Benzovindiflupyr is the provisional International Organization for Standardization name for *N*-[(1*RS*,4*SR*)-9-(dichloromethylidene)-1,2,3,4-tetrahydro-1,4-methanonaphthalen-5-yl]-3-(difluoro-methyl)-1-methyl-1*H*-pyrazole-4-carboxamide (International Union of Pure and Applied Chemistry) (Chemical Abstracts Service No. 1072957-71-1). Technical benzovindiflupyr contains the enantiomers SYN546526 and SYN546527, at a ratio of 50 : 50. Both enantiomers are fungicidally active. No toxicological studies were performed on the individual enantiomers.

Benzovindiflupyr is a new broad-spectrum foliar fungicide of the chemical group pyrazole carboxamide. It 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 pivotal studies were certified as complying with good laboratory practice.

The chemical structure of benzovindiflupyr is shown in Fig. 1.

Fig. 1. Chemical structure of benzovindiflupyr

Evaluation for acceptable daily intake

1. Biochemical aspects

1.1 Absorption, distribution and excretion

A quantitative whole-body autoradiography and expired air study was performed in fasted male and female Han Wistar rats. Single doses of 98.2% radiochemically pure [pyrazole-5-^{14}C]benzovindiflupyr or 98.8% radiochemically pure [phenyl-U-^{14}C]benzovindiflupyr at 1 mg/kg body weight (bw) (5.5 MBq/mg) or 40 mg/kg bw (0.13 MBq/mg) were administered by oral gavage to two animals of each sex per dose and label position. The radioactivity in expired air in the 1 mg/kg bw groups was determined during the 24 hours after dosing. In the 40 mg/kg bw groups, no expired air evaluation was possible because of severe clinical signs observed predominantly in females after 2 hours. The radioactivity remaining in organs and tissues was determined at 1 hour and 72 hours.

Radioactivity in the expired air accounted for less than 0.03% of the dose at 1 mg/kg bw for both label positions; the 40 mg/kg bw groups could not be evaluated because of severe clinical signs. At 1 mg/kg bw, the highest concentrations of radioactivity were present in the Harderian gland and the liver throughout the course of the experiment in both sexes, with lower concentrations in the kidney, brown fat and adrenal glands. The bladder wall had a higher concentration in male rats after pyrazole label administration compared with the males treated with the phenyl label or females treated with either radiolabel. At 72 hours after dosing, the radioactivity had declined in all tissues. The female animals generally showed higher tissue concentrations than the males at the first termination time, with generally similar concentrations achieved by 72 hours after dosing. At 40 mg/kg bw, the highest concentrations were found in the Harderian gland and the liver throughout the course of each experiment in both sexes, with lower concentrations in the adrenal glands, brown fat, kidney and preputial gland. There was a clear difference in brain concentration compared with blood between the sexes within the first hours after dosing. In females, the concentration in brain was approximately 2.5–3.5 times higher than that in blood. By contrast, in males, the brain concentration was lower than that in blood and around 3–4 times lower than that in female brain. Radioactivity in blood was still detectable at 72 hours after dosing in both sexes. These results indicate that redistribution of radioactivity from the tissues into the blood and subsequent excretion were still in progress at this time point. There were no clear differences in tissue distribution profiles between the two radiolabels used (Shaw, 2011a).

To investigate the pharmacokinetics of benzovindiflupyr, single doses of 97.9% radiochemically pure [pyrazole-5-^{14}C]benzovindiflupyr at 1 mg/kg bw (5.46 MBq/mg) or 40 mg/kg bw (0.125 MBq/mg) were administered by oral gavage to nine non-fasted Han Wistar rats of each sex per dose. For pharmacokinetic analyses in blood, three animals of each sex per dose group were allocated to three blood sampling groups covering 120 hours after dosing. To obtain plasma for metabolic profiling, two animals of each sex per dose were terminated at 2 and 4 hours after dosing, and plasma was analysed in a separate study (Green, 2011).

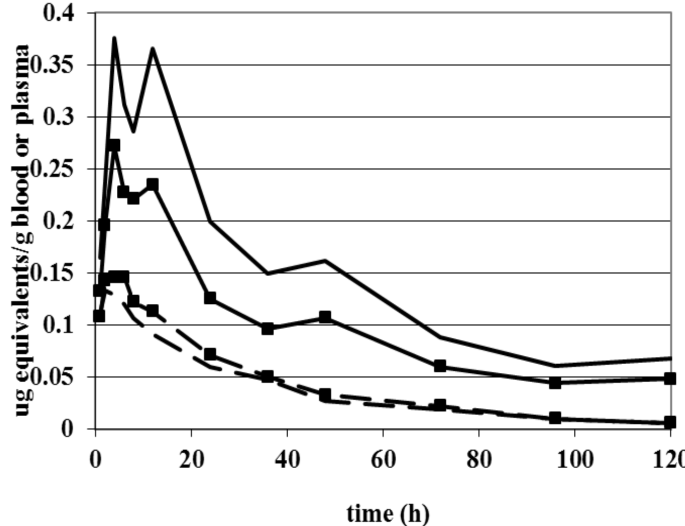
Maximum blood and plasma total radioactive residue (TRR) concentrations were achieved at 2–4 hours in male and female rats after dosing with 1 mg/kg bw (Table 1). At 40 mg/kg bw, maximum blood and plasma TRR concentrations were achieved at 6 hours in males and at 24 hours in females. The mean terminal half-lives in blood and plasma were significantly lower in low-dose females than in low-dose males, but were similar in males and females at the high dose. The area under the plasma concentration–time curve ($AUC_{0-\infty}$) values were lower in females than in males. Excretion was nearly complete within 120 hours (Figs 2 and 3). In plasma and blood at both dose levels, TRR concentrations were higher in males than in females. In females, but not in males, the $AUC_{0-\infty}$ increased proportionally with dose. Radioactivity was excreted via the faeces, accounting for 78.9% and 82.3% at the low dose level and 88.8% and 87.6% at the high dose level in males and females, respectively. Excretion via the urine was 8.9% and 5.1% of the administered dose at the low dose level and 4.9% and 6.4% at the high dose level in males and females, respectively (Shaw, 2011d).

Table 1. Plasma and blood pharmacokinetic parameters in rats following single oral administration of benzovindiflupyr at 1 or 40 mg/kg bw

Parameter	1 mg/kg bw				40 mg/kg bw			
	Blood		Plasma		Blood		Plasma	
	Males	Females	Males	Females	Males	Females	Males	Females
C_{max} (µg eq/mL)	0.272	0.146	0.376	0.133	4.32	3.24	5.42	3.06
T_{max} (h)	4	4	4	2	6	24	6	24
AUC_{0-t} (µg eq·h/mL)	11.6	5.06	17.2	4.4	231.4	180.2	292	174
$AUC_{0-\infty}$ (µg eq·h/mL)	15.9	5.29	22.6	4.63	258.3	194.9	320	197
$t_{½}$ (h)	61.7	26.9	55.2	28.5	34.3	27.8	29.8	33.1

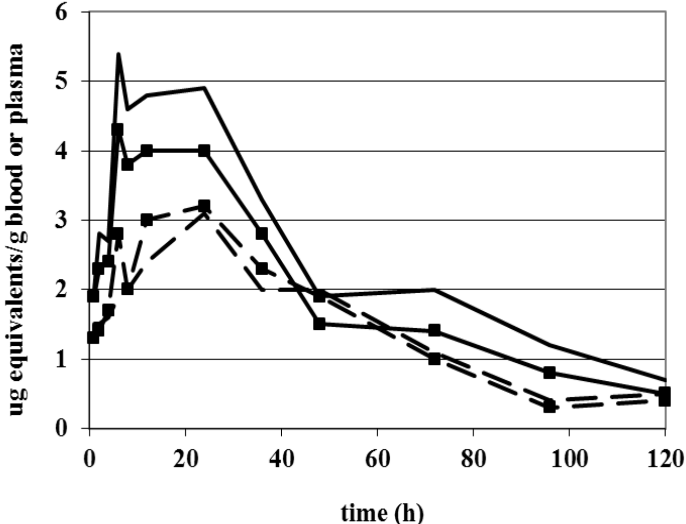
AUC: area under the plasma concentration–time curve; C_{max}, peak concentration; eq, equivalents; $t_{½}$, half-life; T_{max}, time to reach C_{max}
Source: Shaw (2011d)

Fig. 2. Mean total radioactive residue concentrations in plasma and blood of male and female rats following a single oral administration of [pyrazole-5-^{14}C]benzovindiflupyr at 1 mg/kg bw



Solid line: males; dashed line: females; squares: blood; no symbols: plasma
Source: Shaw (2011d)

Fig. 3. Mean total radioactive residue concentrations in plasma and blood of male and female rats following a single oral administration of [pyrazole-5-^{14}C]benzovindiflupyr at 40 mg/kg bw



Solid line: males; dashed line: females; squares: blood; no symbols: plasma
Source: Shaw (2011d)

To investigate the excretion pattern and tissue distribution of benzovindiflupyr, single doses of 97.9% radiochemically pure [pyrazole-5-^{14}C]benzovindiflupyr at 1 mg/kg bw (5.46 MBq/mg) or 40 mg/kg bw (0.125 MBq/mg) were administered by oral gavage to four non-fasted Han Wistar rats of each sex per dose. Urine, faeces and cage washes were collected over 7 days, and animals were then killed. TRR concentrations in the organs were analysed.

No clinical signs were observed in the dosed animals. Recovery of TRR was 98.6–101.6% in the low-dose and the high-dose groups. In the low-dose group, excretion was virtually complete within 96 hours; at day 7 post-dosing, 84.1–90.4% of TRR was recovered in faeces, and 6.3–12.2% in urine. In the high-dose group at 7 days post-dosing, 90.3–92.7% was excreted via faeces, and 6.5–6.8% via urine. At day 7 post-dosing, males in the low-dose group had 0.046 µg equivalents (eq) per gram in the liver and 0.055 µg eq/g in the kidney. In females, the respective values were both 0.016 µg eq/g. The concentrations of TRR in all other organs were lower. In the high-dose group, males had 0.202 µg eq/g in the liver and 0.043 µg eq/g in the kidney. In females, these values were 0.012 and 0.0036 µg eq/g. The concentrations of TRR in all other organs were lower. Generally, the concentrations were 2- to 5-fold higher in organs of males than in organs of females (Shaw, 2011c).

To investigate the tissue depletion of benzovindiflupyr, single doses of 97.9% radiochemically pure [pyrazole-5-^{14}C]benzovindiflupyr at 1 mg/kg bw (5.46 MBq/mg) or 40 mg/kg bw (0.118 MBq/mg) were administered by oral gavage to 15 non-fasted Han Wistar rats of each sex per dose. At 4, 24, 72, 102 and 144 hours post-dosing in the low-dose group and 24, 48, 72, 102 and 144 hours post-dosing in the high-dose group, three animals of each sex per dose were killed, and organs, tissues and body fluids were retained for TRR analysis.

No clinical signs were observed in the dosed animals. At 1 mg/kg bw, the highest levels of TRR were observed in both sexes at the first sampling time of 4 hours. In males at this time point, 19.6% of the dose was found in the carcass, and 5.05% in liver; in females, the respective values were 21.76% and 5.85%. In males, the mean concentrations were 1.17 µg eq/g in the liver and 0.81 µg eq/g in the kidney and the adrenals. In females, the respective values were 1.23 µg eq/g, 1.04 µg eq/g and 1.2 µg eq/g. In both sexes, the concentration of TRR in tissues declined, retaining the relative organ distribution. At 144 hours post-dosing, the TRR remaining in the carcass and tissues accounted for approximately 2.4% of the dose in males and 2.0% in females. In male rats, the elimination half-lives

for most tissues were generally in the range of 50–101 hours, with highest values for pancreas, liver, spleen, adrenals and muscle. In female rats, the elimination half-lives for most tissues were generally in the range of 40–61 hours, with highest values for pancreas, kidney, liver, spleen and heart. At 40 mg/kg bw, the highest levels of TRR were observed in both sexes at the first sampling time of 24 hours. In males at this time point, 7.2% of the dose was found in the carcass, and 1.8% in liver; in females, the respective values were 10.9% and 2.3%. In males, the mean concentrations were 14.1 µg eq/g in the liver, 8.2 µg eq/g in the kidney, 7.8 µg eq/g in the thyroid and 9.7 µg eq/g in the adrenals. In females, the mean concentrations were 25.2 µg eq/g in the liver, 20.2 µg eq/g in the adrenals, 16.6 µg eq/g in the renal fat, 13.3 µg eq/g in the kidney and 12.9 µg eq/g in the pancreas. In both sexes, TRR levels in tissues declined, retaining the relative organ distribution. At 144 hours, detectable TRR levels in males and females were found in liver, kidney and adrenals. In males, the remaining radioactivity accounted for 1.1% of the administered dose, and in females, 3.8%. In male rats, the elimination half-lives for most tissues were generally in the range of 43–210 hours, with highest values for pancreas, thyroid, kidney, liver, bone mineral and brain. In female rats, the elimination half-lives for most tissues were generally in the range of 70–316 hours, with highest values for liver, thymus, muscle and thyroid (Hutton, 2012).

To investigate the excretion and tissue distribution of benzovindiflupyr, 36 male Han Wistar rats were administered by gavage daily doses of 1 mg/kg bw per day (1.909 MBq/mg) of 97.3% radiochemically pure [pyrazole-5-^{14}C]benzovindiflupyr for maximally 14 days. Twenty-four hours after 3, 7, 10 and 14 doses and 3, 7, 10, 14, 18, 21, 28 and 63 days after 14 doses, three animals per time point were killed. To analyse for TRR, organs and blood samples were collected. Additionally, for 24 hours after the first dose or for 24 hours after 14 doses, faeces and urine were collected from three rats to analyse metabolites.

No adverse clinical observations were reported during the study. TRR concentrations in all organs increased during the dosing period. By the end of the dosing period, TRR concentrations in organs appeared to reach steady state. The highest TRR levels were found in liver (0.776 µg eq/g, equivalent to 0.26% of the dose) and kidneys (0.702 µg eq/g, equivalent to 0.03% of the dose). In all other organs, TRR concentrations were lower than in plasma (0.535 µg eq/g) at 24 hours after 14 doses. After cessation of dosing, the TRR concentrations in organs decreased slowly until day 28 after dosing. TRR concentrations in liver, kidney and thyroid were still approximately 0.1 µg eq/g or slightly less. At day 63 post-dosing, TRR concentrations in thyroid, adrenals, spleen, liver and kidney were in the range of 0.023–0.092 µg eq/g. TRR concentrations were lower in all other organs. The half-lives for some tissues, such as adrenals, spleen and whole blood, could not be reliably calculated because of low and variable tissue concentrations due to inter-animal variability and concentrations being at or around the limit of reliable measurement. The calculated terminal half-lives for other tissues were as follows: plasma, 2.49 days; liver, 17.3 days; pancreas, 18.65 days; heart, 19.65 days; kidneys, 21.42 days; bone mineral, 22.05 days; muscle, 24.49 days; lungs, 26.61 days; renal fat, 36.19 days; brain, 49.59 days; thymus, 61.77 days; and testes, 69.06 days. In a 24-hour period after 1 day of exposure, 72% of the administered dose was excreted in faeces, and 5.1% in urine. After a 14-day exposure period, 110.5% was excreted in faeces and 5.9% in urine within a collection period of 24 hours (Shaw, 2012).

To investigate the excretion pattern and tissue distribution of benzovindiflupyr, single doses of 1 mg/kg bw (5.55 MBq/mg) or 40 mg/kg bw (0.14 MBq/mg) of 97.7% radiochemically pure [pyrazole-5-^{14}C]benzovindiflupyr were administered by oral gavage to four bile duct–cannulated non-fasted Han Wistar rats of each sex per dose. Urine, bile, faeces and cage washes were collected over 2 days, after which the rats were terminated. A terminal blood sample was collected, together with the gastrointestinal tract and residual carcass, and each sample taken was then analysed for TRR.

No adverse clinical observations were reported in either dose group during the study. At 1 mg/kg bw, the major routes of elimination in both males and females were the bile (means of 76.1% and 68.5% of the administered dose in males and females, respectively) and the faeces (means of

16.9% and 15.8% of the administered dose in males and females, respectively), recovered over 2 days post-dosing. Urinary excretion accounted for means of 3.6% and 4.0% of the dose in males and females, respectively. The majority of the administered radioactivity was excreted by 48 hours after dosing (97.1% and 88.7% in males and females, respectively). Absorption accounted for 81.1% of the dose in males and 79.0% of the dose in females, as calculated from the biliary and urinary excretion, cage wash and TRR remaining in the carcass. Mean blood TRR concentrations were 0.013 and 0.022 µg eq/g for males and females, respectively. Mean plasma TRR concentrations were 0.017 and 0.023 µg eq/g for males and females, respectively. At 40 mg/kg bw, two females and one male showed problems with the bile flow; the females were excluded from the study. The major routes of elimination in both males and females were the bile (means of 47.3% and 56.5% of the administered dose in males and females, respectively) and the faeces (means of 32.7% and 31.9% of the administered dose in males and females, respectively), recovered over 2 days post-dosing. Urinary excretion accounted for means of 8.6% and 3.8% of the dose in males and females, respectively. The majority of the administered radioactivity was excreted by 48 hours post-dosing (90.0% and 85.7% in males and females, respectively). Absorption accounted for 61% of the dose in males and females, as calculated from the biliary and urinary excretion, cage wash and TRR remaining in the carcass. Mean blood TRR concentrations were 1.18 and 0.50 µg eq/g for males and females, respectively. Mean plasma TRR concentrations were 1.58 and 0.53 µg eq/g for males and females, respectively (Shaw, 2011b).

The pharmacokinetics of TRR was investigated comparatively following oral administration (1 mg/kg bw, 2.49 MBq/mg), intravenous bolus administration (0.25 mg/kg bw, 5.39 MBq/mg) and intravenous 1-hour infusion administration (0.5 mg/kg bw, 5.39 MBq/mg) of 99.5% radiochemically pure [pyrazole-5-^{14}C]benzovindiflupyr to groups of four male and four female Han Wistar rats. Blood samples were obtained over a 4-day period following intravenous and oral administration of benzovindiflupyr.

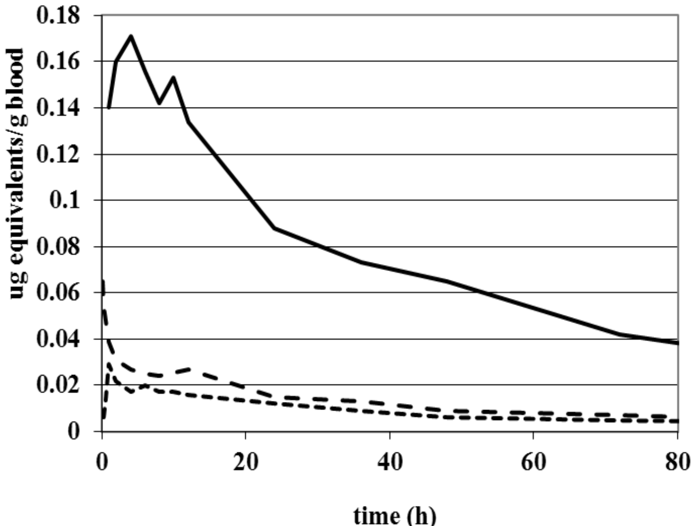
No adverse clinical observations were reported in either dose group during the study. In Fig. 4, blood concentrations of males for the oral, the intravenous bolus and the intravenous infusion applications are shown (blood concentrations of females are not shown, but are very similar to the male profiles). In the study, the intravenous infusion group was not further analysed, as the extremely low blood concentrations were not explainable. In the oral dose group, maximum levels were reached at about 3 hours, with maximum concentrations of 0.172 µg eq/g in males and 0.130 µg eq/g in females. Following the intravenous bolus application of 0.25 mg/kg bw, maximum concentrations in blood were approximately 0.072 µg eq/g for both sexes. The $AUC_{0-\infty}$ of the oral and the intravenous bolus application groups were approximately dose proportional. In both application groups, inter-individual $AUC_{0-\infty}$ variation was up to 200% (Shaw, 2011b).

1.2 Biotransformation

The metabolism of benzovindiflupyr was investigated by analysing bile, faeces, urine and plasma samples retained from the previous studies: single oral administration of 1 or 40 mg/kg bw of [pyrazole-5-^{14}C]benzovindiflupyr (Shaw, 2011b, 2011c, 2011d) and 14 consecutive daily oral doses of 1 or 40 mg/kg bw of [pyrazole-5-^{14}C]benzovindiflupyr (Shaw, 2012).

Benzovindiflupyr was extensively metabolized in rats (see Fig. 5), giving rise to at least eight types of metabolites (desmethyl, hydroxy, dihydroxy, desmethyl hydroxy, desmethyl dihydroxy, bicyclo ring-open, glucuronide conjugate, sulfate conjugate). There was no evidence for cleavage at the pyrazole–phenyl bridge. Faeces from bile duct–cannulated animals contained only benzovindiflupyr (approximately 13–26% of the dose) and a small quantity of SYN546039 (approximately 1–2% of the dose). The majority of metabolites in faeces from non-cannulated rats were hydroxylated metabolites of both benzovindiflupyr and SYN546206 (desmethyl benzovindiflupyr). The major metabolite was identified as SYN546041 (26–56% of the dose). SYN546360 (approximately 2–12% of the dose), SYN546643 (approximately 3–8% of the dose), SYN546645 (approximately 1–8% of the dose) and SYN546619 (approximately 4–5% of the dose)

Fig. 4. Mean blood concentrations in male rats dosed orally with 1 mg/kg bw, by intravenous bolus with 0.25 mg/kg bw and by intravenous infusion with 0.5 mg/kg bw



Solid line: oral, 1 mg/kg bw; long dashed line: intravenous bolus, 0.25 mg/kg bw; short dashed line: intravenous infusion, 0.5 mg/kg bw
Source: Shaw (2011b)

were also present. A sulfate conjugate of SYN546042 (approximately 6–7% of the dose) was detected in faeces from females, but not males. Ring-opening metabolites SYN546634, SYN546706 and SYN546707 (approximately 1–4% of the dose) were detected in males only. At least eight components remained unidentified in faeces from male rats, with no individual components exceeding 1.5% of the dose. All metabolites in faecal samples from female rats were identified. Only about 5–15% of the dose was recovered in urine. The major metabolite in urine was identified as SYN546360 (approximately 0.3–5% of the dose). Other identified components included glucuronide conjugates of desmethyl hydroxy metabolites (hydroxy SYN546206 and SYN546708; approximately 0.5–0.7% of the dose), hydroxy metabolites (SYN546360; approximately 0.6–2.3% of the dose) and dihydroxy metabolites (SYN546619 and dihydroxy benzovindiflupyr; approximately 0.3–0.8% of the dose); and the non-conjugated metabolites SYN546708 (approximately 0.2–1% of the dose), SYN546042 (approximately 0.1% of the dose), SYN546041 (0.6% of the dose) and SYN546643 (approximately 0.2–0.4% of the dose). A sulfate conjugate of SYN546042 (0.9% of the dose) was detected in urine from females, but not males, whereas ring-open metabolites of SYN546706 and SYN546707 (approximately 0.5–1% of the dose) were detected primarily in males. Up to 11 components were unidentified in urine, none of which exceeded 1% of the dose. The metabolites identified in urine from bile duct–cannulated rats were generally similar to those present in urine from non-cannulated animals. Metabolites in bile were glucuronide conjugates of hydroxylated metabolites of benzovindiflupyr and SYN546206 (desmethyl benzovindiflupyr). The major metabolites after hydrolysis were SYN546041 (approximately 29–45% of the dose), SYN546360 (approximately 5–7% of the dose) and SYN546039 (approximately 4–15% of the dose). Other identified metabolites included SYN546643, SYN516619, SYN546645, SYN546708 and hydroxy benzovindiflupyr (approximately 1–3% of the dose). The metabolic profile of plasma collected at time points close to the time at which the maximum concentration (C_{max}) was reached (T_{max}) showed the presence of benzovindiflupyr and hydroxylated metabolites of benzovindiflupyr and SYN546206 (desmethyl benzovindiflupyr). In males, unmetabolized parent was identified, and the major metabolites were SYN546041, SYN546039 and SYN546042, with SYN546206 present at a relatively low concentration. At least five unidentified components and non-migrating material at the origin of the thin-layer chromatography were also detected. In females, the major metabolite was SYN546206. Benzovindiflupyr and SYN546041 were present at a relatively high concentration, whereas

SYN546039 and SYN546042 were present at lower concentrations. Non-migrating material at the origin of the thin-layer chromatography was detected (Green, 2011).

Fig. 5. Metabolism of benzovindiflupyr in the rat

benzovindiflupyr (SYN545192)

SYN546206

SYN546708

SYN546041 SYN546042

SYN546039 SYN546040

SYN546360

SYN546643 SYN546645

SYN546634

SYN546619 SYN546644

SYN546706

SYN546422 (not detected, postulated)

SYN546707

1.3 Effects on enzymes and other biochemical parameters

No information was available.

2. Toxicological studies

2.1 Acute toxicity

The results of studies of acute toxicity with benzovindiflupyr are summarized in Table 2.

Table 2. Acute toxicity of benzovindiflupyr

Species	Strain	Sex	Route	LD_{50} (mg/kg bw)	LC_{50} (mg/L)	Purity (%)	Reference
Rat	Crl:(WI)BR	Female	Oral	55	–	97	Tavaszi (2010)
Rat	Crl:(WI)BR	Male, female	Dermal	> 2 000	–	97	Zelenak (2010a)
Rat	Crl:(WI)BR	Male, female	Inhalation	–	0.56–1.03	97	Nagy (2010)

LC_{50}: median lethal concentration; LD_{50}: median lethal dose

(a) Oral administration

The acute oral toxicity of benzovindiflupyr (purity 97%; batch no. SMU9BP005) was tested at dose levels of 17.5, 55 and 175 mg/kg bw in one, four or three female Crl:(WI)BR rats, respectively. There were no signs of toxicity at 17.5 mg/kg bw. At 55 mg/kg bw, all animals showed decreased activity, dyspnoea and incoordination, and one animal exhibited a hunched back. One animal died within 2 hours after dose administration. At 175 mg/kg bw, all animals showed decreased activity, prone position, incoordination, piloerection, dyspnoea and decreased body temperature, and one animal showed decreased respiratory rate and clonic convulsion. All animals died within 24 hours after dose administration. The median lethal dose (LD_{50}) of benzovindiflupyr in female rats was 55 mg/kg bw (Tavaszi, 2010). During the absorption, distribution, metabolism and excretion (ADME) studies described in section 1.1 (Shaw, 2011a), it was realized that acute toxicity is exaggerated by fasting animals before dosing. Therefore, the discrepancy between the finding of nearly no toxicity at 40 mg/kg bw in ADME studies and severe toxicity at 55 mg/kg bw in the acute oral study is due to the feeding status of the animals.

(b) Dermal application

The acute dermal toxicity of benzovindiflupyr (purity 97%; batch no. SMU9BP005) was tested at 2000 mg/kg bw in five male and five female Crl:(WI)BR rats. There were no mortalities, no systemic clinical signs of toxicity and no local dermal signs of toxicity. The dermal LD_{50} of benzovindiflupyr in male and female rats was greater than 2000 mg/kg bw (Zelenak, 2010a).

(c) Exposure by inhalation

The acute respiratory toxicity of benzovindiflupyr (purity 97%; batch no. SMU9BP005) after a 4-hour nose-only exposure was tested in two female Crl:(WI)BR rats at 1.03 and 2.48 mg/L and in five male and female rats at 0.56 mg/L. Clinical signs noted on the day of exposure at all dose levels included increased or laboured respiratory rate and noisy respiration in many animals. In addition, ataxia, lethargy, crouching, clonic convulsions, comatose state, coldness to touch and prone position were noted in some animals during the 1st week of the observation period. One female died at 0.56 mg/L, and all animals died at higher dose levels. The respiratory median lethal concentration (LC_{50}) of benzovindiflupyr in male and female rats was greater than 0.56–1.03 mg/L (Nagy, 2010).

(d) Dermal and ocular irritation

The primary skin irritation potential of 0.5 g benzovindiflupyr (purity 97%; batch no. SMU9BP005) per animal was tested in male New Zealand White rabbits following exposure for 4 hours. Benzovindiflupyr was initially mildly irritating to the skin in male rabbits (Zelenak, 2010b).

The primary eye irritation potential of 0.1 g benzovindiflupyr (purity 97%; batch no. SMU9BP005) per animal was tested in one male and two female New Zealand White rabbits by instillation into the left eye of the animals. Moderate reddening of the conjunctivae and mild reddening of the sclera were observed. These effects were reversible within the observation period of 14 days. Benzovindiflupyr was classified as moderately irritating to the eye (Mallaun, 2011).

(e) Dermal sensitization

In a mouse local lymph node assay, benzovindiflupyr (purity 97%; batch no. SMU9BP005) was investigated for its skin sensitizing potential in female CBA/J Rj mice. Animals were exposed to approximately 25 µL of 0%, 0.01%, 0.1% or 1.0% (weight per volume) benzovindiflupyr in dimethyl sulfoxide. Benzovindiflupyr exhibited no skin sensitizing potential in female mice (Torok-Batho, 2010).

2.2 Short-term studies of toxicity

Mice

Benzovindiflupyr (purity 98.3%; batch no. TE-6341) was administered for 28 days in the diet to groups of five male and five female 8-week-old Crl:CD-1(ICR) mice. The concentrations in feed were 0, 100, 300 and 500 parts per million (ppm) (equal to 0, 15.6, 47.4 and 81.8 mg/kg bw per day for males and 0, 19.0, 57.9 and 91.5 mg/kg bw per day for females, respectively). For each dose group, a satellite group was allocated for blood toxicokinetic control of exposure. Detailed clinical observations, body weights and feed consumption were recorded weekly. At study termination, haematology, clinical chemistry, organ weights and kidney histology from all animals were investigated. Histological analysis in all other organs was performed only in the control and high-dose groups.

At the end of the study, body weights in male mice were lower by approximately 7% at 300 ppm and by approximately 21% at 500 ppm; in females, body weights at 500 ppm were lower by 11% compared with controls. Feed consumption was not affected. There were no haematological or clinical chemistry findings attributable to treatment in any group. Body weight–adjusted brain weights were statistically significantly increased by 5% in all dosed males. There were no changes in body weight–adjusted organ weights. Histological findings were confined to the kidney. Two males and one female at 500 ppm showed tubulointerstitial nephritis with tubular basophilia accompanied by interstitial inflammatory cell infiltration.

The no-observed-adverse-effect level (NOAEL) was 100 ppm (equal to 15.6 mg/kg bw per day), based on reduced body weight at 300 ppm (equal to 47.4 mg/kg bw per day) (Shearer, 2010).

Benzovindiflupyr (purity 97%; batch no. SMU9BP005) was administered for 13 weeks in the diet to groups of 10 male and 10 female 7-week-old Crl:CD-1(ICR) mice. For each dose group, a satellite group of three animals of each sex was allocated for blood toxicokinetic control of exposure. The concentrations in feed were 0, 100, 300 and 500 ppm (equal to 0, 17.0, 55.6 and 97.9 mg/kg bw per day for males and 0, 20.9, 59.6 and 102.8 mg/kg bw per day for females, respectively). Detailed clinical observations were performed weekly, and body weights and feed consumption were recorded daily at the beginning of the study and twice weekly thereafter. At study termination, haematology, clinical chemistry, organ weights and histology of liver, colon and rectum from all animals were investigated. Histological analysis in all other organs was performed only in the control and high-dose groups.

At the high dose, two main study males and one satellite male were terminated due to severe toxic effects, such as body weight loss. Incidences of clinical observations noted for males and females in all treated groups were higher than those for controls. The observations included piloerection, rolling gait, staggering, circling, irregular respiration and soft faeces. Except for soft faeces, no clear dose–response relationship was evident. At the end of the study, body weights in male mice were lower by 14% at 300 ppm and by 25% at 500 ppm; in females, body weights were lower by 9% at 300 ppm and by 15% at 500 ppm. Reduced body weight gain was observed from the 1st day of treatment. Feed consumption was not affected. There were no haematological findings attributable to treatment in any group. A statistically significant higher plasma globulin level was noted in both sexes at 500 ppm and in males at 300 ppm. Statistically lower plasma triglyceride levels were found in males at 300 and 500 ppm, and higher creatinine levels were observed in males at 500 ppm. A statistically significant increase in plasma calcium levels was noted in females at 300 and 500 ppm. A range of absolute organ weights in males exposed to 300 and 500 ppm was reduced, including liver weights. In females, only absolute thymus weights at 500 ppm were reduced. After body weight correction, only heart weights in males at 500 ppm were still lower by 17% compared with controls. As no histological correlates were noted, the biological significance of this effect is unclear. The incidence of tubular basophilia in the kidney in females was increased at 500 ppm (4/13 versus 1/13 in control animals). Minimal to moderate mucosal hyperplasia was found in the colon and/or rectum in most animals treated at 500 ppm. Minimal or mild mucosal hyperplasia was also found in the colon and/or rectum in some animals at 300 ppm. In one male and one female, the macroscopic finding of distended intestine was also observed at necropsy.

The NOAEL was 100 ppm (equal to 17.0 mg/kg bw per day), based on reduced body weight gains and minor clinical chemistry changes at 300 ppm (equal to 55.6 mg/kg bw per day) (Mackay, 2011).

Rats

In an investigative study, benzovindiflupyr (purity 98.3%; batch No. TE-6341) was administered for maximally 29 days in the diet to groups of 25 male and 25 female 7-week-old Crl:WI(Han) rats. The concentrations in feed were 0, 100, 750 and 1500 ppm (equivalent to 0, 10, 75 and 150 mg/kg bw per day, respectively). At days 3, 4, 8, 15 and 29, five animals of each sex per group were terminated. Viability, body weight, feed and water consumption and general behaviour were recorded daily. At terminal sacrifice, all animals underwent gross necropsy, and organs were weighed and fixed.

In this study, not all recorded data were analysed and discussed further. Body weights were lower by 18% in females treated at 750 ppm and above and by 8% in males treated at 1500 ppm. Feed consumption was lower in males dosed at 1500 ppm for the first 5 days of treatment; thereafter, feed consumption was considered to be comparable with that of control animals. In females, feed consumption was generally lower compared with the control animals throughout the treatment period in animals dosed at 750 ppm and above. At the beginning of the study, feed consumption was reduced by 60%, and at study termination, it was reduced by approximately 20%. At day 3, relative liver weights were statistically significantly higher and relative kidney weights lower in males at 1500 ppm. At day 4, relative liver weights were statistically significantly higher in males at 750 and 1500 ppm. At 1500 ppm, thymus weights in males and heart weights in females were lower. At day 8, relative liver weights were statistically significantly higher in all treated animals. Absolute heart weights were statistically significantly lower in females at 1500 ppm. At day 15, relative liver weights were statistically significantly higher in males treated at 1500 ppm. Relative kidney weights were lower in females at 750 and 1500 ppm. At day 29, relative liver weights were higher in all treated females and in males treated with 1500 ppm. Additionally in females, lower absolute heart weights and ovary weights at 750 and 1500 ppm and lower absolute uterus and kidney weights at 1500 ppm were observed. There were no necropsy findings attributable to treatment.

In this investigative study, no NOAEL was derived (Robertson, 2010b).

After completion of the 2-year study in rats (Mackay, 2012a; see section 2.3) and the observation of thyroid tumours, thyroids of male animals from the investigative rat study discussed above were examined. Thyroid follicular cell hypertrophy was found at day 15 in 2/5 males dosed at 1500 ppm and at day 29 in 2/4 and 1/5 males dosed at 750 and 1500 ppm, respectively. In all other groups at all other time points, no thyroid follicular cell hypertrophy was found (Robertson, 2012b).

Benzovindiflupyr (purity not reported; batch no. TE-6341) was administered for 28 days in the diet to groups of five male and five female 13-week-old HsdRCCHan:WIST rats. The concentrations in feed were 0, 100, 400 and 1200 ppm (equal to 0, 9, 36 and 107 mg/kg bw per day for males and 0, 9, 36 and 90 mg/kg bw per day for females, respectively). Clinical observations were recorded daily, and body weights and feed consumption were recorded twice weekly. Motor activity and grip strength were monitored regularly. At study termination, haematology, clinical chemistry, organ weights and histology (including livers and kidneys of low-dose and mid-dose animals) were investigated in control and high-dose animals.

No treatment-related clinical signs or functional effects were observed at any dose level. Males and females at 1200 ppm had lower body weights (−11%) compared with controls. In females at 1200 ppm, there was a slight decrease in feed consumption. Haematology was not affected by treatment. Statistically significant but not dose-related reductions in plasma total protein levels and albumin levels of about 8% were evident in all treated females. In addition, females treated with 1200 ppm displayed a slight but statistically significant increase (+50%) in plasma aspartate aminotransferase (ASAT) activity. A slight but statistically significant reduction in plasma glucose levels (−15%) was observed in males at 1200 ppm. Minimal tubular basophilia in kidneys was observed in females at 0 (0/5), 100 (1/5), 400 (2/5) and 1200 ppm (4/5), and the incidence was not dose related in males at 0 (1/5), 100 (4/5), 400 (2/5) and 1200 ppm (0/5). Minimal centrilobular hepatocyte hypertrophy was observed in the 400 and 1200 ppm males. Tubular basophilia in kidneys is a very common finding in rats, even in short-term studies (historical control mean 38% in males and 21% in females, median 20% in both sexes).

The NOAEL was 400 ppm (equal to 36 mg/kg bw per day), based on lower body weights in both sexes at 1200 ppm (equal to 90 mg/kg bw per day) (Marr, 2010).

Benzovindiflupyr (purity 98.3%; batch no. TE-6341) was administered in the diet for 13 weeks to groups of 10 male and 10 female 7-week-old Crl:WI(Han) rats. The concentrations in feed were 0, 100, 750 and 1500 ppm (equal to 0, 8.2, 58.8 and 108.8 mg/kg bw per day for males and 0, 9, 36 and 90 mg/kg bw per day for females, respectively). Clinical observations were recorded weekly, and body weights and feed consumption were recorded daily at the beginning of the study and twice weekly thereafter. A functional observational battery was performed at the end of the study. Eyes were examined at study termination; for toxicokinetic control of exposure, blood was collected. Haematology, clinical chemistry and organ weights were recorded for all animals. Histology of organs (including livers of low-dose and mid-dose animals) was examined in control and high-dose animals.

One high-dose male died due to a blood sampling error. There were no treatment-related clinical signs or effects on the functional observational battery at any dose level. At the end of the study, body weights in male rats were lower by approximately 11% at 750 ppm and by approximately 14% at 1500 ppm; in females, body weights were lower by 12% at 750 ppm and by 20% at 1500 ppm. Reduced body weight gain was observed from the 1st week of treatment. Feed utilization was statistically significantly lower compared with controls in both sexes at 750 and 1500 ppm and also in males at 100 ppm. Haematology was not affected by treatment. Statistically significant decreases were noted in alkaline phosphatase (AP) and glucose levels in all animals at 1500 ppm and in females only at 750 ppm. There was a statistically significant increase in urea level in males at 750 ppm and above. In males, relative liver weights were increased at 750 ppm, reaching statistical significance at 1500 ppm. Centrilobular hepatocyte hypertrophy was present in all males treated at 1500 ppm and in 4/10 animals treated at 750 ppm. In females, this finding was recorded in 4/10 animals at 1500 ppm.

The NOAEL was 100 ppm (equal to 8.2 mg/kg bw per day), based on reduced body weight gain at 750 ppm (equal to 58.8 mg/kg bw per day) (Robertson, 2010a).

Benzovindiflupyr (purity 97%; batch no. SMU9BP005) was administered dermally for 4 weeks (5 days/week) to groups of 10 male and 10 female 8-week-old RCCHan:WIST rats at a dose of 0, 100, 300 or 1000 mg/kg bw. General clinical observations and skin reactions were recorded daily, and body weights and feed consumption were measured weekly. At study termination, a functional observational battery was performed, clinical chemistry was analysed and selected organs were weighed and examined macroscopically and microscopically.

No treatment-related effects were observed.

The NOAEL was 1000 mg/kg bw per day, the highest dose tested (Sommer, 2012).

Dogs

Benzovindiflupyr (purity 97%; batch no. SMU9BP005) was administered by gelatine capsule for 90 days to four male and four female 27- to 29-week-old Beagle dogs. The dose levels were 0, 30, 375 and 750 mg/kg bw per day. Detailed clinical observations and body weights were recorded weekly, and feed consumption was recorded daily. Blood samples were taken from all dogs in weeks −2, 4, 8 and 13, and urine samples were collected at weeks −2, 6 and 13. Additionally, for toxicokinetic control of exposure, blood was collected in weeks 1, 7 and 13 at several time points within 24 hours post-dosing. At study termination, eyes were examined, organ weights were recorded and histopathological examinations of organs of all animals were performed.

Salivation was noted in two females at 375 mg/kg bw per day and in all females at 750 mg/kg bw per day, starting at the end of the 1st week. Mean feed consumption was reduced in animals at 375 and 750 mg/kg bw per day during the first 2 weeks of treatment. Feed consumption improved in all animals after the feeding regimen was changed (feeding 3 hours after completion of dosing instead of immediately after dosing). Slight body weight loss was recorded in most of the animals during the 1st week of treatment at 375 and 750 mg/kg bw per day. Reduced body weight gain was observed in males at 375 and 750 mg/kg bw per day from day 22 onwards and sporadically in females at 750 mg/kg bw per day between day 8 and the end of the study. The terminal body weights were 20% and 15% lower in males and females, respectively, compared with controls. There were no treatment-related ophthalmoscopic or haematological changes at any dose level. Urine analysis parameters were not affected by treatment. Increased plasma triglyceride levels were recorded in males and females during the whole treatment period at 750 mg/kg bw per day. Decreased plasma calcium levels were observed in males at 375 and 750 mg/kg bw per day during weeks 8 and 13. Weight analysis and macroscopic and microscopic examination of organs did not reveal any treatment-related effect.

The NOAEL in this study was 30 mg/kg bw per day, based on transient body weight loss due to reduced feed consumption and minor biochemical changes at 375 mg/kg bw per day (Pothmann, 2010).

Benzovindiflupyr (purity 97%; batch no. SMU9BP005) was administered by gelatine capsule to four male and four female 26- to 30-week-old Beagle dogs for 52 weeks. The dose levels were 0, 25, 250 and 500 mg/kg bw per day. Detailed clinical observations and body weights were recorded weekly, and feed consumption was recorded daily. Blood samples were taken from all dogs in weeks −2, 13, 26 and 52, and urine samples were collected in weeks −2, 26 and 52. At study termination, eyes were examined, organ weights were recorded and histopathological examinations of organs of all animals were performed.

Salivation was often noted in both sexes at 250 and 500 mg/kg bw per day, compared with controls. Vomiting was observed in all groups, including the controls, but with an increased incidence in treated animals at 250 and 500 mg/kg bw per day. Salivation and vomiting occurred at the end of the 1st week. Mean feed consumption was not affected at any dose level. Based on group mean and

median body weight values, males had a lower body weight gain at 250 and 500 mg/kg bw per day. In females, the body weight gain at 500 mg/kg bw per day was reduced. The final body weights in both sexes were lower by approximately 10% at the high dose level. There were no treatment-related ophthalmoscopic changes. The only haematological changes were statistically significantly lower eosinophil differential counts (absolute) in 250 and 500 mg/kg bw per day males at week 52. Urine analysis, clinical biochemistry, macroscopic pathology and microscopic pathology were not affected by treatment.

The NOAEL in this study was 25 mg/kg bw per day, based on reduced body weight gain in males at 250 mg/kg bw per day (Braun, 2011).

2.3 Long-term studies of toxicity and carcinogenicity

Mice

Benzovindiflupyr (purity 97%; batch no. SMU9BP005) was administered in the diet to groups of 50 male and 50 female 7-week-old Crl:CD-1(ICR) mice for 80 weeks. The concentrations in feed were 0, 20, 60 and 200 ppm (equal to 0, 2.62, 7.55 and 26.18 mg/kg bw per day for males and 0, 2.89, 8.67 and 29.26 mg/kg bw per day for females, respectively). Detailed clinical observations were recorded weekly. Body weights and feed consumption were recorded twice weekly up to week 4, once weekly up to week 15 and then every other week. For haematology, blood was taken from all animals at week 50 and at study termination. At study termination, organs were weighed and examined histologically.

There were no clinical observations or changes in feed consumption in any of the treated dose groups. Haematology, organ weights and macroscopic appearance of organs were not affected by treatment in any of the dose groups. In high-dose males, the body weight increase was lower by approximately 5% in the first few weeks. In high-dose males, colon mucosal hyperplasia incidence was elevated compared with controls (13/49 versus 1/49). In high-dose females, this finding was also noted at an elevated incidence (10/48 versus 0/48). In high-dose males and females, caecum mucosal hyperplasia was elevated compared with controls (4/50 in males and 2/48 in females, compared with 0/49 and 0/48 in controls). In both sexes at all dose levels, Harderian gland adenoma incidence was higher than in concurrent controls (Table 3). The incidences at the low doses and the high doses in both sexes were well above the historical control incidence, but at the middle dose, the incidences were close to the maximum found in four historical control groups. However, the Harderian gland adenoma incidences are considered not to be treatment related because no dose–response relationship is evident and the concurrent control groups show very low incidences. Furthermore, the historical control data are based on four studies only, suggesting that the natural range of variability for the Harderian gland adenoma incidence is statistically not yet covered and might expand with an increasing database.

Table 3. Incidences of neoplastic changes in male and female mice at terminal kill and preterm deaths combined

	Incidence of neoplastic finding							
	Males (*n* = 50 per group)				Females (*n* = 50 per group)			
	0 ppm	20 ppm	60 ppm	200 ppm	0 ppm	20 ppm	60 ppm	200 ppm
Harderian gland adenocarcinoma	0	1	0	0	0	0	0	0
Harderian gland adenoma	2	7	4	8	0	4	3	5
Harderian gland adenoma historical control incidences from four studies (average and range (%))	5.5 (4–8)	–	–	–	2.5 (2–4)	–	–	–

Source: Mackay (2012b)

The NOAEL was 60 ppm (equal to 7.55 mg/kg bw per day), based on colon/caecum mucosal hyperplasia at 200 ppm (equal to 26.18 mg/kg bw per day) in both sexes (Mackay, 2012b).

Rats

Benzovindiflupyr (purity 97%; batch no. SMU9BP005) was administered in the diet to groups of 64 male and 64 female 6-week-old Crl:WI(Han) rats for 104 weeks. Twelve animals of each sex per dose were investigated at an interim kill at 52 weeks. The concentrations in feed were 0, 25, 100 and 600 ppm for males (equal to 0, 1.2, 4.9 and 30.2 mg/kg bw per day, respectively) and 0, 25, 100 and 400 ppm for females (equal to 0, 1.7, 6.7 and 27.4 mg/kg bw per day, respectively). The eyes of all main study animals were examined pre-experimentally, at week 50 and prior to termination. Detailed clinical observations were recorded weekly, and body weights and feed intake were recorded weekly up to week 15 and then every other week. A functional observational battery of tests and locomotor activity monitoring were performed during week 51/52 on the interim kill animals. For haematology, blood was taken from 13 animals of each sex per dose at weeks 14, 27, 53 and 79. Urine was collected from 13 male and 13 female rats at weeks 13, 26, 52, 78 and 104. At study termination at 52 weeks and 104 weeks, organs were weighed and examined histologically.

There were no clinical observations considered to be related to treatment. Body weights and feed utilization were lower from the 1st week onwards in both sexes at the high dose level (400/600 ppm). Feed utilization was also statistically significantly lower in 100 ppm females in the first 4 weeks of treatment. Terminal body weights were lower by 11% in high-dose males and by 25% in high-dose females. No ophthalmic changes were observed, and the functional observational battery was not affected by treatment. In high-dose females, certain red cell parameters were slightly but statistically significantly reduced in the 1st year, and prothrombin time was slightly but statistically significantly shortened at week 79 only. A few biochemical changes at the high dose levels and sporadically also at the intermediate dose level were observed mainly within the first few weeks of the study. They included decreased AP and ASAT/alanine aminotransferase (ALAT) activities (maximum by 40%) and increased glucose levels in both sexes by approximately 20%. In the 2nd year of the study, the urea level was increased by approximately 30% in high-dose females. Decreased AP and ASAT/ALAT activities were still seen in the 2nd year of the study, mainly in males. At 52 weeks, organ weights were similar to those of controls in all dose groups. At 104 weeks, several absolute organ weights were higher than control animal organ weights. After body weight adjustment, all organ weights were similar. If body weight is used as a covariate, male liver weights at the high dose were statistically significantly higher than those of controls. No macroscopic findings were recorded in any of the treated dose groups. At 52 weeks, the incidence of hepatocellular centrilobular hypertrophy was statistically significantly elevated in males (75% versus 0%) and females (83% versus 0%) receiving 600 and 400 ppm, respectively, and present in animals at 100 ppm (16.6% in males and 8.3% in females). The incidence of minimal centrilobular pigmentation was statistically significantly increased in females at 400 ppm (50% versus 0%). At terminal kill, centrilobular hepatocellular hypertrophy was increased in mid- and high-dose males and high-dose females; additionally in high-dose males, eosinophilic foci of cellular alteration in liver were increased (Table 4). In males at 600 ppm, the incidence of thyroid follicular cell adenoma was statistically significantly increased compared with the concurrent control group. The incidences were 2% at 0 ppm, 7.6% at 25 ppm, 9.6% at 100 ppm and 17.3% at 600 ppm. The incidence for males at 600 ppm was statistically significant and also higher than the historical control incidence range and mean/median values (eight studies, range 2–11.1%, mean and median 5.8%). A dose-related decrease in adrenal cortical adenoma was observed.

The NOAEL for systemic toxicity and carcinogenicity was 100 ppm (equal to 4.9 mg/kg bw per day), based on histological changes in the liver of males, an increased incidence of thyroid follicular cell adenoma in males and reduced body weight gain in both sexes at the high dose (600 ppm in males, equal to 30.2 mg/kg bw per day; and 400 ppm in females, equal to 27.4 mg/kg bw per day) (Mackay, 2012a).

Table 4. Incidences of non-neoplastic and neoplastic changes in male and female rats at terminal kill and preterm deaths combined

	Severity	Incidence of finding							
		Males (*n* = 52 per group)				Females (*n* = 52 per group)			
		0 ppm	25 ppm	100 ppm	600 ppm	0 ppm	25 ppm	100 ppm	400 ppm
Hepatocellular hypertrophy, centrilobular	Minimal	0	0	1	2	0	1	2	3
	Mild	0	1	6*	11**	0	1	3	33**
	Moderate	0	0	1	0	0	0	0	0
	Total	0	1	8**	13**	0	2	5	36**
Pigmentation hepatocytes, centrilobular	Minimal	0	0	0	0	0	0	0	1
	Mild	0	0	0	0	0	0	0	1
	Moderate	0	0	0	0	0	0	0	1
	Total	0	0	0	0	0	0	0	3
Eosinophilic hepatocytes, foci	Minimal	0	2	0	2	0	0	2	1
	Mild	1	2	4	6	2	0	1	2
	Moderate	0	1	0	6*	0	0	0	1
	Marked	0	0	0	1	0	0	0	1
	Total	1	5	4	15**	2	0	3	5
Hepatocellular vacuolation, centrilobular	Minimal	0	0	2	3	0	0	3	0
	Mild	2	2	1	5	0	2	2	0
	Moderate	0	1	2	1	0	0	0	0
	Marked	0	1	0	0	0	0	0	0
	Severe	0	1	0	0	0	0	0	0
	Total	2	5	5	9	0	2	5	0
Thyroid follicular cell adenoma		1	4	5	9*	0	1	1	1

*: $P < 0.05$; **: $P < 0.01$
Source: Mackay (2012a)

2.4 Genotoxicity

Benzovindiflupyr was tested for genotoxicity in a range of assays in vitro and in vivo. No evidence for genotoxicity was observed in any test. The results of the genotoxicity tests are summarized in Table 5. It is concluded that benzovindiflupyr is unlikely to be genotoxic.

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

Rats

Benzovindiflupyr (purity 97%; batch no. SMU9BP005) was administered in the diet to groups of 25 male and 25 female 7-week-old HanRcc:WIST rats. The concentrations in feed were 0, 25, 100 and 600 ppm for males and 0, 25, 100 and 250 ppm for females. The doses achieved are depicted in Table 6. After a 10-week premating phase under exposure, F_0 animals were mated and allowed to rear two F_1 litters. F_{1A} parents were mated after a 10-week premating period and produced a single F_2 generation. Clinical observations, body weight development and feed consumption were recorded regularly (weekly or 3 times weekly). In parental animals in the control and high-dose groups, organs were examined microscopically. Reproductive performance was assessed based on successful mating

Table 5. Results of genotoxicity studies with benzovindiflupyr

End-point	Test system	Concentration	Lot no.; purity	Result	Reference
Reverse mutation (Ames)	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 *Escherichia coli* WP2 and WP2 uvrA	3.0–5000 µg/plate (±S9)	SMU9BP005; 97.0%	Negative	Sokolowsk (2011a)
Mouse lymphoma TK	L5178Y cell line	0.34–10 µg/mL (±S9)	SMU9BP005; 97.0%	Negative	Wollny (2010)
Chromosomal aberration	Human lymphocytes	10–50 µg/mL (±S9)	SMU9BP005; 97.0%	Negative	Bohnenberger (2010)
Micronucleated bone marrow cells	Male and female Wistar (Han) rats	175 mg/kg bw in males and 75 mg/kg bw in females	SMU9BP005; 97.0%	Negative	Innes (2010)

S9, 9000 × *g* supernatant fraction from rat liver homogenate; TK, thymidine kinase

Table 6. Doses achieved in the multigeneration study in rats

	Doses (mg/kg bw per day)					
	Males			Females		
	25 ppm	100 ppm	600 ppm	25 ppm	100 ppm	250 ppm
F_0 parents premating	1.7	6.8	40.5	2.0	8.2	19.4
F_1 parents premating	1.9	7.8	48.0	2.1	8.7	22.0
F_0 females during gestation	–	–	–	1.9	7.6	17.5
F_1 females during gestation	–	–	–	2.0	7.9	18.3
F_0 females postpartum	–	–	–	4.1	16.8	40.2
F_1 females postpartum	–	–	–	4.3	17.2	43.1

Source: Adamska (2012)

(litter with at least one pup alive), length of gestation and precoital interval. F_{1A} pups were separated from the dams at postnatal day (PND) 29 and reared as F_1 parents to produce the F_2 generation. Pups were investigated for clinical condition, survival, body weight development, anogenital distance, vaginal opening and preputial separation. At termination of F_0 and F_1 parents, organ weights and histology, sperm number, sperm morphology, sperm motility and spermatid resistance to homogenization were recorded. In pups, organs were weighed and examined macroscopically.

There were no significant clinical observations in F_0 or F_1 parental animals. Cumulative body weight gain and feed consumption were reduced in 600/250 ppm F_0 and F_1 males and females throughout premating and gestation. Cumulative body weight gain was also low (−20%) in females at 100 ppm in the premating phase. In the F_0 and F_1 250 ppm groups, the mean corpora lutea count at necropsy was statistically significantly lower than in controls (Table 7). Additionally, the mean number of implantations was slightly, but not statistically significantly, lower in the 250 ppm group of the F_0 generation, as was the mean F_0 litter size (10.0 compared with 11.5 in the control group). In the F_0 and F_1 males at 600 ppm and F_1 females at 250 ppm, the weight of the liver adjusted for body weight was statistically significantly higher than in controls. Centrilobular hepatocellular hypertrophy was observed in high-dose males, and hypertrophy in adrenal zona glomerulosa was observed in high-dose females. Based on vaginal micropathology to determine estrous cycle status at the end of lactation, an increased number of animals in lactational diestrus was observed at 250 ppm in F_0 and F_1 females compared with controls. The incidence was also slightly increased at 100 ppm.

Table 7. Parental and pup findings in the multigeneration study in rats

	0 ppm	25 ppm	100 ppm	250/600 ppm
Mean corpora lutea counts				
- F_1	16.7 ± 3.3	16.0 ± 2.5	15.8 ± 2.4	14.3 ± 1.7**
- F_2	14.6 ± 2.9	14.4 ± 2.1	13.9 ± 1.2	13.5 ± 1.3
Mean implantations				
- F_1	13.2 ± 2.87	12.4 ± 2.28	13.1 ± 2.66	11.7 ± 2.9
- F_2	12.7 ± 2.22	12.7 ± 2.21	12.8 ± 1.58	12.4 ± 2.45
Preputial separation (days)				
- F_1	26.5 ± 1.4	26.2 ± 1.0	26.8 ± 1.4	28.4 ± 1.9**
Vaginal opening (days)				
- F_1	33.3 ± 1.6	33.6 ± 1.9	33.5 ± 1.7	34.6 ± 2.4*

*: $P < 0.05$; **: $P < 0.01$
Source: Adamska (2012)

On PNDs 1–4, pup body weights were similar between groups in the F_1 generation and lower at 250 ppm in the F_2 generation. Thereafter, at 250 ppm, F_1 pups had lower body weights (about −10% from PND 7 to PND 21), as did F_2 pups (by 8% at PND 1 and by 14% at PND 21). The time until preputial separation was statistically significantly longer in the males in the 600 ppm dose group. In females at 250 ppm, the time to vaginal opening was slightly, but statistically significantly, longer than in controls. In high-dose F_1 and F_2 females, relative liver weights were increased.

The NOAELs for parental and offspring toxicity were both 100 ppm (equal to 6.8 mg/kg bw per day), based on decreased body weight gain in parental animals and on lower postnatal body weight gain at 250 ppm (equal to 17.5 mg/kg bw per day). The reproductive NOAEL was 250 ppm (equal to 17.5 mg/kg bw per day), the highest dose tested (Adamska, 2012).

(b) Developmental toxicity

Rats

Benzovindiflupyr (purity 97%; batch no. SMU9BP005) was administered by oral gavage to groups of 24 female time-mated 11-week-old RccHan:WIST(SPF) rats from gestation day (GD) 6 to GD 20. The dose levels were 0, 7.5, 15 and 30 mg/kg bw per day. Detailed clinical observations, body weights and feed consumption were recorded. All rats terminated at day 21 of gestation were given a macroscopic examination, including external observation and an examination of the thoracic and abdominal viscera. The following developmental parameters were examined: number of corpora lutea, gravid uterus weight, number and position of implantations, number of live fetuses, number of early and late intrauterine deaths, weight and sex of fetuses and external, visceral and skeletal abnormalities and variations.

At 30 mg/kg bw per day, 14/24 animals showed ataxia, decreased activity, hunched and prostrate posture and ruffled fur from treatment days 6 to 16. High-dose animals showed reduced feed consumption (−33%) from the 1st day and reduced body weights (−9% at study termination) from the 3rd treatment day onwards. High-dose litter weights were statistically significantly lower by 11% compared with the concurrent control group. Abnormalities were seen in five fetuses in five high-dose litters (Table 8). Two fetuses had great vessel abnormalities and an interventricular septal defect of the heart. In three other fetuses, findings were situs inversus totalis, severely thin diaphragm and severely dilated renal pelvis and ureter. The incidence of thymus long cranial (extrathymic tissue, thymic remnant in the neck) was increased statistically significantly on a fetal basis at 15 mg/kg bw per day (18% versus 7% in control fetuses) and on a fetal and litter basis at 30 mg/kg bw per day (24% versus 7% in control fetuses and 74% versus 29% in control litters). Historical control fetuses had a mean fetal incidence of 6% (range 3–9%). Although this variation was statistically significantly

increased at 15 mg/kg bw per day and above, it was considered not relevant for NOAEL setting, as the background incidence is high and the effect not adverse. At 30 mg/kg bw per day, delayed ossification was noted in cervical vertebrae, sternebrae, proximal phalanges of digits and calcanei.

Table 8. Fetal findings in a developmental toxicity study in rats

	Incidence of finding[a]			
	0 mg/kg bw per day	7.5 mg/kg bw per day	15 mg/kg bw per day	30 mg/kg bw per day
No. of litters investigated	*24*	*23*	*21*	*23*
No. of fetuses investigated	*154*	*141*	*134*	*147*
Thoracic and abdominal situs inversus	0	0	0	1
Pituitary small; abdomen internal haemorrhage; origins of ascending aorta and pulmonary trunk malpositioned and heart interventricular septal defect	0	0	0	1
Aortic arch narrow, origins of ascending aorta and pulmonary trunk malpositioned and heart interventricular septal defect; lung two lobes only; azygos vein bilateral, azygos vein persisting into abdomen; abdominal situs inversus and liver lobes misshapen/absent	0	0	0	1
Diaphragm severely thin localized	0	0	0	1
Renal pelvis and ureter severely dilated	0	0	0	1
Renal pelvis dilated	0	0	3 (2)	4 (4*)
Summary incidences of anomalies	0	0	3 (2)	9 (9*)
Thymus long cranial (variation)	11 (7)	16 (10)	24* (11)	35** (17**)

*: $P < 0.05$; **: $P < 0.01$ (Fisher's exact test)
[a] Figures are fetuses affected; affected litters are given in parentheses.
Source: Whitlow (2011)

The NOAEL for maternal toxicity was 15 mg/kg bw per day, based on reduced body weight and clinical signs of toxicity at 30 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 15 mg/kg bw per day, based on lower litter weights, delayed ossification and increased incidences of anomalies at 30 mg/kg bw per day (Whitlow, 2011).

Rabbits

In a dose range–finding developmental toxicity study, benzovindiflupyr (purity 97%; batch no. SMU9BP005) was administered by oral gavage to groups of 10 female (approximately 6 months old) mated New Zealand White rabbits from GD 7 to GD 28. The dose levels were 0, 25, 50 and 100 mg/kg bw per day. Clinical observations, body weights and feed consumption were recorded. All rabbits were terminated at GD 29, and gross macroscopic examination of all internal organs, with emphasis on the uterus, uterine contents, position of fetuses in the uterus and number of corpora lutea, was performed. The uteri (and contents) with live fetuses were weighed. Fetuses were weighed individually and examined for gross external and visceral abnormalities.

The 100 mg/kg bw per day group was terminated at GD 14 because of severe body weight loss and low feed consumption. At 50 mg/kg bw per day, two animals were terminated on GD 18 and GD 20. They had severe body weight loss from GD 7 and low feed consumption from GD 10 onwards. One mid-dose female with severe body weight loss (−22%) aborted on GD 27. In this group, the mean body weight gain was lower than in control animals. Mean feed consumption in this group was slightly lower than that of the control group during GDs 10–13 and GDs 13–21, primarily due to

the females that were euthanized in extremis or that aborted. Mean feed consumption among surviving females was similar to that of the control group during GDs 21–29 and GDs 7–29. Fetal growth and survival were not affected by treatment at the low and middle doses. There were no visceral or skeletal malformations or variations at incidences higher than in controls.

In this dose range–finding study, the NOAEL for maternal toxicity was 25 mg/kg bw per day, based on decreased body weight gain at 50 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 50 mg/kg bw per day, the highest dose that was evaluable (Coder, 2011a).

In a developmental toxicity study, benzovindiflupyr (purity 97%; batch no. SMU9BP005) was administered by oral gavage to groups of 25 female (approximately 5.5 months old) mated Hra:(NZW)SPF rabbits from GD 7 to GD 28. The dose levels were 0, 10, 20 and 35 mg/kg bw per day. Clinical observations, body weights and feed consumption were recorded. All rabbits were terminated at GD 29, and gross macroscopic examination of all internal organs, with emphasis on the uterus, uterine contents, position of fetuses in the uterus and number of corpora lutea, was performed. The uteri (and contents) with live fetuses were weighed. Fetuses were weighed individually and examined for external and visceral abnormalities.

At all dose levels, body weight gain was dose-relatedly lower, statistically significantly at the middle and high doses from GD 13 to GD 21. This effect was observed from the beginning of treatment at 35 mg/kg bw per day and at the lower dose levels from the 2nd week of treatment. From GD 23 onwards, all dosed groups had higher body weight gains compared with controls. At GD 29, body weights of all groups were similar. Although the incidence of late resorptions in the 35 mg/kg bw per day group was statistically significantly higher (2.5% per litter) than in the concurrent control group (0.0% per litter), it was within the range of historical control data (0.0–5.1% per litter). There were no further treatment-related effects on fetal growth or development.

The NOAEL for maternal toxicity was 10 mg/kg bw per day, based on decreased body weight gain at 20 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 35 mg/kg bw per day, the highest dose tested (Coder, 2011b).

2.6 Special studies

(a) Neurotoxicity

Rats

In an acute neurotoxicity study, a single dose of benzovindiflupyr (purity 97%; batch no. SMU9BP005) was administered by gavage to non-fasted groups of 10 male and 10 female 7-week-old RCCHan:WIST(SPF) rats. The dose levels were 0, 10, 30 and 80 mg/kg bw. Animals were observed for 14 days. Detailed clinical observations, body weights and feed consumption were recorded weekly. A functional observational battery was performed and locomotor activity was recorded at days 1, 7 and 14. At day 15, animals were killed, and the brains were weighed and preserved together with spinal cord and samples from the peripheral nervous system for microscopic examination.

In females at 30 and 80 mg/kg bw, feed intake was slightly lower than in controls on days 1 and 2, and body weight gain in the 1st week was slightly lower than in controls. On day 1 at 1 hour post-dosing, decreased activity was noted in 3/10 males and 10/10 females at 80 mg/kg bw and in 5/10 females at 30 mg/kg bw. All 80 mg/kg bw females exhibited swaying gait, abnormal gait, collapse and ruffled fur (Table 9). On day 2, treatment-related clinical signs were limited to the 80 mg/kg bw group and consisted of a swaying gait in 4/10 females, decreased activity in 3/10 females, abnormal gait in 3/10 females and ruffled fur in 5/10 females. On days 3–14, no treatment-related daily observations were noted. On day 1 in females at 30 mg/kg bw and in males and females at 80 mg/kg bw, body temperature was statistically significantly lower than in controls. In females at 30 mg/kg bw and above, forelimb grip strength was reduced on day 1. In females at 30 and 80 mg/kg bw, locomotor activity was reduced on day 1. One 80 mg/kg bw male was noted with single nerve fibre degeneration (minimal severity) in the proximal tibial nerve, and another 80 mg/kg bw male was

noted with single nerve fibre degeneration (minimal severity) in the dorsal root ganglion nerve. Both of these findings were within the range of normal background incidence and therefore are considered not treatment related.

Table 9. Daily clinical signs in female rats in an acute neurotoxicity study

	Incidence of finding (n = 10 per group)[a]			
	0 mg/kg bw per day	10 mg/kg bw per day	30 mg/kg bw per day	80 mg/kg bw per day
Decreased activity	0	0	5 (0)	10 (3)
Swaying gait	0	0	0	10 (4)
Collapse	0	0	0	10 (0)
Abnormal gait	0	0	0	10 (3)

[a] Figures are animals affected on day 1; affected animals on day 2 are given in parentheses.
Source: Sommer (2011b)

The NOAEL for systemic effects was 10 mg/kg bw, based on clinical signs of toxicity at 30 mg/kg bw. The NOAEL for acute neurotoxicity was 80 mg/kg bw, the highest dose tested (Sommer, 2011b).

In a subchronic neurotoxicity study, benzovindiflupyr (purity 97%; batch no. SMU9BP005) was administered for 90 days in the diet to groups of 12 male and 12 female 7-week-old RccHan:WIST (SPF) rats. The concentrations in feed were 0, 100, 400 and 800 ppm (equal to 0, 6.31, 25.95 and 50.67 mg/kg bw per day, respectively) for males and 0, 100, 250 and 500 ppm (equal to 0, 7.48, 19.17 and 37.99 mg/kg bw per day, respectively) for females. The eyes of all animals were examined pre-study, and the eyes of the control and high-dose groups were examined during the week prior to termination. Detailed clinical observations and body weights were recorded weekly, and feed consumption was recorded twice weekly. A functional observational battery was performed and locomotor activity was recorded in weeks 2, 5, 9 and 13. At week 13, animals were killed, and the brains were weighed and preserved together with spinal cord and samples from the peripheral nervous system for microscopic examination.

Mean overall feed consumption was reduced in male and female rats at 800/500 ppm, in males by 4.1% and in females by 10.3%. Females at 100 and 250 ppm also showed reduced feed intake by 4.6%. Mean overall body weight gain was reduced by 12.7% and 14.9% in males at 400 and 800 ppm, respectively, and by 9.8% in females at 500 ppm. No other treatment-related effects were observed.

The NOAEL for systemic toxicity was 100 ppm (equal to 6.31 mg/kg bw per day), based on decreased body weight gain in males at 400 ppm (equal to 25.95 mg/kg bw per day). The NOAELs for subchronic neurotoxicity were 800 ppm (equal to 50.67 mg/kg bw per day) in males and 500 ppm (equal to 37.99 mg/kg bw per day) in females, the highest doses tested (Sommer, 2011a).

(b) Mode of action of thyroid tumours in rats

Benzovindiflupyr (purity 97%; batch no. SMU9BP005) was administered for up to 14 days in the diet to groups of 60 male 6- to 7-week-old Crl:WI(Han) rats. Fifteen additional animals in the control and high-dose groups were kept as 63-day recovery groups. The concentrations in feed were 0, 100, 600 and 1200 ppm (equal to 0, 9.9, 57.7 and 112.8 mg/kg bw per day, respectively). Phenobarbital (PB) sodium salt as positive control was administered at 1200 ppm to a group of 60 male rats for up to 14 days. After 1, 3, 7 and 14 days of treatment, 15 animals were killed 24 hours after the last treatment. Two hours before termination, animals subcutaneously received radioactively

labelled 5-bromo-2′-deoxyuridine (BrdU). Clinical observations, body weight development and feed intake were recorded regularly. Blood samples were taken from animals at the day of scheduled termination and analysed for thyroxine (T_4), triiodothyronine (T_3) and thyroid stimulating hormone (TSH). At terminal sacrifice, animals were subjected to detailed necropsy, and thyroids and livers were weighed and examined histologically.

There were no clinical signs of toxicity in the benzovindiflupyr groups. Animals in the 600 and 1200 ppm groups had lower body weight gains from the 1st day of treatment onwards. The 1200 ppm recovery group had a slightly higher body weight gain than controls; hence, at the end of the recovery period, body weights were similar to those of controls. At day 15, 100 and 600 ppm males had statistically lower mean serum T_3 levels; at 1200 ppm, T_3 was lower from day 2 onwards. T_4 was lower only during the first 4 days of treatment at 1200 ppm. TSH was elevated only at day 15 in the 1200 ppm group. All values in the recovery group were similar to the control values. Treatment with 1200 ppm PB sodium salt resulted in decreases in mean T_3 and T_4 levels and increased mean serum TSH levels after 14 days of treatment. Relative liver weights were statistically significantly increased from day 4 onwards at 1200 ppm benzovindiflupyr and from day 8 onwards in the 600 ppm group. At day 15, relative thyroid weights were increased, although not statistically significantly. In the recovery group, organ weights were similar between the 1200 ppm group and the control group. From day 8 onwards, hepatocellular hypertrophy was observed in the 600 ppm and the 1200 ppm groups. In the PB group, hepatocellular hypertrophy was noted from day 4 onwards; at day 15, minimal follicular cell hypertrophy in the thyroid was also found. In the recovery group, no histological difference was noted compared with control animals. Benzovindiflupyr at 1200 ppm resulted in the induction of hepatic microsomal uridine diphosphate–glucuronosyltransferase (UDPGT) activity towards T_4 as substrate on days 4, 8 and 15. UDPGT activity was increased at 600 ppm on days 4 and 15 and at day 4 in the 100 ppm group. Treatment with 100, 600 or 1200 ppm also produced increases in hepatic microsomal protein content. The increases in hepatic microsomal protein content and UDPGT activity observed after 14 days of treatment at 1200 ppm were fully reversible after 63 days of recovery. Treatment with 1200 ppm PB sodium salt as a positive control resulted in increases in hepatic microsomal protein content and the induction of UDPGT activity on days 4, 8 and 15. On day 15, thyroid follicular cell proliferation was statistically significantly increased to 479% and 471% of the concurrent control value in the 1200 ppm benzovindiflupyr or 1200 ppm PB sodium salt groups. In animals treated with 1200 ppm benzovindiflupyr for 14 days followed by a 63-day recovery period, thyroid follicular cell proliferation was similar to that in the concurrent control (Robertson, 2012a).

(c) Immunotoxicity

Benzovindiflupyr (purity 97.7%; batch no. SMU0FP003) was administered in the diet to groups of 10 female 8-week-old Crl:CD1(ICR) mice for 28 days. The concentrations in feed were 0, 100, 200 and 400 ppm (equal to 0, 26.4, 47.1 and 97.1 mg/kg bw per day, respectively). Cyclophosphamide as immunosuppressant was administered at 50 mg/kg bw per day from day 24 to day 27 to a group of 10 animals on basal diet. Animals were immunized with an intravenous injection of sheep red blood cells on study day 24. Clinical observations, body weight development and feed intake were recorded regularly. Blood samples were collected at the scheduled termination, and liver, spleen and thymus were weighed. Spleen cell suspensions were prepared, spleen cell counts were performed and the number of specific immunoglobulin M (IgM) antibody-forming cells directed towards the sheep red blood cell antigen were determined to measure the humoral immune response using the splenic antibody-forming cell assay.

All animals survived to the scheduled necropsy. Body weight loss was noted in the 400 ppm group on study day 3; body weight gains were generally similar to those of the vehicle control group for the remainder of the study. There were no treatment-related macroscopic findings or effects on organ weights or feed consumption. There were no significant effects on spleen cell number or on the humoral immune response of splenic IgM to the T cell–dependent antigen sheep red blood cells.

For the positive control group administered cyclophosphamide, statistically significantly lower spleen weight, spleen cell numbers, specific activity and total spleen activity of IgM antibody-forming cells were noted when compared with the vehicle control group (Wasil, 2012).

(d) Hepatic UDPGT activity in male rats

Hepatic UDPGT activity with T_4 as substrate was analysed in liver samples from male rats administered benzovindiflupyr in the diet at 0, 100, 750 or 1500 ppm for periods of 3, 7, 14 and 28 days (Robertson, 2010b). For comparison, liver samples of male rats treated with PB sodium salt for 7 days from another study (Robertson, 2012a) were also analysed for hepatic UDPGT activity with T_4 as substrate.

Treatment with 100 ppm benzovindiflupyr for 3, 7, 14 and 28 days had no significant effect on hepatic microsomal protein content and UDPGT activity with T_4 as substrate. After treatment at 750 and 1500 ppm for 7 days, hepatic microsomal protein content was significantly increased to 122% and 136% of control values, respectively. After 3, 7 and 14 days, hepatic UDPGT activity with T_4 as substrate was significantly increased to 189%, 199% and 211% of control values, respectively, by treatment with 750 ppm benzovindiflupyr and to 182%, 297% and 201% of control values, respectively, by treatment with 1500 ppm benzovindiflupyr. After 28 days of treatment, UDPGT activity was significantly increased to 243% of the control value by treatment with 1500 ppm benzovindiflupyr. The treatment of male rats with 1200 ppm PB sodium salt for 7 days significantly increased hepatic microsomal protein content to 150% of the control value. Hepatic UDPGT activity per gram of liver was significantly increased to 177% of the control value (Lake, 2012a).

(e) In vitro male rat thyroid peroxidase inhibition assay

Benzovindiflupyr (purity 97%; batch No. SMU9BP005) concentrations of 0.01, 0.1, 1.0 and 10 µmol/L were assayed for in vitro thyroid peroxidase inhibitory activity in male Wistar Han rats. A pooled thyroid gland microsomal preparation from five rats was assayed for thyroid peroxidase activity by determining the monoiodination of L-tyrosine. As a positive control, the effect of 6-propyl-2-thiouracil (10 µmol/L) on rat thyroid peroxidase activity was also determined.

Benzovindiflupyr had no significant effect on rat thyroid peroxidase activity at any concentration tested. 6-Propyl-2-thiouracil resulted in 100% inhibition of thyroid peroxidase activity (Lake, 2012b).

(f) Studies with metabolites SYN546039 and SYN545720

SYN546039 (CSCD695908; racemic mixture of 3-difluoromethyl-1-methyl-1*H*-pyrazole-4-carboxylic acid: [(1*S*,2*S*,4*R*)-9-dichloromethylene-2-hydroxy-1,2,3,4-tetrahydro-1,4-methano-naphthalene-5-yl]-amide and [(1*R*,2*R*,4*S*)-9-dichloromethylene-2-hydroxy-1,2,3,4-tetrahydro-1,4-methano-naphthalene-5-yl]-amide) and SYN545720 (CSCD465008; 3-(difluoromethyl)-1*H*-pyrazole-4-carboxylic acid) were investigated in acute and subacute or subchronic toxicity studies and genotoxicity studies. SYN546039 was also investigated in a developmental toxicity study in rabbits. The metabolite structures are illustrated in Fig. 6. SYN546039 was found in rats, plants, soil and surface water, and SYN545720 was found in plants and soil.

Fig. 6. Structures of metabolites SYN546039 (CSCD695908) and SYN545720 (CSCD465008)

SYN546039
CSCD695908

SYN545720
CSCD465008

SYN545720 (CSCD465008)

Acute toxicity. The acute oral toxicity of SYN545720 (purity 94.0 ± 2%; batch no. MES-103/1) at 2000 mg/kg bw was tested in five female HanRCC Wistar rats. No mortalities were seen, but ruffled fur, slight sedation and hunched posture were observed within the first few hours after treatment. The LD_{50} of CSCD465008 in female rats was greater than 2000 mg/kg bw (Simon, 2008).

Subchronic toxicity. SYN545720 (purity 94.0 ± 2%; batch no. MES-103/1) was administered for 28 days in the diet to groups of five male and five female 7-week-old Crl:WI(Han) rats. The concentrations in feed were 0, 2000, 6000 and 12 000 ppm (equal to 0, 175, 497 and 1018 mg/kg bw per day for males and 0, 176, 525 and 1107 mg/kg bw per day for females, respectively). Clinical observations were recorded daily, and body weights and feed consumption were recorded weekly. At week 3, all animals were subjected to a functional observational battery, and locomotor activity was monitored within the detailed clinical observation. In week 3, all animals underwent an ophthalmic examination. At study termination, haematology, urine and clinical chemistry parameters were analysed. Organs of all animals were examined macroscopically and weighed. A range of tissues from control and high-dose animals was examined histologically. Livers and duodenum were investigated microscopically, and the ethoxyresorufin *O*-deethylase (EROD; cytochrome P450 [CYP] 1A1/2) and pentoxyresorufin *O*-depentylase (PROD; CYP2B1/2) activities of liver microsomes were analysed. Hepatic microsomes from β-naphthoflavone-treated (100 mg/kg bw for 4 days) and PB sodium salt–treated (80 mg/kg bw for 4 days) male and female Sprague-Dawley rats were used as positive controls.

None of the investigated parameters showed any treatment-related changes, and no induction of CYP1A or CYP2B activity was noted (Table 10).

Table 10. CYP1A and CYP2B activities in hepatic microsomes of male and female rats treated with SYN545720

Treatment	Activity relative to control (fold change)			
	CYP1A		CYP2B	
	Males	Females	Males	Females
2 000 ppm SYN545720	0.9	0.9	1.1	1.0
6 000 ppm SYN545720	0.7	0.8	0.9	0.9
12 000 ppm SYN545720	0.7	0.9	1.2	1.0
PB sodium salt (80 mg/kg bw)	2.8	2.8	23.1	53.8
β-Naphthoflavone (100 mg/kg bw)	11.9	15.9	1.7	4.9

Source: Walraven (2008)

The NOAEL was 12 000 ppm (equal to 1018 mg/kg bw per day), the highest dose tested (Walraven, 2008).

Genotoxicity in vitro. SYN545720 (purity 94.0 ± 2%; batch no. MES-103/1) was tested for genotoxicity in a range of guideline-compliant assays in vitro. No evidence for genotoxicity was observed in any test (Table 11).

SYN546039 (CSCD695908)

Acute toxicity. The acute oral toxicity of SYN546039 (purity 98%; batch no. MES 139/4) at dose levels of 175, 550 and 2000 mg/kg bw was tested in one female RccHan:WIST rat at each of 175 and 550 mg/kg bw and in three animals at 2000 mg/kg bw. Ruffled fur was observed in the first 3 days after treatment. There were no further signs of toxicity. The LD_{50} of SYN546039 in female rats was greater than 2000 mg/kg bw (Sieber, 2011).

Table 11. Results of genotoxicity studies with SYN545720

End-point	Test system	Concentration	Batch no.; purity	Result	Reference
Reverse mutation (Ames)	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537; *Escherichia coli* WP2, WP2 uvrA	3–5 000 µg/plate	MES-103/1; 94%	Negative	Sokolowski (2008)
Mouse lymphoma TK	L5178Y cell line	110–1 760 µg/mL	MES-103/1; 94%	Negative	Wollny (2008)
Chromosomal aberration	Human lymphocytes	574.7–1 760 µg/mL	MES-103/1; 94%	Negative	Bohnenberger (2008)

TK: thymidine kinase

Subchronic toxicity. SYN546039 (purity 98.5%; batch no. L80-26) was administered for 90 days in the diet to groups of 10 male and 10 female 6-week-old Crl:WI(Han) rats. The concentrations in feed were regularly adjusted to provide 0, 100, 300 and 1000 mg/kg bw per day to males and females. Detailed clinical observations and body weights and feed consumption were recorded weekly. At study termination, all animals were subjected to a functional observational battery, locomotor activity monitoring and an ophthalmic examination. At study termination, haematology, urine and clinical chemistry parameters were analysed. Organs of all animals were examined macroscopically and weighed. Organs from the control and high-dose groups were investigated microscopically.

In all examined parameters, there were no treatment-related effects.

The NOAEL was 1000 mg/kg bw per day, the highest dose tested (Kaspers, 2009).

Developmental toxicity. In a developmental toxicity study, SYN546039 (purity 99.3%; batch no. L80-66) was administered by oral gavage from GD 6 to GD 28 to groups of 60 female mated 14- to 16-week-old Crl:KBL(NZW) rabbits in the control group, 25 animals in the low-dose and mid-dose groups and 35 animals in the high-dose group. The dose levels were 0, 100, 300 and 1000 mg/kg bw per day. Clinical observations, body weights and feed consumption were recorded. All rabbits were terminated at GD 29; macroscopic examination of the uterus, uterine contents and positions of fetuses in the uterus was performed, and the number of corpora lutea was recorded. The uteri (and contents) with live fetuses were weighed. Fetuses were weighed individually and examined for gross external, visceral and skeletal abnormalities.

In high-dose animals, signs of severe toxicity and an increased incidence of mortalities and abortions were noted (Table 12). The body weight development of dams was not affected by treatment, but the feed consumption in high-dose animals was slightly lower than in controls. At necropsy, the incidence of findings was increased in the high-dose group and was mostly related to the gastrointestinal tract. The incidences of external and visceral malformations and variations were similar. The incidence of misshapen interparietal skull bone was increased at 1000 mg/kg bw per day above the concurrent and the historical control incidence. Additionally, the incidence of malformed vertebral column and/or ribs was increased above the concurrent and the historical control incidence at the middle and the high doses.

The maternal NOAEL was 300 mg/kg bw per day, based on an increased incidence of mortalities and abortions at 1000 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 300 mg/kg bw per day, based on the increased incidence of misshapen interparietal skull bone at 1000 mg/kg bw per day (Schneider, 2009).

Genotoxicity in vitro. SYN546039 (purity 98%; batch no. MES 139/4) was tested in vitro at concentrations up to 5000 µg/plate for reverse mutagenicity in an Ames test in *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 and in *Escherichia coli* strains WP2 and WP2 uvrA. No evidence for genotoxicity was observed (Sokolowski, 2011b).

Table 12. Findings in the developmental toxicity study in rabbits with SYN546039

	0 mg/kg bw per day	100 mg/kg bw per day	300 mg/kg bw per day	1000 mg/kg bw per day	Historical control data
No. of animals	60	25	25	35	–
No. died or sacrificed moribund	0	1	0	5	–
No. not pregnant	14	6	8	5	–
No. of abortions	2	1	0	5	–
No. of litters	41	17	17	18	–
No. of fetuses	351	153	152	138	–
Incidence of misshapen interparietal skull bone (%)	0.3	0.7	0.7	2.2	0.5 (0.0–1.5)
Incidence of malformed vertebral column and/or ribs (%)	0.3	0.0	2.6	0.7	0.1 (0.0–0.5)
Incidence of incomplete ossification of cervical centrum (% fetuses affected/litter)	35.0	37.0	31.2	50.5	8.4 (0.0–29.0)

Source: Schneider (2009)

3. Observations in humans

No reports were submitted.

Comments

Biochemical aspects

In ADME studies, overnight-fasted animals showed clinical signs at doses that were non-toxic to fed animals. Therefore, most of the ADME studies were performed in fed animals. Absorption of benzovindiflupyr was approximately 80% at the low dose (1 mg/kg bw) and showed saturation at the high dose (approximately 60% absorption at 40 mg/kg bw). Peak levels of radiolabel occurred in plasma within 2–4 hours (1 mg/kg bw) or within 6–24 hours (40 mg/kg bw) post-dosing. Peak levels were approximately 2-fold higher in males than in females. The apparent plasma half-life of the labelled material in rats was 28–55 hours. In low-dose animals, 4% was found in urine and 17% in faeces; in high-dose animals, 9% was found in urine and 32% in faeces. However, at both dose levels, 86–97% of the absorbed dose was excreted in bile duct–cannulated rats within 48 hours after administration. For tissues, the elimination half-lives were in the range of 40–316 hours. Highest residues were identified in the liver, kidney, adrenals, thyroid and heart. The major route of excretion was by bile, accounting for approximately 69–76% of the administered dose at the low dose and 47–57% at the high dose. After repeated daily dosing, levels of radioactivity in tissues appeared to have reached steady-state concentrations after 14 days. The predominant metabolic pathway for benzovindiflupyr is *N*-demethylation, phenyl and/or bicyclo hydroxylation and opening of the bicyclo system. Additionally, subsequent formation of glucuronic acid or sulfate conjugates was observed. The amide bond of benzovindiflupyr is preserved.

Toxicological data

The oral LD_{50} was 55 mg/kg bw in fasted female rats. The dermal LD_{50} was greater than 2000 mg/kg bw, and the LC_{50} in an inhalation study was 0.56 mg/L. Benzovindiflupyr was mildly irritating to the skin and moderately irritating to the eyes. Benzovindiflupyr showed no skin sensitizing potential in a mouse local lymph node assay.

In repeated-dose toxicity studies in mice, rats and dogs, the main effects were on the liver (hepatocellular hypertrophy, increased organ weight) and body weight, with minor changes in clinical chemistry parameters.

In a 4-week mouse feeding study with dietary concentrations of 0, 100, 300 and 500 ppm (equal to 0, 15.6, 47.4 and 81.8 mg/kg bw per day for males and 0, 19.0, 57.9 and 91.5 mg/kg bw per day for females, respectively), body weight at 300 ppm was reduced by 7% in the absence of reduced feed consumption. Both sexes showed tubular basophilia with interstitial inflammatory cell infiltration at 500 ppm. The NOAEL was 100 ppm (equal to 15.6 mg/kg bw per day), based on reduced body weight at 300 ppm (equal to 47.4 mg/kg bw per day).

In a 13-week mouse feeding study with dietary concentrations of 0, 100, 300 and 500 ppm (equal to 0, 17.0, 55.6 and 97.9 mg/kg bw per day for males and 0, 20.9, 59.6 and 102.8 mg/kg bw per day for females, respectively), 3 of 13 males showing severe toxic effects, such as body weight loss, at 500 ppm were terminated early. Reduced body weight gains without reduced feed consumption were noted from the 1st day of treatment at 300 and 500 ppm. Body weights at study termination were lower compared with controls by 14% at 300 ppm and by 25% at 500 ppm in males and by 9% and 15% in females, respectively. At 300 ppm and above, some changes in clinical chemistry parameters were observed, including globulin, triglyceride, creatinine and calcium levels. The incidence of tubular basophilia in the kidney in females was increased at 500 ppm. The NOAEL was 100 ppm (equal to 17.0 mg/kg bw per day), based on reduced body weight gains and minor clinical chemistry changes at 300 ppm (equal to 55.6 mg/kg bw per day).

In a 4-week rat feeding study with dietary concentrations of 0, 100, 400 and 1200 ppm (equal to 0, 9, 36 and 107 mg/kg bw per day for males and 0, 9, 36 and 90 mg/kg bw per day for females, respectively), the NOAEL was 400 ppm (equal to 36 mg/kg bw per day), based on lower body weights in both sexes at 1200 ppm (equal to 90 mg/kg bw per day).

In a 13-week rat feeding study with dietary concentrations of 0, 100, 750 and 1500 ppm (equal to 0, 8.2, 58.8 and 108.8 mg/kg bw per day for males and 0, 9, 36 and 90 mg/kg bw per day for females, respectively), the NOAEL was 100 ppm (equal to 8.2 mg/kg bw per day), based on reduced body weight gain at 750 ppm (equal to 58.8 mg/kg bw per day).

In studies in which dogs were administered benzovindiflupyr by gelatine capsule gavage for 13 weeks (0, 30, 375 and 750 mg/kg bw per day) and 52 weeks (0, 25, 250 and 500 mg/kg bw per day), the major effects were salivation and vomiting starting at the end of the 1st week and reduced body weight gain, with an overall NOAEL of 30 mg/kg bw per day and an overall lowest-observed-adverse-effect level (LOAEL) of 250 mg/kg bw per day.

In an 18-month feeding study in mice with dietary concentrations of 0, 20, 60 and 200 ppm (equal to 0, 2.62, 7.55 and 26.18 mg/kg bw per day for males and 0, 2.89, 8.67 and 29.26 mg/kg bw per day for females, respectively), high-dose males and females had higher incidences of colon and caecum mucosal hyperplasia. There was no compound-related increase in tumours. The NOAEL was 60 ppm (equal to 7.55 mg/kg bw per day), based on colon/caecum mucosal hyperplasia in both sexes at 200 ppm (equal to 26.18 mg/kg bw per day).

The Meeting concluded that benzovindiflupyr is not carcinogenic in mice.

In a 24-month feeding study in rats with dietary concentrations of 0, 25, 100 and 600 ppm in males (equal to 0, 1.2, 4.9 and 30.2 mg/kg bw per day, respectively) and 0, 25, 100 and 400 ppm in females (equal to 0, 1.7, 6.7 and 27.4 mg/kg bw per day, respectively), body weights and feed efficiency were lower from the 1st week onwards in both sexes at the high dose level. In high-dose females, some red blood cell parameters were slightly, but statistically significantly, reduced in the 1st year, and prothrombin time was slightly, but statistically significantly, shortened at week 79 only. The incidence of minimal hepatocellular centrilobular pigmentation was statistically significantly increased in females at 400 ppm at terminal kill. Centrilobular hepatocellular hypertrophy was increased in mid- and high-dose males and high-dose females; additionally, the incidence of eosinophilic foci of cellular alteration in liver was increased in high-dose males. In males at 600 ppm, the incidence of thyroid follicular cell adenoma was statistically significantly increased compared

with the concurrent control group. The incidences were 2% at 0 ppm, 7.6% at 25 ppm, 9.6% at 100 ppm and 17.3% at 600 ppm. The incidence for males at 600 ppm was also higher than the historical control incidence range (2–11.1%). The NOAEL for systemic toxicity and carcinogenicity was 100 ppm (equal to 4.9 mg/kg bw per day), based on histological changes in the liver of males, an increased incidence of thyroid follicular cell adenoma in males and reduced body weight gain in both sexes at the high dose (600 ppm in males, equal to 30.2 mg/kg bw per day; and 400 ppm in females, equal to 27.4 mg/kg bw per day).

The Meeting concluded that benzovindiflupyr is carcinogenic in male rats at the highest dose tested.

The potential genotoxicity of benzovindiflupyr was tested in an adequate range of in vitro and in vivo studies, providing no evidence of genotoxic potential.

The Meeting concluded that benzovindiflupyr is unlikely to be genotoxic.

In a mechanistic study to investigate possible modes of action for the thyroid follicular cell adenomas, male rats were fed diets with 0, 100, 600 and 1200 ppm (equal to 0, 9.9, 57.7 and 112.8 mg/kg bw per day, respectively) for up to 14 days, and T_4, T_3 and TSH levels were measured at several time points. Reversible decreases in T_3 levels were noted at the high dose from day 2 onwards, and T_4 was reduced only in the first 4 days of treatment. TSH was elevated only in the 1200 ppm group at termination. Benzovindiflupyr at 1200 ppm induced UDPGT activity towards T_4 on days 4, 8 and 15. UDPGT activity was increased also at 600 ppm on days 4 and 15 and in the 100 ppm group on day 4. Treatment with 100, 600 or 1200 ppm also produced increases in hepatic microsomal protein content. Thyroid follicular cell proliferation was increased at 1200 ppm. In a further mechanistic study on UDPGT activity against T_4 in livers from benzovindiflupyr-treated rats, UDPGT activity was increased from the first measurement on treatment day 3 onwards at dose levels of 750 ppm and above. In an in vitro assay, benzovindiflupyr had no significant effect on rat thyroid peroxidase activity at concentrations up to 10 μmol/L.

Decreased T_4 levels, hepatocellular hypertrophy and increased thyroid follicular cell proliferation were identified in a mechanistic study as early events in male rats exposed to benzovindiflupyr. A mode of action for thyroid follicular cell adenoma based on decreased T_3/T_4 levels due to increased liver enzyme activity and a compensatory activity of the thyroid could be proposed. This would be supported by UDPGT activity, hepatocellular hypertrophy and reduced T_3/T_4, for which there was good dose concordance. However, elevated TSH levels were observed only at higher dose levels, and, apart from thyroid follicular cell adenoma, no histological changes in the thyroid were observed at 600 ppm in the rat carcinogenicity study. Therefore, this proposed mode of action is not completely supported by the data available. However, on the basis of the lack of genotoxicity, the absence of carcinogenicity in mice and the fact that only thyroid follicular cell adenomas were observed and that these were increased only in male rats, the Meeting concluded that benzovindiflupyr is unlikely to pose a carcinogenic risk to humans from the diet.

In a two-generation study of reproductive toxicity in rats at dietary concentrations of 0, 25, 100 and 600 ppm (equal to 0, 1.7, 6.8 and 40.5 mg/kg bw per day, respectively, in the premating period) for males and 0, 25, 100 and 250 ppm (equal to 0, 1.9, 7.6 and 17.5 mg/kg bw per day, respectively, in the gestation period) for females, body weight gain and feed consumption were reduced at 600/250 ppm in F_0 and F_1 males and females throughout premating and gestation. In the F_0 and F_1 250 ppm groups, the mean corpora lutea count at necropsy was statistically significantly lower than in controls. In the F_0 and F_1 males at 600 ppm and F_1 females at 250 ppm, the relative liver weight was higher than in controls. Centrilobular hepatocellular hypertrophy in high-dose males and hypertrophy in adrenal zona glomerulosa in high-dose females were observed. An increased number of animals in lactational diestrus was observed at 250 ppm in F_0 and F_1 females compared with controls. At 250 ppm, pup body weights at birth were lower in F_2 animals and postnatal body weight gains were lower in F_1 and F_2 animals compared with controls. The time until preputial separation and vaginal opening was statistically significantly longer in the high-dose animals. The NOAELs for parental and offspring toxicity were both 100 ppm (equal to 6.8 mg/kg bw per day), based on decreased body weight gain in parental animals and on lower postnatal body weight gain at 250 ppm

(equal to 17.5 mg/kg bw per day). The reproductive NOAEL was 250 ppm (equal to 17.5 mg/kg bw per day), the highest dose tested.

In a study on the developmental toxicity of benzovindiflupyr in rats at dose levels of 0, 7.5, 15 and 30 mg/kg bw per day administered by gavage, 14/24 high-dose animals showed ataxia, decreased activity, hunched and prostrate posture and ruffled fur from treatment days 6 to 16. High-dose animals showed reduced feed consumption from the 1st treatment day and reduced body weights from the 3rd treatment day onwards. High-dose litter weights were statistically significantly lower than those of controls. Five fetuses from five different high-dose litters showed soft tissue anomalies, with no clear pattern. The incidence of renal pelvis dilatation was statistically significantly increased on a litter basis at 30 mg/kg bw per day. The incidence of extrathymic tissues was increased statistically significantly at 15 and 30 mg/kg bw per day. As there is a high background incidence for this non-adverse variation, it was not used as a basis for the NOAEL. At 30 mg/kg bw per day, delayed ossification was noted in cervical vertebrae, sternebrae, proximal phalanges of digits and calcanei. The NOAEL for maternal toxicity was 15 mg/kg bw per day, based on reduced body weight and clinical signs of toxicity at 30 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 15 mg/kg bw per day, based on lower litter weights, delayed ossification and increased incidences of anomalies at 30 mg/kg bw per day.

In a study on the developmental toxicity of benzovindiflupyr in rabbits at dose levels of 0, 10, 20 and 35 mg/kg bw per day, body weight gain was lower in a dose-related manner at all dose levels, statistically significantly at the middle and high doses from GDs 13 to 21. This effect was observed from the beginning of treatment at 35 mg/kg bw per day and from the 2nd week of treatment at the lower dose levels. At GD 29, body weights of all groups were similar. Although the incidence of late resorptions in the 35 mg/kg bw per day group was statistically significantly higher than in the concurrent control group, it was within the range of historical control data. There were no further treatment-related effects on fetal growth or development. The NOAEL for maternal toxicity was 10 mg/kg bw per day, based on decreased body weight gain at 20 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 35 mg/kg bw per day, the highest dose tested.

The Meeting concluded that benzovindiflupyr is not teratogenic in rats or rabbits.

In an acute neurotoxicity study in rats administered benzovindiflupyr at a dose of 0, 10, 30 or 80 mg/kg bw, decreased locomotor activity was noted in females at 30 mg/kg bw and in males and females at 80 mg/kg bw at 1 hour post-dosing on the 1st day. All 80 mg/kg bw females also exhibited swaying gait, abnormal gait, collapse and ruffled fur. On days 3–14, no treatment-related daily observations were noted. On day 1 in females at 30 mg/kg bw and in males and females at 80 mg/kg bw, body temperature was statistically significantly lower than in controls. In females at 30 and 80 mg/kg bw, forelimb grip strength was reduced on day 1. The NOAEL for systemic effects was 10 mg/kg bw, based on clinical signs of toxicity at 30 mg/kg bw. The NOAEL for acute neurotoxicity was 80 mg/kg bw, the highest dose tested.

In a 13-week rat feeding study of the neurotoxicity of benzovindiflupyr with dietary concentrations of 0, 100, 400 and 800 ppm (equal to 0, 6.31, 25.95 and 50.67 mg/kg bw per day, respectively) for males and 0, 100, 250 and 500 ppm (equal to 0, 7.48, 19.17 and 37.99 mg/kg bw per day, respectively) for females, no effects were observed except for reduced feed intake and reduced body weight gain. The NOAEL for systemic toxicity was 100 ppm (equal to 6.31 mg/kg bw per day), based on decreased body weight gain in males at 400 ppm (equal to 25.95 mg/kg bw per day). The NOAELs for subchronic neurotoxicity were 800 ppm (equal to 50.67 mg/kg bw per day) in males and 500 ppm (equal to 37.99 mg/kg bw per day) in females, the highest doses tested.

The Meeting concluded that benzovindiflupyr is not neurotoxic.

In a 4-week mouse feeding study on immunotoxicity with dietary concentrations of 0, 100, 200 and 400 ppm (equal to 0, 26.4, 47.1 and 97.1 mg/kg bw per day, respectively), there were no significant effects on spleen cell number or on the humoral immune response of splenic IgM to the T cell–dependent antigen sheep red blood cells.

Toxicological data on metabolites and/or degradates

In studies of benzovindiflupyr, SYN546039 (CSCD695908; racemic mixture of 3-difluoro-methyl-1-methyl-1*H*-pyrazole-4-carboxylic acid: [(1*S*,2*S*,4*R*)-9-dichloromethylene-2-hydroxy-1,2,3,4-tetrahydro-1,4-methano-naphthalene-5-yl]-amide and [(1*R*,2*R*,4*S*)-9-dichloromethylene-2-hydroxy-1,2,3,4-tetrahydro-1,4-methano-naphthalene-5-yl]-amide) was found in rats, plants, soil and surface water, and SYN545720 (CSCD465008; 3-(difluoromethyl)-1*H*-pyrazole-4-carboxylic acid) was found in plants and soil.

SYN546039 and SYN545720 were both of low acute oral toxicity in rats, with LD_{50} values greater than 2000 mg/kg bw, and did not give any evidence of genotoxic potential.

In a 4-week rat feeding study with SYN545720 at dietary concentrations of 0, 2000, 6000 and 12 000 ppm (equal to 0, 175, 497 and 1018 mg/kg bw per day for males and 0, 176, 525 and 1107 mg/kg bw per day for females, respectively), no evidence of toxicity was observed. The NOAEL was 12 000 ppm (equal to 1018 mg/kg bw per day), the highest dose tested.

In a 13-week rat feeding study with SYN546039 at dietary concentrations yielding 0, 100, 300 and 1000 mg/kg bw per day, no evidence of toxicity was observed. The NOAEL was 1000 mg/kg bw per day, the highest dose tested.

In a study on the developmental toxicity of SYN546039 in rabbits at dose levels of 0, 100, 300 and 1000 mg/kg bw per day, the maternal NOAEL was 300 mg/kg bw per day, based on the increased incidence of mortalities and abortions at 1000 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 300 mg/kg bw per day, based on the increased incidence of misshapen interparietal skull bone at 1000 mg/kg bw per day.

Human data

No information was provided on the health surveillance of workers involved in the manufacture or use of benzovindiflupyr.

The Meeting concluded that the existing database on benzovindiflupyr was adequate to characterize the potential hazard to fetuses, infants and children.

Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) of 0–0.05 mg/kg bw, based on the NOAEL of 4.9 mg/kg bw per day in the 2-year rat feeding study for decreased body weight gain and increased incidences of eosinophilic foci of cellular alteration in the liver at 27.4 mg/kg bw per day. A safety factor of 100 was applied. The ADI is supported by the NOAEL of 7.55 mg/kg bw per day in the mouse 80-week feeding study, based on colon/caecum mucosal hyperplasia at 26.18 mg/kg bw per day. The margin between the upper bound of the ADI and the LOAEL of 30.2 mg/kg bw per day for thyroid follicular cell adenoma in male rats is approximately 600.

The Meeting established an acute reference dose (ARfD) of 0.1 mg/kg bw, derived from the NOAEL of 10 mg/kg bw in the acute neurotoxicity study in rats, on the basis of decreased locomotor activity at 1 hour post-dosing at the LOAEL of 30 mg/kg bw per day. A safety factor of 100 was applied. This ARfD is supported by the NOAEL of 15 mg/kg bw per day in a developmental toxicity study in rats with clinical signs at 30 mg/kg bw per day starting on the 1st day of treatment.

Levels relevant to risk assessment of benzovindiflupyr

Species	Study	Effect	NOAEL	LOAEL
Mouse	Eighteen-month study of toxicity and carcinogenicity[a]	Toxicity	60 ppm, equal to 7.55 mg/kg bw per day	200 ppm, equal to 26.18 mg/kg bw per day
		Carcinogenicity	200 ppm, equal to 26.18 mg/kg bw per day[b]	–
Rat	Acute neurotoxicity[c]	Toxicity	10 mg/kg bw	30 mg/kg bw
	Two-year study of toxicity and carcinogenicity[a]	Toxicity	100 ppm, equal to 4.9 mg/kg bw per day	400 ppm, equal to 27.4 mg/kg bw per day
		Carcinogenicity	100 ppm, equal to 4.9 mg/kg bw per day	600 ppm, equal to 30.2 mg/kg bw per day
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	250 ppm, equal to 17.5 mg/kg bw per day[b]	–
		Parental toxicity	100 ppm, equal to 6.8 mg/kg bw per day	250 ppm, equal to 17.5 mg/kg bw per day
		Offspring toxicity	100 ppm, equal to 6.8 mg/kg bw per day	250 ppm, equal to 17.5 mg/kg bw per day
	Developmental toxicity study[c]	Maternal toxicity	15 mg/kg bw per day	30 mg/kg bw per day
		Embryo and fetal toxicity	15 mg/kg bw per day	30 mg/kg bw per day
Rabbit	Developmental toxicity study[c]	Maternal toxicity	10 mg/kg bw per day	20 mg/kg bw per day
		Embryo and fetal toxicity	35 mg/kg bw per day[b]	–
Dog	Thirteen-week and 1-year studies of toxicity[c,d]	Toxicity	30 mg/kg bw per day	250 mg/kg bw per day
SYN545720				
Rat	Four-week study of toxicity[a]	Toxicity	12 000 ppm, equal to 1 018 mg/kg bw per day[b]	–
SYN546039				
Rat	Thirteen-week study of toxicity[a]	Toxicity	1 000 mg/kg bw per day[b]	–
Rabbit	Developmental toxicity study[c]	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	1 000 mg/kg bw per day

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

Estimate of acceptable daily intake

0–0.05 mg/kg bw

Estimate of acute reference dose

0.1 mg/kg bw

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

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

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

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid, 80% at low dose (1 mg/kg bw), 60% at higher dose (40 mg/kg bw)
Dermal absorption	No data (probably low, dermal LD_{50} high compared with oral LD_{50})
Distribution	Extensive, highest levels in liver
Potential for accumulation	No evidence of accumulation
Rate and extent of excretion	Rapid, 86–97% within 48 h, mainly via bile
Metabolism in animals	Extensive, primarily via hydroxylation at bicyclo and phenyl moieties
Toxicologically significant compounds in animals, plants and the environment	Benzovindiflupyr
Acute toxicity	
Rat, LD_{50}, oral	55 mg/kg bw
Rat, LD_{50}, dermal	> 2 000 mg/kg bw
Rat, LC_{50}, inhalation	0.56 mg/L
Rabbit, dermal irritation	Mildly irritating
Rabbit, ocular irritation	Moderately irritating
Dermal sensitization	No sensitizing potential (mouse local lymph node assay)
Short-term studies of toxicity	
Target/critical effect	Body weight (rat)
Lowest relevant oral NOAEL	8.2 mg/kg bw per day (rat)
Lowest relevant dermal NOAEL	No data
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Liver, body weight, thyroid
Lowest relevant NOAEL	4.9 mg/kg bw per day (rat)
Carcinogenicity	Unlikely to pose a carcinogenic risk from the diet
Genotoxicity	
	Not genotoxic
Reproductive toxicity	
Target/critical effect	No reproductive toxicity
Lowest relevant parental NOAEL	6.8 mg/kg bw per day
Lowest relevant offspring NOAEL	6.8 mg/kg bw per day
Lowest relevant reproductive NOAEL	17.5 mg/kg bw per day, the highest dose tested

Developmental toxicity	
Target/critical effect	Fetal weights and anomalies at maternally toxic doses
Lowest relevant maternal NOAEL	15 mg/kg bw per day (rat)
Lowest relevant embryo/fetal NOAEL	15 mg/kg bw per day (rat)
Neurotoxicity	
Acute and subchronic neurotoxicity	Not neurotoxic
Other toxicological studies	
Immunotoxicity	Not immunotoxic
Studies on metabolites	In oral studies, SYN546039 and SYN545720 less toxic than the parent
Medical data	
	No reports submitted

LC_{50}: median lethal concentration; LD_{50}: median lethal dose; NOAEC: no-observed-adverse-effect concentration; NOAEL: no-observed-adverse-effect level

Summary

	Value	Study	Safety factor
ADI	0–0.05 mg/kg bw	Two-year toxicity study in rats	100
ARfD	0.1 mg/kg bw	Acute neurotoxicity study in rats	100

ADI: acceptable daily intake: ARfD: acute reference dose

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BIXAFEN

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Explanation

Bixafen is the International Organization for Standardization–approved common name for *N*-(3′,4′-dichloro-5-fluorobiphenyl-2-yl)-3-(difluoromethyl)-1-methyl-1*H*-pyrazole-4-carboxamide (International Union of Pure and Applied Chemistry) (Chemical Abstracts Service No. 581809-46-3), a novel fungicide from the pyrazole-carboxamide class. Bixafen exhibits broad fungicidal activity in various crops by inhibition of succinate dehydrogenase, an enzyme of complex II within the mitochondrial respiration chain.

Bixafen 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 were certified as complying with good laboratory practice (GLP).

Evaluation for acceptable daily intake

Unless otherwise stated, the studies evaluated in this monograph were performed by laboratories that were certified for GLP and complied 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. As these guidelines specify the clinical pathology tests normally performed and the tissues normally examined, only significant exceptions to these guidelines are reported here, to avoid repetitive listing of study parameters.

1. Biochemical aspects

The studies on absorption, distribution, metabolism and excretion (ADME) of bixafen in rats were conducted using the radiolabels shown in Fig. 1.

Fig. 1. Structure of bixafen and position of radiolabels

[pyrazole-5-^{14}C]bixafen

[dichlorophenyl-U-^{14}C]bixafen

1.1 Absorption, distribution and excretion

In an ADME study conducted according to OECD Test Guideline 417, groups of male and female Wistar Unilever HsdCpb:WU rats were administered by oral gavage a suspension of [dichlorophenyl-U-^{14}C]bixafen (purity of non-radiolabelled test material > 99%; radiochemical purity > 98%) in 0.5% aqueous tragacanth. The dosing regimens and sacrifice times are presented in Tables 1 and 2. The rats were sacrificed 3 days after dosing except for the animals used for the bile duct cannulation investigations, which were sacrificed 2 days after dosing. The total radioactivity, which included the test item and metabolites, was determined in plasma samples, bile, urine and faeces, as well as in organs and tissues at sacrifice. The metabolism was investigated by radio–high-performance liquid chromatography (HPLC) and spectroscopic methods in selected bile and urine samples and in faecal extracts (Bongartz, 2008).

Recovery. Between 93.5% (group 4) and 106.6% (group 7) of the administered dose was recovered by measurement of the total radioactivity in plasma samples, bile, urine and faeces, as well as in organs and tissues at sacrifice. The radioactivity recovered as a percentage of the administered dose is given in Table 3.

Absorption. The absorption of bixafen started immediately after administration, as was shown by the concentration of radioactivity in plasma. The maximum plasma concentration (C_{max}) was reached approximately 2–8 hours after the administration (T_{max}). The absorption rate of [dichlorophenyl-U-^{14}C]bixafen was calculated from the recoveries in bile, urine and body, excluding gastrointestinal tract, and was 86.3% of the recovered dose for males (group 5) and 82.9% for females (group 7), leading to the conclusion of an almost complete absorption (Table 4). It is assumed that the absorbed dose becomes systemically available.

Table 1. Treatment schedule in the ADME rat study with [dichlorophenyl-U-^{14}C]bixafen

Group	Dose (mg/kg bw)	No. of animals	Sex	Duration (h)	Treatment
1	2	4	Male	72	Single low dose
2	2	4	Female	72	Single low dose
3	50	4	Male	72	Single high dose
4	50	4	Female	72	Single high dose
5	2	5	Male	48	Single low dose/bile duct cannulation
6	2	4	Male	72	Pretreatment (14 × non-radiolabelled and 1 × radiolabelled test item)
7	2	5	Female	48	Single low dose/bile duct cannulation

ADME: absorption, distribution, metabolism, excretion; bw: body weight
Source: Bongartz (2008)

Table 2. Sampling details in the ADME rat study with [dichlorophenyl-U-^{14}C]bixafen

Group	Urine (h)	Faeces (h)	Bile (h)	Organs (h)	Blood (min or h)
1	4, 8, 12, 24, 48 & 72	24, 48 & 72	–	72	Minutes: 10, 20, 40, 60 & 90 Hours: 2, 3, 4, 6, 8, 24, 28, 32, 48, 56 & 72
2	4, 8, 24, 48 & 72	24, 48 & 72	–	72	Minutes: 10, 20, 40, 60 & 90 Hours: 2, 3, 4, 6, 8, 24, 28, 32, 48, 56 & 72
3	4, 8, 24, 48 & 72	24, 48 & 72	–	72	Minutes: 10, 20, 40, 60 & 90 Hours: 2, 3, 4, 6, 8, 24, 28, 32, 48, 56 & 72
4	4, 8, 24, 48 & 72	24, 48 & 72	–	72	Minutes: 10, 20, 40, 60 & 90 Hours: 2, 3, 4, 6, 8, 24, 28, 32, 48, 56 & 72
5	4, 8, 24, 32 & 48	24 & 48	4, 8, 24, 32 & 48	48	Not performed
6	4, 8, 24, 48 & 72	24, 48 & 72	–	72	Minutes: 10, 20, 40, 60 & 90 Hours: 2, 3, 4, 6, 8, 24, 28, 32, 48, 56 & 72
7	4, 8, 24, 32 & 48	24 & 48	4, 8, 24, 32 & 48	48	Not performed

ADME: absorption, distribution, metabolism, excretion
Source: Bongartz (2008)

Distribution and plasma kinetics. The distribution of bixafen from the central compartment to the organs and tissues was followed by measuring the concentration of the total radioactivity in plasma. The pharmacokinetic parameters (two- or three-compartment modelling using the TOPFIT software) are given in Table 5.

The maximum equivalent concentration in plasma (C_{max}) ranged between 0.4 and 6.6 µg/mL, depending on the dose. The maximum was reached approximately 2–4 hours after dosing for the low-dose groups and the test with pretreated rats. In the high-dose groups, the maximum plasma concentration was observed approximately 8 hours after dosing.

The radioactivity in the plasma declined to approximately 1% of the maximum value of radioactivity within 72 hours post-administration. That indicated no retention of the compound-related residues in the body of the animals.

Table 3. Recovery of radioactivity in the ADME rat study (as a % of administered dose) with [dichlorophenyl-U-^{14}C]bixafen

	Recovery of radioactivity (% of administered dose)						
	2 mg/kg bw		50 mg/kg bw		2 mg/kg bw		
	Group 1 / male	Group 2 / female	Group 3 / male	Group 4 / female	Group 5 / male	Group 6 / male	Group 7 / female
Urine	1.41	2.87	0.69	1.67	0.71	1.92	0.83
Bile	ND	ND	ND	ND	83.04	ND	55.93
Faeces	92.37	91.25	98.45	91.39	7.41	104.0	6.26
Total excreted	93.78	94.12	99.14	93.06	91.16	105.92	63.02
Skin	0.028	0.201	0.002	0.028	0.387	0.014	4.073
Sum in organs	0.300	1.373	0.105	0.174	2.348	0.165	28.03
Body, excluding gastrointestinal tract	0.328	1.573	0.106	0.202	2.734	0.179	32.11
Gastrointestinal tract	0.202	1.377	0.031	0.207	6.341	0.142	11.46
Total in body	0.530	2.950	0.137	0.409	9.075	0.321	43.57
Total recovery	94.31	97.07	99.28	93.47	100.2	106.2	106.6

ADME: absorption, distribution, metabolism, excretion; bw: body weight; ND: not determined
Source: Bongartz (2008)

Table 4. Recovery of radioactivity in the ADME rat study (as a % of total radioactivity recovered) with [dichlorophenyl-U-^{14}C]bixafen

	Recovery of radioactivity (% of total radioactivity recovered)						
	2 mg/kg bw		50 mg/kg bw		2 mg/kg bw		
	Group 1 / male	Group 2 / female	Group 3 / male	Group 4 / female	Group 5 / male	Group 6 / male	Group 7 / female
Urine	1.50	2.96	0.69	1.78	0.71	1.81	0.78
Bile	ND	ND	ND	ND	82.86	ND	51.47
Faeces	97.94	94.00	99.17	97.78	7.39	97.89	5.84
Total excreted	99.44	96.96	99.86	99.56	90.96	99.70	58.09
Skin	0.030	0.208	0.002	0.030	0.385	0.013	3.960
Sum in organs	0.318	1.416	0.105	0.187	2.334	0.156	26.71
Body, excluding gastrointestinal tract	0.348	1.624	0.107	0.217	2.719	0.169	30.67
Gastrointestinal tract	0.212	1.419	0.031	0.224	6.322	0.133	11.25
Total in body	0.560	3.043	0.138	0.441	9.041	0.302	41.92
Absorption (sum of urine, bile and body, excluding gastrointestinal tract)					86.29	ND	82.92

ADME: absorption, distribution, metabolism, excretion; bw: body weight; ND: not determined
Source: Bongartz (2008)

Compared with the low-dose tests, the plasma concentration curves of the high-dose groups and the test with pretreated rats featured a very fast elimination phase at the beginning of the test (= $t_{½\ \text{elim}\ 1}$) followed by a slower terminal elimination phase (= $t_{½\ \text{elim}\ 2}$). Thus, calculations of plasma concentrations for these tests were performed with a three-compartment model by TOPFIT. Plasma concentrations of both low-dose groups showed one terminal elimination phase. They were calculated with a two-compartment model by TOPFIT.

Table 5. Pharmacokinetic parameters in the ADME rat study with [dichlorophenyl-U-^{14}C]bixafen

Parameter	2 mg/kg bw		50 mg/kg bw		2 mg/kg bw
	Group 1 / male	Group 2 / female	Group 3 / male	Group 4 / female	Group 6 / male
T_{max} (h), measured	2.0	4.0	8.0	8.0	2.0
T_{max} (h), calculated	2.03	5.06	5.18	9.15	2.66
C_{max} (µg/mL), measured	0.49	0.56	6.55	5.39	0.42
C_{max} (µg/mL), calculated	0.51	0.54	5.72	5.56	0.42
$t_{½ abs}$ (h)	0.46	0.61	0.52	0.05	0.19
$t_{½ elim 1}$ (h)	8.42	9.36	3.48	2.87	0.95
$t_{½ elim 2}$ (h)	ND	ND	21.7	6.31	25.0
$AUC_{0–∞}$ (mg/L·h)	7.3	14.3	82.6	139.0	5.1
MRT_{tot} (h)	12.9	19.3	12.1	18.3	12.1
MRT_{abs} (h)	0.72	9.34	5.14	9.31	8.07
MRT_{disp} (h)	12.10	9.92	6.95	9.02	4.03

abs: absorption; ADME: absorption, distribution, metabolism, excretion; AUC: area under the plasma concentration–time curve; bw: body weight; C_{max}: maximum equivalent plasma concentration; disp: disposition; elim: elimination; MRT: mean residence time; ND: not determined; $t_{½}$: half-life; T_{max}: time to reach C_{max}; tot: total

Source: Bongartz (2008)

Regarding all tests, there was a faster elimination of bixafen for males compared with females. Calculated area under the plasma concentration–time curve ($AUC_{0–∞}$) values for females were approximately twice as high as those for males. This indicates a higher systemic exposure for female rats. Ten times higher $AUC_{0–∞}$ values were calculated for the high-dose tests compared with the low-dose tests.

The mean residence time (MRT_{tot}) of bixafen-related radioactivity was short for all groups included in the study, ranging from 12 to 19 hours.

Excretion. For all treatments, the renal excretion was very low (< 2.9% of the administered dose). The major radioactivity (> 91% of the administered dose) was excreted via faeces. Bile duct–cannulated rats (low dose, 2 mg/kg body weight [bw]) showed a high excretion via bile, 83.0% for male rats and 55.9% for female rats (Table 3). For female bile duct–cannulated rats, a significant portion of radioactivity was still present in the body at the time of sacrifice (48 hours after administration).

The excretion was fast and almost complete 72 hours after administration. At this time, more than 93% of the administered dose had been excreted via urine and faeces in the tests with low, high and repeated doses. No significant dose-related differences were observed. During the first sampling period (0–24 hours), the excretion via faeces was approximately twice as fast for male rats as for female rats. Tests with bile duct–cannulated rats showed a total excretion of approximately 91% of the administered dose for male rats and approximately 63% for female rats (Tables 3 and 6).

Residues in organs and tissues at sacrifice. At sacrifice 72 hours after oral administration of bixafen (groups 1–4 and 6), between approximately 0.1% and 3.0% of the administered dose was found in the bodies (including gastrointestinal tract) of the rats. For bile duct–cannulated rats, approximately 9.1% (group 5, males) and 43.6% (group 7, females) were detected in the bodies (Table 3).

Table 6. Cumulative excretion of radioactivity in the ADME rat study with [dichlorophenyl-U-^{14}C]bixafen

Time (h)	Cumulative excretion of radioactivity (% of administered dose)						
	2 mg/kg bw		50 mg/kg bw		2 mg/kg bw		
	Group 1 / male	Group 2 / female	Group 3 / male	Group 4 / female	Group 5 / male	Group 6 / male	Group 7 / female
Urine							
4	0.21	0.12	0.10	0.09	0.04	0.24	0.03
8	0.42	0.37	0.17	0.19	0.15	0.54	0.12
12	0.61	ND	ND	ND	ND	ND	ND
24	1.19	1.67	0.57	0.85	0.47	1.59	0.36
32	ND	ND	ND	ND	0.53	ND	0.55
48	1.37	2.59	0.68	1.57	0.71	1.88	0.83
72	1.41	2.87	0.69	1.67	ND	1.92	ND
Faeces							
24	71.51	43.44	78.57	50.56	5.34	82.74	3.65
48	88.99	82.38	96.89	86.52	7.41	102.1	6.26
72	92.37	91.25	98.45	91.39	ND	104.0	ND
Bile							
4	ND	ND	ND	ND	11.05	ND	2.87
8	ND	ND	ND	ND	24.12	ND	7.05
24	ND	ND	ND	ND	67.23	ND	27.43
32	ND	ND	ND	ND	74.71	ND	36.27
48	ND	ND	ND	ND	83.04	ND	55.93
Total excreted	93.78	94.12	99.14	93.06	91.16	105.92	63.02

ADME: absorption, distribution, metabolism, excretion; bw: body weight; ND: not determined
Source: Bongartz (2008)

The highest equivalent concentrations were detected in the liver (approximately 0.05–0.84 μg/g) and the kidneys (approximately 0.01–0.20 μg/g) (Table 7). Higher concentrations were found in the perirenal fat and adrenal gland of female rats, especially in the high-dose tests (approximately 0.05–0.20 μg/g). The concentrations in the other organs and tissues were low and ranged between 0.001 and 0.09 μg/g for the low-dose tests and the test with pretreated rats and between 0.01 and 0.10 μg/g for the high-dose tests. From the renal and faecal excretion and from the elimination kinetics of total radioactivity from plasma, it was concluded that the small amounts of residual radioactivity in organs and tissues are subject to further elimination. Residues in the organs and tissues for female rats were in most cases higher than those for male rats (Table 7).

In a study on ADME conducted according to OECD Test Guideline 417, a group of four male Wistar Unilever HsdCpb:WU rats was administered by oral gavage a suspension of [pyrazole-5-^{14}C]bixafen (purity of non-radiolabelled test material > 99%; radiochemical purity > 99%) in 0.5% aqueous tragacanth at a single dose of 2 mg/kg bw. The rats were sacrificed 3 days after dosing. The total radioactivity, which included the parent compound and metabolites, was determined in plasma samples, urine and faeces, as well as in organs and tissues at sacrifice. The metabolism was investigated by radio-HPLC and spectroscopic methods in selected urine samples and in faecal extracts.

A total of 98.1% of the administered radioactivity was recovered from plasma, urine and faeces as well as organs and tissues at sacrifice (Table 8).

Table 7. Equivalent concentrations of residues in organs and tissues from the ADME rat study with [dichlorophenyl-U-^{14}C]bixafen

	Equivalent concentrations of residues (µg/g)						
	2 mg/kg bw		50 mg/kg bw		2 mg/kg bw		
	Group 1 / male	Group 2 / female	Group 3 / male	Group 4 / female	Group 5 / male	Group 6 / male	Group 7 / female
Erythrocytes	0.005 5	0.009 7	0.074 2	0.066 1	0.015 3	0.005 9	0.098 3
Plasma	0.002 7	0.009 8	0.053 9	0.050 6	0.017 6	0.001 8	0.135 7
Spleen	0.003 5	0.021 4	0.022 4	0.069 0	NA	0.003 0	NA
Liver	0.061 3	0.138 1	0.810 8	0.837 7	NA	0.052 3	NA
Kidney	0.012 3	0.053 5	0.099 6	0.203 1	NA	0.008 4	NA
Perirenal fat	0.005 0	0.052 0	0.024 9	0.115 5	NA	0.002 4	NA
Adrenal gland	0.008 7	0.088 2	0.051 9	0.199 3	NA	0.003 9	NA
Testis	0.001 8	NA	0.006 0	NA	NA	0.000 8	NA
Ovary	NA	0.038 5	NA	0.096 0	NA	NA	NA
Uterus	NA	0.019 8	NA	0.062 9	NA	NA	NA
Skeletal muscle	0.002 2	0.022 0	NC	0.043 1	NA	0.000 8	NA
Bone femur	0.001 9	0.008 4	NC	0.035 5	NA	0.000 9	NA
Heart	0.004 8	0.046 7	0.017 1	0.089 2	NA	0.001 9	NA
Lung	0.004 5	0.032 2	0.024 7	0.081 4	NA	0.002 2	NA
Brain	0.001 3	0.011 8	NC	0.025 1	NA	0.000 4	NA
Thyroid gland	0.009 9	0.040 0	NC	NC	NA	0.008 6	NA
Skin	0.002 2	0.017 3	NC	0.057 6	0.032 7	0.001 2	0.360 1
Carcass	0.004 2	0.033 6	0.012 1	0.065 6	0.074 1	0.001 4	0.879 5

ADME: absorption, distribution, metabolism, excretion; bw: body weight; NA: not analysed; NC: calculation of mean was not possible, as only one value was above the limit of detection
Source: Bongartz (2008)

Table 8. Recovery of radioactivity in the ADME study with [pyrazole-5-^{14}C]bixafen administered to rats at a dose of 2 mg/kg bw

	Recovery of radioactivity	
	% of dose administered	% of dose recovered
Urine	4.34	4.44
Faeces	93.37	95.20
Total excreted	97.71	99.64
Skin	0.030	0.031
Sum in organs	0.187	0.191
Body, excluding gastrointestinal tract	0.217	0.222
Gastrointestinal tract	0.134	0.137
Total in body	0.351	0.358
Balance	98.07	100

ADME: absorption, distribution, metabolism, excretion; bw: body weight
Source: Bongartz (2007)

The absorption of bixafen started immediately after administration, as shown by the concentration of radioactivity in the plasma. The C_{max} (0.42 µg/mL) was reached approximately 3 hours after administration (T_{max}). The distribution of the test substance from the central compartment to the different organs and tissues was followed by measuring the concentration of the total radioactivity in plasma. The pharmacokinetic parameters (two-compartment modelling using the TOPFIT software) are given in Table 9. There was a rapid elimination of the test item. The radioactivity in the plasma declined to approximately 0.5% of the maximum value of radioactivity within 72 hours post-administration. Plasma concentrations showed one terminal elimination phase (= $t_{½\ elim\ 1}$). Plasma concentrations were calculated with a two-compartment model by TOPFIT. The calculated $AUC_{0-\infty}$ value amounted to 6.5 h·mg/L. The mean residence time (MRT_{tot}) of bixafen-related radioactivity was short and amounted to 13 hours.

Table 9. Pharmacokinetic parameters in the ADME study with [pyrazole-5-^{14}C]bixafen administered to rats at a dose of 2 mg/kg bw

Parameter	Value
T_{max} (h), measured	3.0
T_{max} (h), calculated	2.4
C_{max} (µg/mL), measured	0.42
C_{max} (µg/mL), calculated	0.43
$t_{½\ abs}$ (h)	1.12
$t_{½\ elim\ 1}$ (h)	8.64
$AUC_{0-\infty}$ (h·mg/L)	6.5
MRT_{tot} (h)	13.3
MRT_{abs} (h)	0.88
MRT_{disp} (h)	12.5

abs: absorption; ADME: absorption, distribution, metabolism, excretion; AUC: area under the plasma concentration–time curve; bw: body weight; C_{max}: maximum equivalent plasma concentration; disp: disposition; elim, elimination; MRT: mean residence time; $t_{½}$: half-life; T_{max}: time to reach C_{max}; tot: total
Source: Bongartz (2007)

The renal excretion was very low (4.34% of the administered dose). The major radioactivity (93.4% of administered dose) was excreted via faeces (Table 8).

Low amounts of residue were determined in organs and tissues of the animals at sacrifice, 72 hours after the oral administration of a single dose of 2 mg/kg bw. A negligible amount of radioactivity was found in the gastrointestinal tract, showing that the elimination of the compound-related radioactivity was nearly complete. The highest equivalent concentration was detected in the liver (0.03 µg/g).The concentrations in the other organs and tissues were low and ranged between 0.001 and 0.009 µg/g (Table 10). From the renal and faecal excretion and from the elimination kinetics of total radioactivity from plasma, it may be concluded that the small amounts of residual radioactivity in organs and tissues are subject to further elimination (Bongartz, 2007).

In a study conducted according to OECD Test Guideline 417, the distribution of [dichlorophenyl-U-^{14}C]bixafen (purity of non-radiolabelled test material > 99%; radiochemical purity > 98%) in male rats was investigated by quantitative whole-body autoradiography using the radioluminography technique. Eight male Wistar HsdCpb:WU rats were orally administered a target dose of 3 mg/kg bw of radiolabelled bixafen suspended in 0.5% aqueous tragacanth. One control animal was administered the same dose of unlabelled test material and sacrificed at 4 hours after dosing. The distribution of residues in tissues was determined at 1, 4, 8, 24, 48, 72, 120 and 168 hours after administration.

Table 10. Radioactivity residues in organs and tissues from the ADME study with [pyrazole-5-^{14}C]bixafen administered to rats at a dose of 2 mg/kg bw

Organ or tissue	Equivalent concentration (µg/g)	% of administered dose
Erythrocytes	0.001 9	0.001 6
Plasma	0.001 7	0.001 0
Spleen	0.002 6	0.000 3
Liver	0.026 6	0.071 5
Kidney	0.006 6	0.002 9
Perirenal fat	0.003 3	0.000 9
Adrenal gland	0.008 3	0.000 1
Testis	0.001 7	0.001 2
Skeletal muscle	0.002 3	0.001 0
Bone femur	0.001 9	0.000 3
Heart	0.004 2	0.000 9
Lung	0.003 5	0.001 3
Brain	0.001 2	0.000 5
Thyroid gland	0.009 3	< 0.000 1
Skin	0.002 2	0.030 0
Carcass	0.003 5	0.103 6

ADME: absorption, distribution, metabolism, excretion; bw: body weight
Source: Bongartz (2007)

Bixafen was absorbed quickly from the gastrointestinal tract. The maximum concentration of radioactivity in almost all organs and tissues was detected 1 hour after administration. The absorbed radioactivity was distributed rather quickly and evenly in the body, with a preference for liver, kidney, fat (brown and perirenal fat) and infraorbital, Harderian and adrenal glands at the early time points (Table 11).

Radioactive residues in all organs and tissues decreased rapidly between 8 and 48 hours. In nearly all organs and tissues, residues were below the limit of detection (LOD) or limit of quantification (LOQ) at later time points between 72 and 168 hours after dosing. At the end of the test period, only liver, kidney and nasal mucosa showed negligible residues, which were all below 0.05 mg/kg.

There was no sign of a significant retention of radioactivity in specific organs or tissues. Residues in glandular organs or tissues responsible for hormonal regulation (e.g. testis, adrenal or thyroid gland) were rapidly depleted in parallel with the depletion from the other organs and tissues. This is evident from the ratio of the maximum concentration in plasma to the concentration after 24 hours. In all cases – except in nasal mucosa and vitreal body – the ratio was greater than 5, indicating that bioaccumulation following daily administration of the product is unlikely.

Bixafen was rapidly eliminated from the body, predominantly via faecal excretion. Excretion was nearly complete 48 hours after administration. No significant expiration of ^{14}C-labelled volatiles was observed (Spiegel, 2007).

In a study conducted according to OECD Test Guideline 417, the distribution of [pyrazole-5-^{14}C]bixafen (purity of non-radiolabelled test material > 99%; radiochemical purity > 98%) in male rats was investigated by quantitative whole-body autoradiography using the radioluminography technique. Eight male Wistar HsdCpb:WU rats were orally administered a target dose of 3 mg/kg bw of radiolabelled bixafen suspended in 0.5% aqueous tragacanth. One control animal was administered

Table 11. Distribution of total radioactive residues in organs and tissues of rats after a single oral dose of [dichlorophenyl-U-^{14}C]bixafen

Organ or tissue	1 h	4 h	8 h	24 h	48 h	72 h	120 h	168 h	R/B	$C_{max}/C_{24\ h}$
Blood	0.499	0.413	0.357	0.063	0.013	0.008	< LOQ	< LOQ	1.000	7.955
Liver	4.843	4.141	3.104	0.623	0.105	0.067	0.031	0.016	9.703	7.771
Renal cortex	3.025	2.688	2.228	0.321	0.029	0.015	0.007	0.005	6.060	9.419
Renal medulla	2.389	2.303	1.507	0.259	0.031	0.015	0.007	< LOQ	4.786	9.217
Brown fat	5.231	4.616	4.031	0.580	0.020	#	#	#	10.480	9.025
Perirenal fat	3.965	4.837	3.611	0.270	0.012	< LOD	< LOD	#	9.691	17.913
Skeletal muscle	1.447	1.272	1.191	0.164	0.008	< LOD	< LOD	< LOD	2.899	8.846
Myocardium	3.188	2.816	2.415	0.380	0.018	< LOD	< LOD	< LOD	6.387	8.392
Lung	0.888	0.898	0.511	0.106	0.010	< LOQ	#	< LOD	1.799	8.459
Spleen	1.266	1.149	0.965	0.136	0.009	< LOQ	< LOQ	< LOD	2.536	9.295
Pancreas	3.364	3.369	2.413	0.385	0.018	< LOQ	< LOD	< LOD	6.750	8.762
Bone marrow	1.023	0.936	0.859	0.123	0.008	#	#	#	2.049	8.328
Testes	0.864	0.917	0.843	0.116	0.006	< LOD	< LOD	< LOD	1.838	7.881
Brain	1.691	1.105	0.825	0.098	< LOQ	< LOD	< LOD	< LOD	3.388	17.256
Spinal cord	1.977	1.313	0.998	0.109	< LOQ	#	#	#	3.960	18.112
Pituitary gland	2.023	1.703	1.493	0.210	0.012	#	#	#	4.052	9.611
Pineal body	1.994	1.857	1.474	0.186	#	#	#	#	3.995	10.739
Adrenal gland	4.160	3.915	3.155	0.412	0.025	0.010	< LOQ		8.334	10.102
Thymus	1.165	1.114	0.962	0.132	0.007	< LOD	< LOD	#	2.335	8.819
Thyroid gland	1.988	1.891	1.774	0.235	0.014	#	#	#	3.984	8.456
Salivary gland	3.021	2.716	2.293	0.347	0.018	< LOQ	#	#	6.052	8.713
Nasal mucosa	1.152	1.199	1.201	0.450	0.110	0.135	0.044	0.035	2.407	2.673
Skin	1.115	1.036	1.085	0.114	0.008	#	#	#	2.234	9.769
Vitreal body	0.095	0.181	0.139	0.043	0.009	0.008	#	#	0.362	4.165
Harderian gland	4.348	5.166	4.364	0.560	0.016	#	#	#	10.349	9.220
Infraorbital gland	4.691	4.906	4.897	0.615	0.028	#	#	#	9.829	7.975

#: organ or tissue usually visible in the rat sections, but not discernible in the radioluminograms; C_{max}: maximum equivalent concentration; $C_{24\ h}$: concentration after 24 h; LOD: limit of detection; LOQ: limit of quantification; R/B: ratio of maximum concentration in tissue to maximum concentration in blood
Source: Spiegel (2007)

the same dose of unlabelled test material and sacrificed at 4 hours after dosing. The distribution of residues in tissues was determined at 1, 4, 8, 24, 48, 72, 120 and 168 hours after administration.

Bixafen was absorbed quickly from the gastrointestinal tract. The maximum concentration of radioactivity in almost all organs and tissues was detected 1 hour after administration. The absorbed radioactivity was distributed rather quickly and evenly in the body, with a preference for liver, fat, infraorbital gland, Harderian gland and adrenal gland at the early time points (Table 12).

Radioactive residues in all organs and tissues decreased rapidly between 8 and 48 hours. In nearly all organs and tissues, residues were below the LOD or LOQ at later time points between 72 and 168 hours after dosing. At the end of the test period, only liver and nasal mucosa showed negligible residues above the LOD (LOQ). Both residues were below 0.05 mg/kg.

There was no sign of a significant retention of radioactivity in specific organs or tissues. Residues in glandular organs or tissues responsible for hormonal regulation (e.g. adrenal, testis or

thyroid gland) were rapidly depleted in parallel with the depletion from the other organs and tissues. This is evident from the ratio of the maximum concentration in plasma to the concentration after 24 hours. In all cases – except in nasal mucosa – the ratio was greater than 5, indicating that bioaccumulation following daily administration of the product is unlikely.

Bixafen was rapidly eliminated from the body, predominantly via faecal excretion. Excretion was nearly complete 48 hours after administration. No significant expiration of ^{14}C-labelled volatiles was observed (Spiegel, 2006).

Table 12. Distribution of total radioactive residues in organs and tissues of rats after a single oral dose of [pyrazole-5-^{14}C]bixafen

Organ or tissue	1 h	4 h	8 h	24 h	48 h	72 h	120 h	168 h	R/B	$C_{max}/C_{24\ h}$
Blood	0.388	0.349	0.321	0.045	0.009	#	#	1.0	1.0	8.7
Liver	4.228	3.349	2.577	0.363	0.073	0.025	0.012	0.006	10.9	11.7
Renal cortex	2.457	2.144	1.936	0.221	0.035	0.007	< LOQ	< LOQ	6.3	11.1
Renal medulla	1.659	1.507	1.390	0.158	0.023	0.005	< LOD	< LOD	4.3	10.5
Brown fat	4.124	3.361	3.251	0.252	0.041	#	#	#	10.6	16.4
Perirenal fat	2.140	2.640	2.924	0.258	0.016	#	#	#	7.5	11.3
Skeletal muscle	1.196	1.130	1.078	0.126	0.014	#	#	#	3.1	9.5
Myocardium	2.925	2.465	1.819	0.252	0.027	#	#	#	7.5	11.6
Lung	0.844	0.776	0.784	0.064	0.014	#	#	#	2.2	13.2
Spleen	1.003	0.895	0.884	0.093	0.011	< LOD	< LOD		2.6	10.8
Pancreas	2.776	2.584	2.603	0.243	0.031	#	#	#	7.1	11.4
Bone marrow	0.900	0.725	0.695	0.084	0.010	#	#	#	2.3	10.8
Testes	0.687	0.719	0.606	0.077	0.008	< LOD	#	#	1.9	9.3
Brain	1.286	0.793	0.596	0.054	0.006	< LOD	#	#	3.3	23.8
Spinal cord	1.555	0.871	0.748	0.060	0.006	#	#	#	4.0	25.7
Pituitary gland	1.556	1.248	1.172	0.132	0.016	#	#	#	4.0	11.7
Pineal body	1.697	1.320	0.976	0.112	0.015	#	#	#	4.4	15.1
Adrenal gland	3.731	3.000	2.163	0.269	0.030	< LOQ	#	#	9.6	13.9
Thymus	0.977	0.878	0.735	0.088	0.009	#	#	#	2.5	11.1
Thyroid gland	2.156	1.519	1.367	0.167	0.020	#	#	#	5.6	12.9
Salivary gland	2.470	2.097	1.765	0.210	0.024	#	#	#	6.4	11.8
Nasal mucosa	1.358	1.084	0.708	0.411	0.172	0.064	0.074	0.032	3.5	3.3
Skin	0.876	0.667	0.635	0.094	0.012	#	#	#	2.3	9.4
Vitreal body	0.166	0.126	0.176	0.034	0.015	#	#	#	0.5	5.1
Harderian gland	2.816	3.842	3.140	0.379	0.043	#	#	#	9.9	10.1
Infraorbital gland	3.379	3.871	2.954	0.401	0.047	#	#	#	10.0	9.6

#: organ or tissue usually visible in the rat sections, but not discernible in the radioluminograms; C_{max}: maximum equivalent concentration; $C_{24\ h}$: concentration after 24 h; LOD: limit of detection; LOQ: limit of quantification; R/B: ratio of maximum concentration in tissue to maximum concentration in blood
Source: Spiegel (2006)

1.2 Biotransformation

For the investigation of the metabolism of [dichlorophenyl-U-^{14}C]bixafen, bile and urine samples as well as extracts of faeces were taken from the ADME study described above (Bongartz, 2008) and analysed and quantified by radio-HPLC. Faeces were conventionally extracted with

different mixtures of acetonitrile/water and pure acetonitrile followed by an exhaustive extraction (microwave assistance) with a mixture of acetonitrile/water (1 : 1). Approximately 90% of the faecally excreted radioactivity could be extracted.

Parent compound and metabolites were identified in bile (groups 5 and 7) and faecal extract (group 3) by spectroscopic investigations. The compounds in the other samples were assigned by comparison of the metabolite pattern and of the retention times of the identified metabolites. Unassigned (unidentified) metabolites were characterized by their extraction and chromatographic behaviour. An overview of the metabolites detected in bile, urine and faeces is given in Table 13.

Table 13. Metabolites detected in bile, faeces and urine in the ADME rat study with [dichlorophenyl-U-^{14}C]bixafen

Metabolite[a,b]	% of administered dose						
	2 mg/kg bw		50 mg/kg bw		2 mg/kg bw		
	Group 1 / male	Group 2 / female	Group 3 / male	Group 4 / female	Group 5 / male	Group 6 / male	Group 7 / female
B1	NC	NC	NC	NC	0.85	NC	0.65
B2	NC	NC	NC	NC	0.61	NC	0.86
B3	NC	NC	NC	NC	1.54	NC	0.93
B4 and B5	NC	NC	NC	NC	2.00	NC	13.24
B6, F1	0.74	0.34	0.99	NC	25.34	0.91	13.22
B8, F4	1.79	3.07	1.62	0.22	10.97	1.08	4.46
B10	NC	NC	NC	NC	2.67	NC	1.45
B12	NC	NC	NC	NC	1.17	NC	0.70
B13	NC	NC	NC	NC	5.88	NC	1.83
B15	NC	NC	NC	NC	2.85	NC	0.74
B16	NC	NC	NC	NC	4.30	NC	1.70
B18	NC	NC	NC	NC	1.50	NC	0.41
B19	NC	NC	NC	NC	1.24	NC	2.06
B20	NC	NC	NC	NC	12.24	NC	8.42
B21	NC	NC	NC	NC	2.53	NC	0.71
F8	1.23	2.56	0.89	1.16	NC	2.04	NC
F10	1.63	3.43	1.07	1.47	NC	2.70	NC
F11	2.23	1.66	0.90	NC	NC	1.89	NC
F12	NC	NC	NC	0.46	NC	1.76	NC
F13	2.93	2.80	1.10	1.00	NC	3.39	NC
F15	1.94	1.20	1.61	0.53	NC	2.46	NC
F17	1.73	0.86	1.05	0.40	NC	1.80	NC
F18	14.25	34.67	10.65	16.01	NC	14.73	NC
F19	7.41	5.44	3.51	1.84	NC	5.70	NC
F20	1.34	0.65	1.84	3.70	NC	1.79	NC
F21	2.30	0.18	2.66	2.94	NC	3.36	NC
F22	13.69	6.68	7.76	2.94	NC	12.80	NC
F25	2.38	NC	1.93	0.92	NC	2.83	NC
F26	10.96	11.91	7.07	6.26	NC	12.03	NC
F27	5.35	2.39	3.43	1.51	NC	3.81	NC

Metabolite[a,b]	% of administered dose						
	2 mg/kg bw		50 mg/kg bw		2 mg/kg bw		
	Group 1 / male	Group 2 / female	Group 3 / male	Group 4 / female	Group 5 / male	Group 6 / male	Group 7 / female
F29	4.06	2.01	45.96	44.19	NC	9.88	NC
Total identified	75.95	79.85	94.03	85.54	75.68	84.95	51.38
Total characterized[c]	7.55	4.29	2.07	1.88	7.36	7.23	4.55
Solids of faeces	8.88	7.11	2.35	3.96	NC	11.77	NC
Urine, not subquantified	1.41	2.87	0.69	1.67	0.71	1.92	0.83
Faeces, not analysed	NC	NC	NC	NC	7.41	NC	6.26
Total	93.78	94.12	99.14	93.06	91.16	105.87	63.02

ADME: absorption, distribution, metabolism, excretion; bw: body weight; NC: calculation of mean was not possible, as only one value was above the LOD

[a] Identified metabolites, peak identification and report name: B1: bixafen-desmethyl-hydroxy-5-hydroxyphenyl-6-glutathionyl (isomer 1); B2: bixafen-desmethyl-hydroxy-5-hydroxyphenyl-6-glutathionyl (isomer 2); B3: bixafen-desmethyl-5-hydroxyphenyl-6-cysteinyl-glucuronide; B4 and B5: bixafen-desmethyl-5-hydroxyphenyl-6-(glutathionyl-glutamic acid) and bixafen-desmethyl-5-hydroxyphenyl-6-glutathionyl; B6, F1: bixafen-desmethyl-5-hydroxyphenyl-6-cysteinyl; B8, F4: bixafen-5-hydroxyphenyl-6-cysteinyl; B10: bixafen-4-fluoro-5-hydroxyphenyl-glucuronide; B12: bixafen-6-hydroxyphenyl-glucuronide; B13: bixafen-desmethyl-*N*-*O*-glucuronide; B15: bixafen-*N*-*O*-glucuronide; B16: bixafen-4-hydroxyphenyl-glucuronide; B18: bixafen-3-hydroxyphenyl-glucuronide; B19: bixafen-desmethyl-hydroxypyrazole-glucuronide; B20: bixafen-desmethyl-*N*-glucuronide (isomer 1); B21: bixafen-desmethyl-*N*-glucuronide (isomer 2); F8: bixafen-desmethyl-5-hydroxyphenyl-6-(methylsulfinyl); F10: bixafen-desmethyl-5-hydroxyphenyl; F11: bixafen-desmethyl-6-fluoro-5-hydroxyphenyl; F12: bixafen-5-hydroxyphenyl-6-(methylsulfinyl); F13: bixafen-desmethyl-4-fluoro-5-hydroxyphenyl and bixafen-desmethyl-5-hydroxyphenyl-deschloro-(methylthio); F15: bixafen-5-hydroxyphenyl; F17: bixafen-6-fluoro-5-hydroxyphenyl; F18: bixafen-desmethyl-5-hydroxyphenyl-6-(methylthio); F19: bixafen-4-fluoro-5-hydroxyphenyl and bixafen-5-hydroxyphenyl-6-thiol-acetaldehyde; F20: bixafen-desmethyl-3-hydroxyphenyl; F21: bixafen-desmethyl-6′-hydroxy; F22: bixafen-5-hydroxyphenyl-6-(methylthio) and bixafen-4-hydroxyphenyl; F25: bixafen-3-hydroxyphenyl; F26: bixafen-desmethyl; F27: bixafen-*N*-hydroxy- and bixafen-desmethyl-hydroxypyrazole; F29: bixafen (parent compound).

[b] B indicates a metabolite in bile, F in faeces.

[c] Unidentified metabolites were characterized by their extraction and chromatographic behaviour: 8 metabolites in bile (B7, B9, B11, B14, B17, B22, B23, B24), 11 metabolites in faeces (F2, F3, F5, F6, F7, F9, F14, F16, F23, F24, F28).

Source: Bongartz (2008)

No significant sex-related differences were observed. Metabolites originating from molecular cleavage were not detected in this study. High amounts of parent compound were detected in faeces of rats in both high-dose tests and the test with pretreated rats.

The most important metabolic reaction was the demethylation in the pyrazole ring, forming bixafen-desmethyl. Females showed a higher demethylation rate compared with males, especially in the faeces. Hydroxylation of parent compound and bixafen-desmethyl led to hydroxylated compounds. The hydroxylation took place in different positions of the molecule. Elimination of the fluoro atom and a subsequent hydroxylation were also detected. Finally, an NIH shift of the fluorine atom was observed.

Most of the hydroxy compounds were conjugated with glucuronic acid. An *N*-conjugation of bixafen-desmethyl with glucuronic acid was also found. Conjugation with glutathione was a major metabolic reaction in the bile. Glutathione conjugates were further degraded to cysteine conjugates and methylthio, methylsulfinyl and thiol-acetaldehyde compounds.

A minor reaction was the elimination of one of the chloro atoms of bixafen-desmethyl-5-hydroxyphenyl and a further conjugation with methylthio (Bongartz, 2008).

In addition, for the investigation of the metabolism of [pyrazole-5-^{14}C]bixafen, urine samples as well as extracts of faeces were taken from the ADME study described above (Bongartz, 2007) and analysed and quantified by radio-HPLC. Faeces were conventionally extracted with different mixtures of acetonitrile/water and pure acetonitrile followed by an exhaustive extraction (microwave assistance) with a mixture of acetonitrile/water (1 : 1). Approximately 85% of the faecally excreted radioactivity could be extracted.

Parent compound and metabolites were assigned by comparison of the metabolite pattern and of the retention times of the metabolites, which were identified in the study with the dichlorophenyl label (Bongartz, 2008). Low amounts of label-specific metabolites were detected in urine samples. These metabolites were identified by spectroscopic investigations or by comparison with the non-radiolabelled reference compound. Unassigned metabolites were characterized by their extraction and chromatographic behaviour. An overview of the metabolites detected in urine and faeces is given in Table 14.

The main metabolic routes observed for male rats of the dichlorophenyl and the pyrazole labels are identical. Parent compound was detected in faeces only.

The most important metabolic reaction is the demethylation in the pyrazole ring, forming bixafen-desmethyl. Hydroxylation of parent compound and bixafen-desmethyl led to hydroxylated compounds. The hydroxylation took place in different positions of the molecule. Elimination of the fluoro atom and a subsequent hydroxylation were also detected. Finally, an NIH shift of the fluoro atom was observed. Conjugation with glutathione was a major metabolic reaction and led to an intermediate glutathione conjugate, which was further degraded to cysteine conjugates and methylthio, methylsulfinyl and thiol-acetaldehyde compounds.

A minor reaction, observed in urine, is the molecule cleavage forming pyrazole-4-carboxamide and desmethyl-pyrazole-4-carboxamide. An oxidation of bixafen-pyrazole-4-carboxamide led to bixafen-pyrazole-4-carboxylic acid. Another minor reaction is the elimination of one of the chloro atoms of bixafen-desmethyl-5-hydroxyphenyl and a further conjugation with methylthio (Bongartz, 2007).

In conclusion, the results obtained for male and female rats in the ADME study with the dichlorophenyl label (Bongartz, 2008) correspond well with the findings in the ADME study with the pyrazole label (Bongartz, 2007). Based on these results, the metabolic pathway as outlined in Figs 2 and 3 is proposed.

2. Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

Results of studies of the acute toxicity of bixafen are summarized in Table 15.

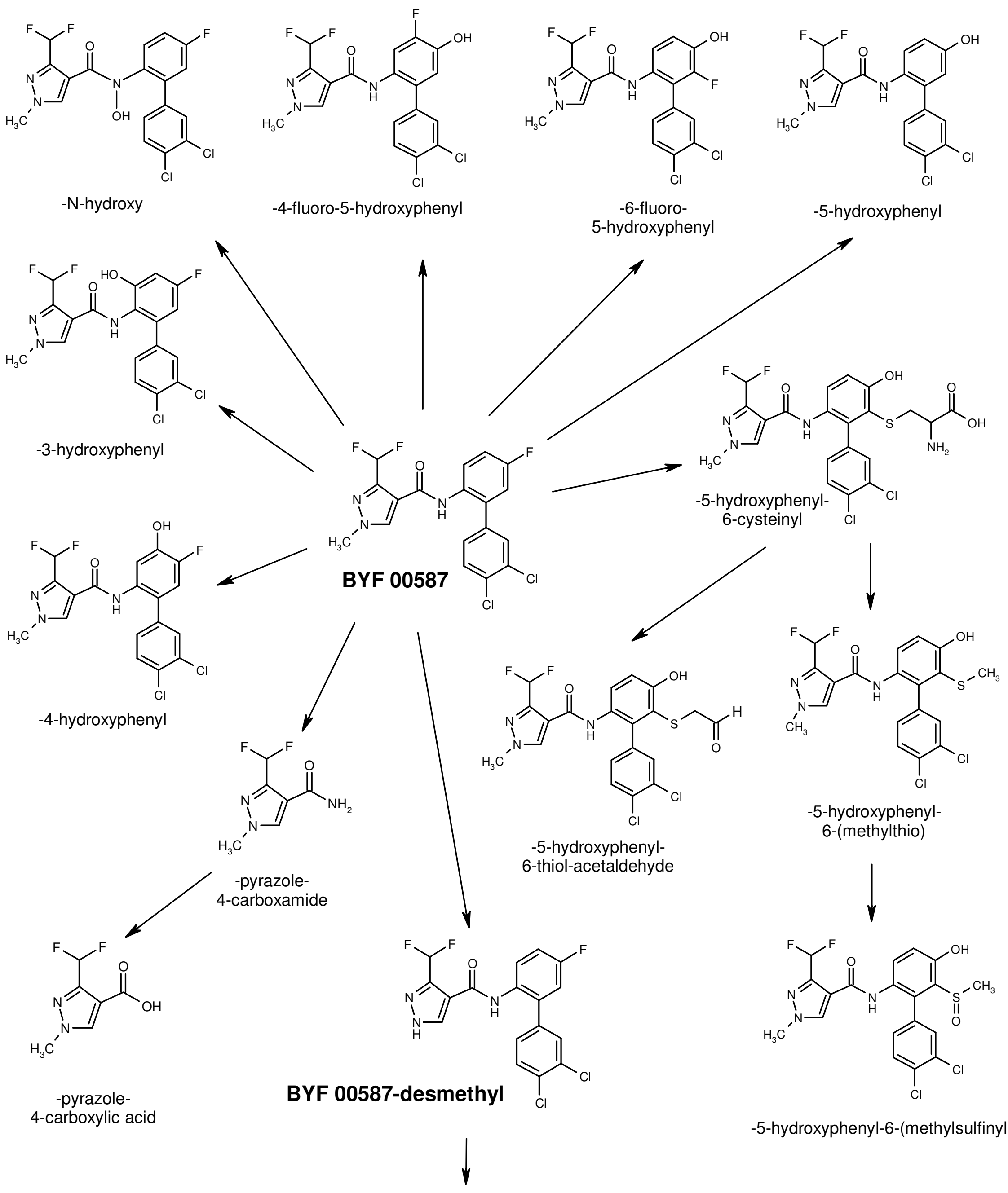
In an acute oral toxicity study conducted according to OECD Test Guideline 423, two groups of three fasted, young female Wistar rats (HsdCpd:Wu) received successively a single bixafen (purity 95.8%) dose of 2000 mg/kg bw by gavage in 2% Cremophor EL in demineralized water (as a vehicle and/or positive control) at a volume of 10 mL/kg bw. Clinical signs and mortality rates were determined several times on the day of administration and subsequently at least once daily for an observation period of at least 14 days, and body weights were recorded on days 1, 8 and 15. On day 15, surviving animals were killed, necropsied and examined for gross pathological changes.

Table 14. Metabolites detected in faeces and urine in the ADME rat study with [pyrazole-5-^{14}C]bixafen

Peak identification[a]	Metabolite report name	% of administered dose
U1	Bixafen-desmethyl-pyrazole-4-carboxamide	2.78
U3	Bixafen-pyrazole-4-carboxamide	0.97
U4	Bixafen-pyrazole-4-carboxylic acid	0.09
U2, U5–U17	Not identified	0.50
Total identified in urine	–	**3.84**
Total in urine	–	**4.34**
F1	Bixafen-desmethyl-5-hydroxyphenyl-6-cysteinyl	1.47
F4	Bixafen-5-hydroxyphenyl-6-cysteinyl	1.84
F8	Bixafen-desmethyl-5-hydroxyphenyl-6-(methylsulfinyl)	1.38
F10	Bixafen-desmethyl-5-hydroxyphenyl	2.31
F11	Bixafen-desmethyl-6-fluoro-5-hydroxyphenyl	1.53
F12	Bixafen-5-hydroxyphenyl-6-(methylsulfinyl)	0.43
F13	Bixafen-desmethyl-4-fluoro-5-hydroxyphenyl and bixafen-desmethyl-5-hydroxyphenyl-deschloro-(methylthio)	3.55
F15	Bixafen-5-hydroxyphenyl	1.92
F17	Bixafen-6-fluoro-5-hydroxyphenyl	1.99
F18	Bixafen-desmethyl-5-hydroxyphenyl-6-(methylthio)	14.13
F19	Bixafen-4-fluoro-5-hydroxyphenyl and bixafen-5-hydroxyphenyl-6-thiol-acetaldehyde	6.97
F20	Bixafen-desmethyl-3-hydroxyphenyl	1.55
F21	Bixafen-desmethyl-6′-hydroxy	2.19
F22	Bixafen-5-hydroxyphenyl-6-(methylthio) and bixafen-4-hydroxyphenyl	10.34
F25	Bixafen-3-hydroxyphenyl	1.94
F26	Bixafen-desmethyl	10.47
F27	Bixafen-*N*-hydroxy- and bixafen-desmethyl-hydroxypyrazole	2.83
F29	Bixafen (parent compound)	8.57
F2–F3, F5–F7, F9, F14, F24, F28	Not identified	9.60
Total identified in faeces	–	**75.42**
Total identified in urine and faeces	–	**79.27**
Total characterized in urine and faeces	–	**10.09**
Solids of faeces	–	6.36
Faeces not analysed[b]	–	1.98
Total in urine and faeces	–	**97.71**

[a] U indicates a metabolite in urine, F in faeces.
[b] Faecal samples 48–72 hours not analysed due to low amount of radioactivity in the pool.
Source: Bongartz (2007)

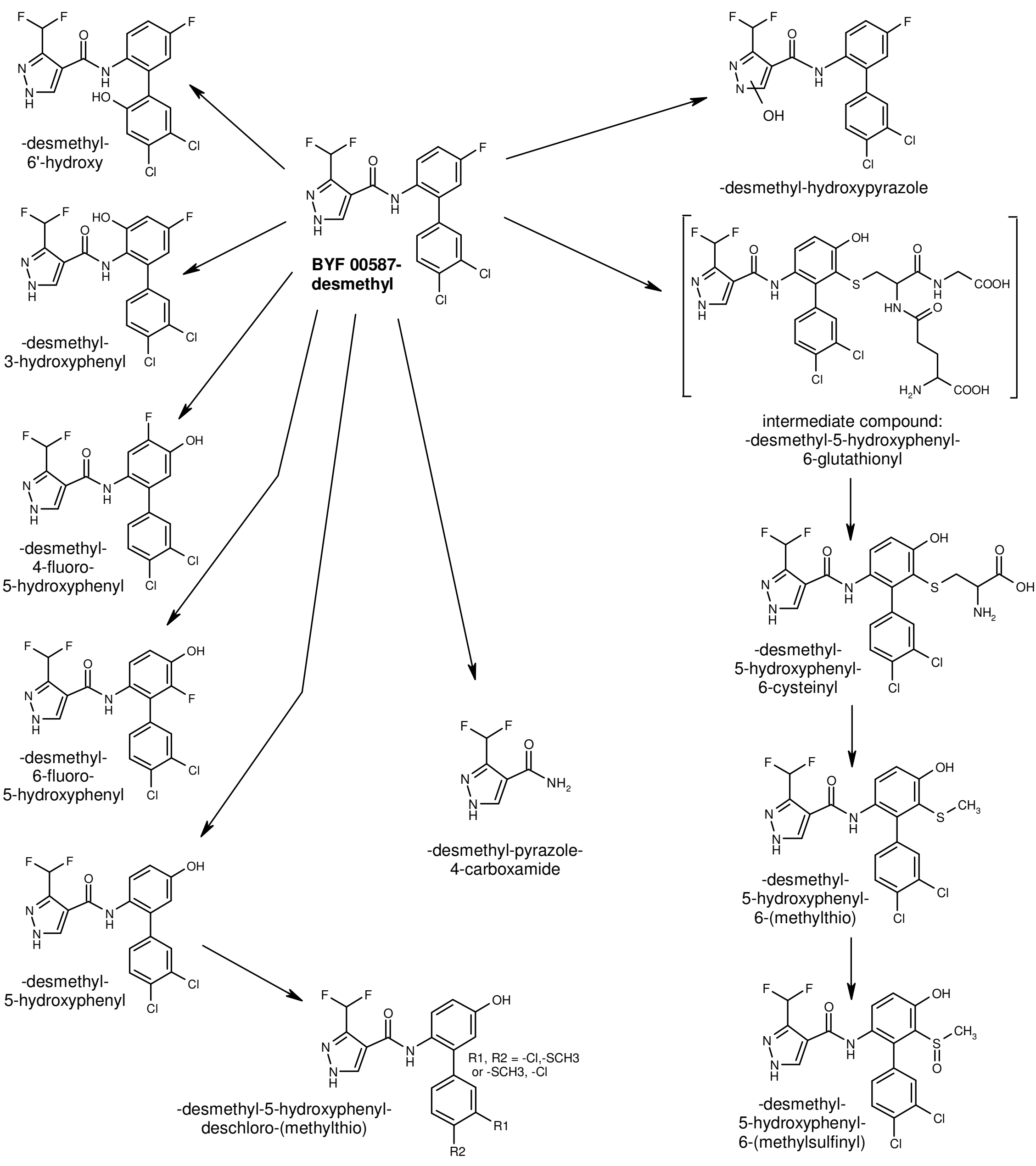
Fig. 2. Proposed metabolic pathway of [pyrazole-5-^{14}C]bixafen in rats



BYF 00587 = bixafen

Source: Bongartz (2007)

Fig. 3. Continued metabolic pathway of [pyrazole-5-14C]bixafen in rats



BYF 00587 = bixafen
Source: Bongartz (2008)

Table 15. Summary of acute oral, dermal and inhalation toxicity of bixafen

Species	Strain	Sex	Route	Purity (%)	LD_{50} (mg/kg bw) or LC_{50} (mg/L)	Reference
Rat	HsdCpd:Wu	F	Oral	95.8	> 2 000	Schüngel (2005a)
Rat	HsdCpd:Wu	M & F	Dermal	95.8	> 2 000	Schüngel (2005b)
Rat	HsdCpd:Wu	M & F	Inhalation	95.8	> 5.383	Pauluhn (2006)

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

No mortalities occurred at 2000 mg/kg bw, the only dose tested. The oral LD_{50} cut-off is greater than or equal to 5000 mg/kg bw, according to OECD Test Guideline 423. There was no toxicological effect on body weight or body weight gain. No clinical signs were observed, and no abnormalities were observed at gross necropsy (Schüngel, 2005a).

In an acute dermal toxicity study conducted according to OECD Test Guideline 402, groups of young adult Wistar rats (five of each sex) were administered bixafen (purity 95.8%) at a single dose of 2000 mg/kg bw. The pure solid test substance was applied semiocclusively for 24 hours to 10% of each animal's body surface moistened with distilled water. After an exposure period of 24 hours, the occlusion was removed, residual test material was removed with tepid water using soap and the area was gently patted dry. The rats were observed for clinical signs and mortality several times on the day of dosing and subsequently at least once daily, for an observation period of at least 14 days. Individual body weights were recorded on days 1, 8 and 15. On day 15, surviving animals were terminated, necropsied and examined for gross pathological changes.

Bixafen was regarded as being of very low acute toxicity after dermal application. No clinical signs were observed in rats of either sex. Body weight and body weight gain were not affected by treatment. There were no gross pathological findings.

The dermal LD_{50} of bixafen was greater than 2000 mg/kg bw (Schüngel, 2005b).

In an acute inhalation toxicity study conducted according to OECD Test Guideline 403, groups of young adult Wistar rats (five of each sex) were exposed by the inhalation route to bixafen (purity 95.8%) in air for 4 hours at a concentration of 5.383 mg/L. Animals were exposed to the aerosolized test substance in Plexiglas exposure tubes applying a directed-flow nose-only exposure principle. A concurrent control group was exposed to an atmosphere using similar exposure conditions (15 L/minute; conditioned dry air). Rats were examined carefully several times on the day of exposure and at least once daily thereafter for 14 days. Visual placing response, grip strength on wire mesh, abdominal muscle tone, corneal and pupillary reflexes, pinnal reflex, righting reflex, tail pinch response and startle reflex with respect to behavioural changes stimulated by sounds (finger snapping) and touch (back) were the reflexes tested. The temperatures were measured shortly after cessation of exposure, and individual body weights were recorded before exposure and on days 3, 7 and 14. On day 15, surviving animals were terminated, necropsied and examined for gross pathological changes.

The method of dust generation employed a "Bayer Generator" system, which gave stable analysed concentrations in the range of 1500–20 000 mg/m^3. The bixafen concentration was determined by gravimetric analysis, where chamber samples were collected after the equilibrium had been attained in hourly intervals. Two samples during each exposure were also taken for analysis of the particle size distribution using an Andersen cascade impactor. At 5383 mg/m^3, the mass median aerodynamic diameter was 6.9 µm (standard deviation = 2.3 µm); 15.3% of the total particulate mass had an aerosol mass less than 3 µm, and 34.3% of the total particulate mass had an aerosol mass less than 5 µm.

Bixafen (solid aerosol) proved to be non-toxic to rats via the inhalation route. No mortality occurred up to the maximum technically attainable concentration. Clinical signs from exposure to bixafen were seen to include bradypnoea, laboured breathing patterns, reduced motility, piloerection, ungroomed hair-coat, limpness, giddiness, high-legged gait, flaccid paralysis of hindlegs and mydriasis. These effects were rapidly reversible, and all animals appeared normal by the 4th post-exposure day. Several animals displayed reduced tonus and horizontal grip strength. One female had an impaired righting reflex. Rectal temperature was lowered by approximately 20% in both sexes with the treatment. No significant treatment-related effects on body weight gain were noted. At necropsy, mild discolouration of the lung was observed, but this was considered not to be toxicologically relevant.

The acute inhalation LC_{50} of bixafen was in excess of 5.383 mg/L (Pauluhn, 2006).

(b) Dermal and ocular irritation and dermal sensitization

Results of studies on dermal and eye irritation and dermal sensitization of bixafen are summarized in Table 16.

Table 16. Summary of irritation and skin sensitization potential of bixafen

Species	Strain	Sex	End-point	Purity (%)	Result	Reference
Rabbit	Crl:KBL(NZW)BR	F	Skin irritation	95.8	Not irritating	Schüngel (2005c)
Rabbit	Esd:NZW	F	Eye irritation	95.8	Not irritating	Schüngel (2005d)
Mouse	Hsd:Win NMRI	F	Skin sensitization (LLNA)	95.8	Not sensitizing	Vohr (2005)

F: female; LLNA: local lymph node assay; M: male

In a study of skin irritation potential conducted according to OECD Test Guideline 404, 0.5 g of pulverized bixafen (purity 95.8%) moistened with distilled water was applied to the shorn dorsal skin of three young adult female New Zealand White rabbits under a gauze patch (placed on the dorsolateral areas of the trunk of each animal and held in place with non-irritating tape for the duration of the exposure period). The contralateral skin area not treated with bixafen served as the control. In the first instance, only one rabbit was used, and three patches were applied successively to it for exposure periods of 3 minutes, 1 hour and 4 hours, respectively. The test was completed using two additional rabbits exposed for 4 hours. The responses were graded 1 hour later. Skin reactions were scored at 1, 24, 48 and 72 hours post-treatment.

No signs of systemic toxicity or mortality were observed during the study period. No erythema, eschar or oedema was seen in any animals at any time point. It was concluded that bixafen is non-irritating to rabbit skin (Schüngel, 2005c).

In a study of eye irritation potential conducted according to OECD Test Guideline 405, 0.1 mL of pulverized bixafen (purity 95.8%) was placed into the conjunctival sac of one eye of each of three male New Zealand White rabbits. The other eye, which was untreated, served as control. The treated eye was not rinsed for at least 24 hours following instillation. Ocular lesions were scored at 1, 24, 48 and 72 hours post-instillation.

No signs of systemic toxicity were observed during the study period. A slight redness of the conjunctivae was observed after 1 hour in all females and at 24 hours in one female (grade 1, having resolved by 2 days post-treatment). It was concluded that bixafen is not irritating to the eyes (Schüngel, 2005d).

In a study of skin sensitization potential conducted according to OECD Test Guideline 429, bixafen (purity 95.8%) was applied epicutaneously to the dorsal surface of each ear of female NMRI mice. Groups of six mice per dose were dosed once daily for 3 consecutive days with 25 µL of suspensions of bixafen at a concentration of 0%, 3%, 10% or 30% in dimethyl sulfoxide (DMSO). The rabbits were anaesthetized by inhalation of carbon dioxide and killed 1 day after the last application (day 4). The appropriate organs were then removed, and lymphatic organs (the auricular lymph nodes) were transferred into physiological (phosphate-buffered) saline. Animals were checked for mortality and clinical signs at least daily during the study. In particular, the site of application was examined for signs of local irritation. Individual body weights were measured at the start of the study and at scheduled termination.

No mortality or clinical signs were observed during the study. The mice did not show an increase in the stimulation indices for cell counts or for weight of the draining lymph nodes after application of bixafen. No substance-specific effects were determined for ear weights, and body weights of the animals were not affected by any treatment.

In conclusion, no activation of the cells of the immune system via the dermal route was seen by the local lymph node assay method. Thus, bixafen did not show any sensitization potential (Vohr, 2005).

2.2 Short-term studies of toxicity

Mice

In a range-finding study of toxicity not conducted according to GLP or OECD test guidelines, groups of five male and five female C57BL/6J mice were fed diets containing bixafen (purity 98.7%) at a concentration of 0, 100, 500 or 2500 parts per million (ppm) (equal to 0, 17, 81 and 305 mg/kg bw per day for males and 0, 21, 103 and 424 mg/kg bw per day for females, respectively) for 28 days. Morbidity/mortality checks were performed twice daily, and a detailed clinical examination was performed weekly. Body weights and feed consumption were recorded weekly throughout the study. Clinical chemistry analyses were performed at the end of the study. Any animals dying prematurely were necropsied, and selected tissues were examined microscopically. At study termination, all animals were necropsied, selected organs were weighed and a range of tissues was taken, fixed and examined microscopically.

At 2500 ppm, between days 7 and 14, all males were killed for humane reasons or were found dead, and 3/5 females were killed for humane reasons. Prior to necropsy, the majority of animals of both sexes had reduced motor activity, 2/5 males and 1/3 females had hunched posture, 3/5 males and 1/3 females were cold to touch, 2/3 females had tremors and piloerection, and there were isolated instances of slow respiration, half-closed eyes and wasted appearance. No mortalities or clinical signs occurred at 500 or 100 ppm.

Body weight at 2500 ppm was reduced by 18% in males due to a marked body weight loss during the 1st week of the study, whereas in females, body weight gain was reduced by 73% and 67% in weeks 1 and 3, respectively, and overall cumulative body weight gain was reduced by 25%. Feed consumption at 2500 ppm was reduced by 38% in males and by 16% in females during the 1st week of the study. Thereafter, feed consumption by females was reduced by 11% and 21% during weeks 3 and 4, respectively. No significant effects on body weight or feed consumption occurred at 500 or 100 ppm.

At the clinical chemistry assessment, treatment-related changes at 2500 ppm consisted of an increase in total cholesterol concentration (106%) in the two surviving females, whereas a reduced albumin concentration (22%) and an increase in alanine aminotransferase activity (371%) were observed in one of the two surviving females. Statistically significantly lower mean albumin concentrations were also noted at 500 ppm in both sexes (23% and 18% in males and females, respectively) and at 100 ppm in females (14%).

At necropsy, enlarged and dark livers and pallor in other organs were noted at 2500 ppm. Enlarged livers were also observed in 3/5 males and 2/5 females at 500 ppm. Minimal to slight hypertrophy of centrilobular hepatocytes was observed in the liver of the two surviving females at 2500 ppm and in 4/5 males and 4/5 females at 500 ppm. In addition, minimal or slight focal coagulative necrosis was seen in the two surviving females at 2500 ppm and in one male and all the females at 500 ppm (Table 17).

The no-observed-adverse-effect level (NOAEL) was 100 ppm (equal to 17 mg/kg bw per day for males and 21 mg/kg bw per day for females), based on liver toxicity (increased liver weight, clinical chemistry changes, focal coagulative necrosis) in both sexes at 500 ppm (equal to 81 mg/kg bw per day for males and 103 mg/kg bw per day for females) and above (Steiblen, 2004).

Table 17. Summary of selected findings in the 28-day mouse study

	Males				Females			
	0 ppm	100 ppm	500 ppm	2 500 ppm	0 ppm	100 ppm	500 ppm	2 500 ppm
Terminal body weight (g)	19.5	19.9	19.0	ND	15.2	15.7	15.7	16.2
Absolute liver weight (g)	0.85	0.96	1.11*	ND	0.70	0.76	0.88*	1.39*
Relative liver weight (% of body weight)	4.34	4.83	5.64*	ND	4.62	4.84	5.58	8.55*
Liver: centrilobular hypertrophy, minimal to slight	0/5	0/5	4/5	[1/5][a]	0/5	0/5	4/5	2/2 [3/3][a]
Liver: focal coagulative necrosis, minimal to slight	0/5	0/5	1/5	[3/5][a]	0/5	0/5	5/5	2/2 [1/3][a]

ND: not determined; ppm: parts per million; * *P*: < 0.05

[a] Premature deaths.

Source: Steiblen (2004)

In a study of toxicity conducted according to OECD Test Guideline 408, groups of 10 male and 10 female C57BL/6J mice were fed diets containing bixafen (purity 99.2%) at a concentration of 0, 50, 200 or 500 ppm (equal to 0, 8.5, 34.3 and 88 mg/kg bw per day for males and 0, 10.4, 42.9 and 110 mg/kg bw per day for females, respectively) for at least 90 days. Morbidity/mortality checks were performed twice daily, and a detailed clinical examination was performed weekly. Body weights and feed consumption were recorded weekly throughout the study. Clinical chemistry analyses were performed at the end of the study. Any animals dying prematurely were necropsied, and selected tissues were examined microscopically. At study termination, all animals were necropsied, selected organs were weighed and a range of tissues was taken, fixed and examined microscopically.

No treatment-related mortalities or clinical signs were noted during the study. One female at 500 ppm was killed for humane reasons; the moribund state of this animal was due to a hydrocephaly and was therefore not related to treatment. Body weight parameters and feed consumption were unaffected by treatment.

Treatment-related effects on clinical chemistry parameters involved an increase in alanine aminotransferase and alkaline phosphatase activity (72% and 28%, respectively) in males at 500 ppm and a decrease in total cholesterol concentration in males at 500 and 200 ppm (47% and 37%, respectively) (Table 18).

At necropsy at 500 ppm, mean absolute and relative liver weights were increased (21–23% in males, 16–20% in females). Enlarged livers were observed in 4/10 males and 4/9 females. At the histopathological examination, slight to moderate centrilobular to panlobular hepatocellular hypertrophy was noted in all males and in 8/9 females. A loss of centrilobular hepatocellular vacuolation was noted in all males, with a concomitant increase of diffuse hepatocellular vacuolation noted in 8/10 males. This change was interpreted to have occurred subsequent to the presence of centrilobular hepatocellular hypertrophy. In the stomach, a higher incidence of focal/multifocal squamous cell hyperplasia was noted in 3/10 males and 4/9 females; this finding was associated with a higher incidence of pustules noted in 3/10 males. At 200 ppm, necropsy revealed a slight increase in mean absolute and relative liver weight (11–12% in males, 8–10% in females). Histopathological examination showed minimal to slight centrilobular to panlobular hepatocellular hypertrophy in 7/10 males (Table 18).

The NOAEL was 50 ppm (equal to 8.5 mg/kg bw per day for males and 10.4 mg/kg bw per day for females), based on liver toxicity in males (increased weight, clinical chemistry changes and diffuse hepatocellular vacuolation) and focal/multifocal squamous cell hyperplasia of the stomach in both sexes at 200 ppm (equal to 34.3 mg/kg bw per day for males and 42.9 mg/kg bw per day for females) and above (Steiblen, 2005a).

Table 18. Summary of selected findings in the 90-day mouse study

	Males				Females			
	0 ppm	50 ppm	200 ppm	500 ppm	0 ppm	50 ppm	200 ppm	500 ppm
ALAT (IU/L)	43	41	38	74**	42	35	40	62
AP (IU/L)	94	101	104**	120**	149	129*	128*	145
Cholesterol (mmol/L)	1.79	1.49*	1.13**	0.94**	1.30	1.36	1.19	1.16
Albumin (g/L)	43	41	39**	38**	44	43	39**	40*
Terminal body weight (g)	23.3	23.2	23.1	23.4	118.6	18.6	18.9	19.1
Absolute liver weight (g)	0.96	0.96	1.07	1.18**	0.82	0.83	0.90	0.98**
Relative liver weight (% of body weight)	4.13	4.12	4.63*	5.06**	4.39	4.44	4.76	5.11**
Liver								
No. examined	10	10	10	10	10	10	10	9
Hepatocellular hypertrophy; centrilobular	0	0	7	10	0	0	0	8
Hepatocellular vacuolation; centrilobular	10	10	8	0	6	7	9	7
Hepatocellular vacuolation; diffuse	0	0	2	8	4	2	1	2
Stomach								
No. examined	10	10	10	10	10	10	10	9
Squamous cell hyperplasia	1	1	2	3	1	0	2	4

ALAT: alanine aminotransferase; AP: alkaline phosphatase; IU: international units; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$
Source: Steiblen (2005a)

Rats

In a range-finding toxicity study not conducted according to GLP or OECD test guidelines, groups of five male and five female Wistar Rj: WI (IOPS HAN) rats were fed diets containing bixafen (purity 99.7%) at a concentration of 0, 50, 350 or 2000 ppm (equal to 0, 3.5, 25 and 137 mg/kg bw per day for males and 0, 4.1, 28 and 138 mg/kg bw per day for females, respectively) for 28 days. Morbidity/mortality checks were performed twice daily, and a detailed clinical examination was performed weekly. Body weights and feed consumption were recorded weekly throughout the study. Haematology and clinical chemistry analyses were performed at the end of the study. On study day 29, all animals were necropsied, selected organs were weighed and a range of tissues was taken and fixed. Histopathological examinations were performed on all tissues from all the animals in the control and high-dose groups, whereas liver, kidney, lung and thyroid gland were also examined in all animals in the intermediate-dose group. Also at necropsy, the remaining portions of the liver from all surviving animals were homogenized for microsomal preparations in order to determine total cytochrome P450 content and specific cytochrome P450 isoenzyme profile.

There were no deaths and no treatment-related clinical signs at any dose level. Body weight gain was reduced at 2000 ppm in both sexes, by 21% (males: after 1–15 days) to 26% (females: after 1–22 days). Body weight was unaffected at 50 or 350 ppm in either sex. At 2000 ppm, feed intake was reduced by 19% in males from days 1 to 8, but returned to normal levels thereafter, whereas in females, there was a reduction in feed intake of 25% on days 1–8 and 9–13% from days 8 to 28. There was no impact on feed consumption in either sex at a dietary concentration of 350 or 50 ppm.

Haematological examination showed an increase in platelets of 21% and 22% in males and females, respectively, at 2000 ppm, the effect being statistically significant only in females. Males at 2000 ppm had a slightly longer mean prothrombin time of 32%, but this was not statistically

significant. Clinical chemistry findings included decreased bilirubin of 54% or 65% in males and females, respectively, at 350 ppm and of 85% and 59% in males and females, respectively, at 2000 ppm. Total serum cholesterol was elevated by 71% in females in the 2000 ppm dose group.

Terminal body weights were not affected by treatment. Liver weights were increased in the 350 and 2000 ppm dose groups in both sexes compared with controls, the effect being more pronounced in females (Table 19). At 350 ppm, females had an increase in liver weight of more than 25%, with 3/5 livers darkened and enlarged grossly. At 2000 ppm, dark livers were observed in 4/5 males. Dark and enlarged livers were observed in all females. Histologically, centrilobular hypertrophy (minimal to slight) was seen in all animals at the highest dose and in 1/5 females and 4/5 males in the intermediate-dose group. Minor, diffuse hypertrophy of the follicular cells in the thyroid gland was seen in 2/5 males at 2000 ppm.

A slight, dose-related increase in benzyloxyresorufin *O*-dealkylase (BROD) and pentoxyresorufin *O*-depentylase (PROD) activities was observed in males, with no significant effect noted at 50 ppm. In females, BROD activity was considered slightly increased at 2000 ppm only; the per cent increase compared with the control female mean appeared high due to a low control value. No significant effect was noted in PROD activity for the females or in ethoxyresorufin *O*-deethylase (EROD) activity for both sexes (Table 19).

Table 19. Summary of selected findings in the 4-week rat study

	Males				Females			
	0 ppm	50 ppm	350 ppm	2 000 ppm	0 ppm	50 ppm	350 ppm	2 000 ppm
Total bilirubin (µmol/L)	1.3	0.9	0.6**	0.2**	1.7	1.2	0.6**	0.7**
Cholesterol (mmol/L)	1.7	1.6	1.6	2.1	1.8	1.9	2.2	3.1**
Terminal body weight (g)	393	377	390	379	231	234	234	225
Absolute liver weight (g)	10.7	10.3	11.6	12.8**	5.8	6.2	7.4*	9.0**
Relative liver weight (% of body weight)	2.71	2.74	2.98*	3.36**	2.52	2.65	3.15**	3.99**
Total P450 (nmol/mg protein)	1.05	1.23	1.30	1.34	0.74	0.74	0.81	1.00
EROD	60.7	53.4	56.6	50.1	60.5	62.6	41.1	47.8
BROD	10.1	11.8	47.2	58.7	1.6	3.4	6.4	15.6
PROD	4.8	5.7	13.5	17.1	3.0	3.6	3.5	5.3
Hepatocellular hypertrophy; centrilobular	0	0	4	5	0	0	1	5

BROD: benzyloxyresorufin *O*-dealkylase; EROD: ethoxyresorufin *O*-deethylase; ppm: parts per million; PROD: pentoxyresorufin *O*-depentylase; *: $P < 0.05$; **: $P < 0.01$
Source: Langrand-Lerche (2004)

The NOAEL was 350 ppm (equal to 25 mg/kg bw per day for males and 28 mg/kg bw per day for females), based on reduced body weight gain, reduced feed consumption and liver toxicity (increased liver weight, increased cholesterol level) and thyroid effects (hypertrophy of follicular cells) at 2000 ppm (equal to 137 mg/kg bw per day for males and 138 mg/kg bw per day for females) (Langrand-Lerche, 2004).

In a study of toxicity conducted according to OECD Test Guideline 408, groups of 10 male and 10 female Wistar Rj: WI (IOPS HAN) rats were fed diets containing bixafen (purity 99.2%) at a concentration of 0, 50, 200, 800 or 2000 ppm (equal to 0, 3.2, 12.9, 50.4 and 130 mg/kg bw per day for males and 0, 3.9, 15.0, 59.2 and 153 mg/kg bw per day for females, respectively) for at least 90

days. An additional 10 males and 10 females fed test diet containing bixafen at a concentration of either 0 or 2000 ppm for at least 90 days were maintained on control diet for a further 28 days to examine the reversibility of any effects seen. Clinical signs were recorded daily, and body weight and feed consumption were measured weekly. A detailed physical examination was performed once during the acclimatization phase and weekly throughout the study. All surviving animals (except for animals of the recovery groups) were subjected to a neurotoxicity assessment (motor activity, sensory reactivity and grip strength) during weeks 11–12 of the study. Additionally, during week 4 of the recovery phase, motor activity was recorded for all surviving animals. Ophthalmological examinations were performed on all animals during the acclimatization phase and on all surviving animals of the control and high-dose groups during week 13. Urine samples were collected overnight before necropsy on selected animals. A blood sample was collected on selected animals for haematology and clinical chemistry determinations before necropsy. In addition, a blood sample was collected on selected animals for triiodothyronine (T_3), thyroxine (T_4) and thyroid stimulating hormone (TSH) analysis during weeks 3–4 and 13 of the study and in week 5 of the recovery phase. All animals were necropsied, selected organs were weighed and a range of tissues was taken, fixed and examined microscopically.

No treatment-related mortalities or clinical signs (including the neurotoxicity assessment) were noted during the study. One female at 2000 ppm was terminated prematurely for humane reasons on day 73. Prior to necropsy, this animal had noisy respiration and a soiled nose. At necropsy, this animal was found to have a nasal cavity fracture, and histopathology revealed a minimal diffuse follicular cell hypertrophy in the thyroid gland.

Feed consumption of females at 800 and 2000 ppm was reduced by 8% or 11%, respectively, between days 1 and 8 and was comparable with that of the control group thereafter.

Clinical pathology determinations at 2000 ppm revealed an increase in the prothrombin time by 51% in males, an increase in total cholesterol concentration by 40% in females and a slight tendency towards higher gamma-glutamyltransferase activity in 5/9 females. Total bilirubin concentration was decreased by 50% in males and 43% in females at 2000 ppm, by 40% in males and 48% in females at 800 ppm and by 29% in females at 200 ppm (Table 20). Hormonal analysis revealed that TSH levels at 2000 ppm increased during weeks 3–4 by 64% in males and by 42% in females when compared with the controls. At week 13, no increase in TSH level was observed; only a slight (32%) increase in T_3 level in females was noted.

At necropsy, mean absolute and relative liver weights were increased by 18–24% in males and 33–41% in females at 2000 ppm and by 7–12% in males and 17–19% in females at 800 ppm (Table 20). At 2000 ppm, enlarged and/or dark and prominent lobulation of the liver was observed in some animals of both sexes and was associated with a minimal to slight centrilobular hepatocellular hypertrophy noted in all males and in 6/9 females at microscopic examination. Also at 2000 ppm, minimal to slight diffuse hypertrophy of the follicular cells was noted in the thyroid gland of 9/10 males and 5/9 females at scheduled termination. This finding was also observed in the female prematurely terminated due to accidental trauma.

At 800 ppm, enlarged and/or prominent lobulation of the liver was observed in some animals of both sexes and was associated with a minimal to slight centrilobular hepatocellular hypertrophy noted in 7/10 males at microscopic examination. Also at 800 ppm, minimal to slight diffuse hypertrophy of follicular cells was observed in the thyroid gland of 6/10 males and 2/10 females (Table 20).

The NOAEL was 200 ppm (equal to 12.9 mg/kg bw per day for males and 15.0 mg/kg bw per day for females), based on liver effects (enlarged livers, increased liver weight) and thyroid effects (hypertrophy of follicular cells) at 800 ppm (equal to 50.4 mg/kg bw per day for males and 59.2 mg/kg bw per day for females) and above (Steiblen, 2005b).

Table 20. Summary of selected findings in the 90-day rat study

	Males					Females				
	0 ppm	50 ppm	200 ppm	800 ppm	2 000 ppm	0 ppm	50 ppm	200 ppm	800 ppm	2 000 ppm
Body weight (g), day 90	553	537	540	534	540	295	292	296	297	288
Body weight gain (g), days 1–90	340	326	329	322	329	121	120	125	125	117
Feed consumption (g), day 8	25.6	25.7	25.6	25.7	25.1	19.9	21.1	18.9	18.3*	17.8**
Prothrombin time (s), day 90	15.0	14.7	16.0	16.4	22.7**	14.2	14.5	13.5	12.8*	12.5**
Total bilirubin (µmol/L), day 90	2.0	1.7	1.6	1.2**	1.0**	2.1	1.7	1.5**	1.1**	1.2**
Cholesterol (mmol/L), day 90	1.97	1.98	1.86	1.96	2.31	2.10	2.31	2.23	2.54	2.94**
TSH (ng/mL), weeks 3–4	6.1	5.7	6.7	6.7	10.0*	3.8	4.3	3.6	4.7	5.4
TSH (ng/mL), week 13	5.6	5.6	6.8	7.2	7.2	3.8	3.4	4.3	4.1	7.7
Terminal body weight (g)	521	514	512	506	505	286	277	279	281	271
Absolute liver weight (g)	11.5	11.7	11.9	12.2	13.5**	6.5	6.4	6.7	7.6**	8.7**
Relative liver weight (% of body weight)	2.19	2.28	2.32	2.41*	2.68**	2.28	2.31	2.40	2.71**	3.22**
Liver: enlarged	0/10	0/10	1/10	3/10	7/10	0/10	0/10	2/10	7/10	8/9
Liver: dark	0/10	0/10	0/10	0/10	3/10	0/10	0/10	0/10	0/10	2/9
Liver: prominent lobulation	0/10	1/10	0/10	3/10	4/10	0/10	0/10	0/10	3/10	2/9
Liver: centrilobular hepatocellular hypertrophy, diffuse										
- Minimal	0/10	0/10	0/10	6/10	4/10	0/10	0/10	0/10	0/10	5/9
- Slight	0/10	0/10	0/10	1/10	6/10	0/10	0/10	0/10	0/10	1/9
- Total	0/10	0/10	0/10	7/10	10/10	0/10	0/10	0/10	0/10	6/9
Thyroid: follicular cell hypertrophy, diffuse										
- Minimal	0/10	0/10	0/10	6/10	4/10	0/10	0/10	0/10	1/10	4/9
- Slight	0/10	0/10	0/10	0/10	5/10	0/10	0/10	0/10	1/10	1/9
- Total	0/10	0/10	0/10	6/10	9/10	0/10	0/10	0/10	2/10	5/9

ppm: parts per million; TSH: thyroid stimulating hormone; *: $P < 0.05$; **: $P < 0.01$
Source: Steiblen (2005b)

Dogs

In a study of toxicity conducted according to OECD Test Guideline 409, groups of four male and four female Beagle dogs were administered bixafen (purity 95.8%) in 0.5% aqueous carboxymethyl cellulose at a dosing volume of 5 mL/kg bw by oral gavage at a dose of 0, 100, 300 or 1000 mg/kg bw per day for 91–94 days. Clinical observations were conducted daily, feed consumption was measured daily and body weights were taken weekly. Clinical chemistry, urine analysis and complete blood count, including differentials, were performed on all animals once prior to administration of the test substance. Following initiation of dosing, haematological, clinical chemistry and urine analysis data were collected from all animals during study weeks 5, 9 and 13.

Ophthalmic examinations were performed pre-exposure and pretermination. A gross necropsy was performed, organ weights were taken and tissues were examined microscopically.

One female in the high-dose group was sacrificed in extremis on study day 87 due to aspiration of the dosing material.

Pale faeces, observed in males and females in the high-dose group, were the only compound-related clinical finding. However, this finding was not of toxicological significance.

There were no treatment-related effects on body weight or feed consumption. No treatment-related ocular abnormalities were observed at ophthalmic examination.

Haematological examination showed a non-statistical decrease in red blood cell counts and statistically significant decreases in haemoglobin and haematocrit on days 58 and 86 in males in the high-dose group (Table 21); no effects were seen at 100 or 300 mg/kg bw per day.

There were no treatment-related clinical chemistry changes or urine analysis findings at any dose level.

Terminal body weights were not affected by compound administration. Compound-related increases in both absolute (non-statistically significant) and relative (statistically significant) liver weights occurred in males and females in the mid- and high-dose groups (Table 21).

Table 21. Summary of selected findings in the 90-day dog study

	Males				Females			
	0 mg/kg bw per day	100 mg/kg bw per day	300 mg/kg bw per day	1 000 mg/kg bw per day	0 mg/kg bw per day	100 mg/kg bw per day	300 mg/kg bw per day	1 000 mg/kg bw per day
Red blood cells (10^6/mL)								
- Day 58	6.91	6.41	6.51	5.99	6.72	6.76	6.60	6.68
- Day 86	7.19	6.86	6.68	6.10	7.02	6.84	6.62	7.06
Haemoglobin (g/dL)								
- Day 58	15.8	14.9	14.8	13.5*	15.1	15.4	14.7	15.3
- Day 86	16.6	16.1	15.5	13.9*	15.8	15.7	15.1	16.3
Haematocrit (%)								
- Day 58	46.2	43.4	43.4	39.5*	44.8	45.1	43.3	45.1
- Day 86	48.5	46.7	45.0	40.5*	46.3	45.7	43.7	47.9
Terminal body weight (kg)	11.15	10.08	11.10	10.43	8.03	7.76	8.08	7.55
Absolute liver weight (g)	351	337	415	432	258	280	316	309
Relative liver weight (% of body weight)	3.14	3.35	3.73*	4.15*	3.22	3.60	3.90*	4.09*
Liver: cytoplasmic vacuolization	0/4	0/4	0/4	2/4	0/4	0/4	0/4	3/4

bw: body weight; *: $P < 0.05$
Source: Sheets (2009)

Micropathological evaluation was conducted on all tissues from the control and high-dose groups. Based on increased liver weights in males and females in the mid- and high-dose groups and on micropathological changes noted in males and females in the high-dose group, livers from animals in the mid- and high-dose groups were also processed and evaluated microscopically. Compound-related micropathological lesions were limited to livers of two males and three females in the high-dose group. The compound-related morphological change, coded as “cytoplasmic vacuolization”, consisted of enlarged hepatocytes, primarily centrilobular, with a vacuolated cytoplasm that did not displace the nucleus (Table 21). This cytoplasmic vacuolization would be more consistent with

glycogen than with fat. These enlarged hepatocytes correlate with the increased liver weights noted in the high-dose group.

The NOAEL was 100 mg/kg bw per day, based on an increase (> 20%) in absolute and relative liver weights of females at 300 mg/kg bw per day and above (Sheets, 2009).

In a study of toxicity conducted according to OECD Test Guideline 452, groups of four male and four female Beagle dogs were administered bixafen (purity 95.8%) in 0.5% aqueous carboxymethyl cellulose at a dosing volume of 5 mL/kg bw by oral gavage at a dose of 0, 10, 100 or 1000 mg/kg bw per day for 365–367 days. Clinical observations were conducted daily, feed consumption was measured daily and body weights were recorded weekly. Clinical chemistry, urine analysis and complete blood count, including differentials, were performed on all animals once prior to administration of the test substance and during study weeks 14, 27, 40 and 52. Ophthalmic examinations were performed pre-exposure and pretermination. A gross necropsy was performed, organ weights were recorded and tissues were examined microscopically.

One male from the high-dose group was sacrificed prematurely for humane reasons on day 247. This animal had approximately 20 mL of blood-tinged fluid oozing from the mouth. Grossly, this animal was noted to have severe, bilateral, red-discoloured zones with clear foam in the lungs. The condition of this animal was attributable to interstitial pneumonia, as microscopic examination of the lungs revealed a mild degree of haemorrhage, oedema and inflammatory cells in the interstitium. The finding was not considered to be treatment related.

The only compound-related clinical finding was pale faeces, observed in males and females in the high-dose group. However, this finding was not of toxicological significance, as the pale faeces were considered to be due to unabsorbed test compound.

There were no treatment-related effects on body weight or feed consumption. No treatment-related ocular abnormalities were observed at ophthalmic examination.

Treatment-related haematological changes were limited to red blood cell counts, haemoglobin and haematocrit values, which were statistically significantly decreased in males at 100 and 1000 mg/kg bw per day on days 92 and/or 183 (Table 22).

Treatment-related clinical chemistry changes were limited to alkaline phosphatase, which was increased in females at 1000 mg/kg bw per day (outside the historical control data range) and more subtly at 100 mg/kg bw per day (within the normal control range), and cholesterol levels, which were slightly increased in females at 100 and 1000 mg/kg bw per day (Table 22). None of the changes noted in the urine analysis parameters was considered to be treatment related.

Terminal body weights for males and females were not statistically significantly different from those of controls. However, there was a minimal trend of decreased mean terminal body weights (8.35%) in the high-dose females. Compound-related organ weight changes for the high-dose males and females were limited to increased relative liver weights with associated liver hypertrophy. Slight, centrilobular hypertrophy of the liver was present in one male and two females in the high-dose group (Table 22). These animals had increased absolute liver weights (non-statistically significant) and relative liver weights (statistically significant) at necropsy. The liver hypertrophy, coded as "centrilobular and midzonal hypertrophy, hepatocellular", was observed and was characterized by enlarged cells primarily involving the cytoplasm. The cytoplasmic appearance of hepatocytes varied from granular to intensively eosinophilic to pale. Often the intensively eosinophilic staining cytoplasmic organelles appeared to be compressed towards the outer edges of the cell.

The NOAEL was 10 mg/kg bw per day, based on haematological effects (decrease in red blood cell count, haemoglobin and haematocrit) in males and liver toxicity (increased liver weight, increased alkaline phosphatase and cholesterol levels) in females at 100 mg/kg bw per day (Eigenberg, 2008).

Table 22. Summary of selected findings in the 1-year dog study

	Males				Females			
	0 mg/kg bw per day	10 mg/kg bw per day	100 mg/kg bw per day	1 000 mg/kg bw per day	0 mg/kg bw per day	10 mg/kg bw per day	100 mg/kg bw per day	1 000 mg/kg bw per day
Red blood cells (10^6/mL)								
- Day 92	7.41	7.20	6.99	6.30*	6.94	7.21	6.37	6.84
- Day 183	7.82	7.24*	7.02*	6.50*	7.07	7.27	6.69	6.68
- Day 359	8.11	7.54	7.71	7.13	7.56	7.80	7.12	7.03
Haemoglobin (g/dL)								
- Day 92	17.3	17.1	16.4*	15.2*	16.2	16.6	15.4	16.9
- Day 183	18.6	17.6	16.7*	15.7*	17.0	16.9	16.5	16.6
- Day 359	19.1	18.2	18.1	17.1	18.1	18.1	17.2	17.4
Haematocrit (%)								
- Day 92	47.4	47.0	44.7*	41.3*	44.3	45.0	41.8	45.4
- Day 183	49.4	46.8	44.3*	42.0*	44.9	44.7	43.9	43.8
- Day 359	51.9	49.6	49.1	46.6	49.0	48.6	47.0	46.8
Alkaline phosphatase (U/L)								
- Day 183	59	89	102	112	66	74	84	152*
- Day 274	59	106	94	100	57	89	82	246*
- Day 359	53	98	90	105	42	77	87*	169*
Cholesterol (mg/dL)								
- Day 92	154	152	162	161	145	163	177*	187*
- Day 359	155	167	158	168	176	190	200	212
Terminal body weight (kg)	11.10	11.98	11.45	11.41	9.96	9.39	9.28	9.13
Absolute liver weight (g)	348	338	367	463	280	325	328	374
Relative liver weight (% of body weight)	3.14	2.81	3.22	4.04*	2.79	3.45*	3.55*	4.08*
Liver: hepatocellular hypertrophy	0	0	0	1	0	0	0	2
Liver: cytoplasmic pigmentation	1	2	4	3	1	2	1	3
Liver: cytoplasmic vacuolation	0	0	0	1	1	0	2	1

bw: body weight; U: units; *: $P < 0.05$
Source: Eigenberg (2008)

2.3 Long-term studies of toxicity and carcinogenicity

Mice

In a study of carcinogenicity conducted according to OECD Test Guideline 451, groups of 60 male and 60 female C57BL/6J mice were given diets containing bixafen (purity 95.8%) at a concentration of 0, 50, 150 or 500 ppm (equal to 0, 6.7, 20.4 and 69.0 mg/kg bw per day for males and 0, 8.6, 25.5 and 85.0 mg/kg bw per day for females, respectively) for at least 78 weeks. An additional 10 animals of each sex per group, similarly treated, were killed after at least 52 weeks of treatment for interim evaluation. After 20 weeks of treatment, the rodent diet was found not to contain a sufficient level of vitamin K_3 and was consequently supplemented to ensure that a minimum level of 2.5 ppm vitamin K_3 was present in the diet for the remainder of the study. Mortality and clinical signs were checked daily. Additionally, detailed physical examinations, including palpation for masses, were performed weekly throughout treatment. Body weight and feed consumption were measured weekly for the first 13 weeks of the study, then approximately monthly thereafter. Haematological

determinations were performed at approximately 12 and 18 months on blood from designated animals. Where possible, blood smears were prepared from moribund animals just before termination. At scheduled termination, body weights were recorded prior to necropsy. All animals were subjected to necropsy, with selected organs weighed at scheduled interim kill and final termination. Designated tissues were fixed and examined microscopically.

There were no treatment-related effects on mortality, clinical signs, body weight parameters, feed consumption or haematological parameters in females at any dose level (Table 23). There was no evidence of a treatment-related effect on tumour incidence in either sex.

In males, during the first 20 weeks of treatment, a number of effects were observed at 500 and 150 ppm, consisting of increased mortality rate at 500 ppm, with eight premature deaths attributed to a haemorrhagic syndrome (as evidenced macroscopically/microscopically by red foci, blood clots, red liquid content, haemorrhage or haematoma in one or more organs), clinical signs at 500 ppm (wasted appearance, reduced motor activity, hunched posture, cold to touch, limited use of limbs, tremors, rapid respiration, prostration and/or absence of righting reflex) and a reduction in mean cumulative body weight gain from weeks 9 to 22 at 500 and 150 ppm. These effects were attributed to the treatment, but were most likely exacerbated by the abnormally low vitamin K_3 content in the rodent diet. This relationship was supported by the absence of persisting effects after the change to a vitamin K_3–supplemented diet, designed to ensure an adequate vitamin K_3 supply to all animals for the remainder of the study.

The cause of death in 3/5 males at 500 ppm allocated to the interim (12-month) kill phase of the study that died or were humanely killed during the first 20 weeks of treatment was attributed to a haemorrhagic syndrome. One male and one female that died before the interim (12-month) kill presented a centrilobular hepatocellular hypertrophy, similar to the treatment-related effect observed at the terminal kill. After the first 20 weeks of study, the mortality rate was not affected by treatment, and no treatment-related clinical signs were recorded in males.

At 500 ppm, body weight was decreased in males at most time points from weeks 9 to 62 by up to 6% ($P < 0.05$ or $P < 0.01$ on most occasions), compared with controls. Cumulative body weight gain was lower than that of controls by 10% ($P < 0.05$) between weeks 1 and 14 and by 33% ($P < 0.01$) between weeks 14 and 26. Thereafter, body weight gain was similar to or higher than that of controls throughout the study. Body weight on day 540, overall cumulative body weight gain between days 1 and 540 and feed consumption throughout treatment were similar to those of the controls in males (Table 23). At 150 ppm, body weight was decreased in males at most time points from weeks 9 to 62 by up to 4% ($P < 0.05$ or $P < 0.01$ on a number of occasions), compared with controls. Cumulative body weight gain was lower than that of controls by 7% between weeks 1 and 14 and by 29% ($P < 0.01$) between weeks 14 and 26. Body weight on day 540 and overall cumulative body weight gain between days 1 and 540 were similar to those of the controls in males.

Haematological evaluation in males at 500 ppm revealed slightly higher platelet counts at month 12 (+20%, $P < 0.01$) and month 18/19 (+22%, $P < 0.01$). In males also, higher haemoglobin concentration and higher mean corpuscular volume were noted for the two sampling periods when compared with the control group. As a consequence, haematocrit and mean corpuscular haemoglobin were higher than in the controls. These variations were slight, their magnitude often being lower at month 18/19 (4–6%, $P < 0.01$) than at month 12 (5–7%, $P < 0.01$). In males at 150 ppm, the same tendencies were observed; however, in view of their low magnitude (3–4%), they were considered not to be biologically relevant. There were no treatment-related changes in haematological parameters in females at any dose level.

At the 12-month interim kill, absolute and relative liver weights were increased by 17–23% in males and 28–31% in females ($P < 0.01$) at 500 ppm, when compared with controls, and by 9–13% in both sexes at 150 ppm, the effect being statistically significant in males only ($P < 0.05$ or $P < 0.01$). Minimal to moderate centrilobular hepatocellular hypertrophy was seen in 6/10 males and 2/10 females at 500 ppm, associated with a decreased incidence of diffuse hepatocellular vacuolation in 5/10 males, an increased incidence of hepatocellular single-cell degeneration/necrosis in 6/10 males,

Table 23. Summary of selected findings in the 78-week mouse study

	Males				Females			
	0 ppm	50 ppm	150 ppm	500 ppm	0 ppm	50 ppm	150 ppm	500 ppm
Unscheduled deaths								
- Week 53	3/60	5/60	3/60	15/60	6/60	5/60	5/60	7/60
- Week 78	8/50	7/50	9/50	12/50	10/50	8/50	12/50	13/50
Body weight (g)								
- Week 26	30.2	30.1	29.1**	28.9**	23.3	23.3	23.3	23.2
- Week 54	32.0	32.0	30.9*	30.9*	25.1	25.9*	25.7	26.1**
- Week 78	31.8	32.2	31.1	31.4	26.5	26.9	27.0	26.8
Terminal body weight (g), 12 months	27.1	28.0	27.6	26.1	22.0	22.1	21.6	22.3
Absolute liver weight (g), 12 months	1.18	1.22	1.31**	1.40**	1.00	1.04	1.11	1.30**
Relative liver weight (% of body weight), 12 months	4.36	4.35	4.75**	5.34**	4.54	4.71	5.12	5.81**
Microscopic findings at the 12-month kill								
No. of animals examined	10	10	10	10	10	10	10	10
Centrilobular hepatocellular hypertrophy, diffuse								
- Minimal	0	0	5	1	0	0	0	1
- Slight	0	0	0	3	0	0	0	1
- Moderate	0	0	0	2	0	0	0	0
- Total	0	0	5	6	0	0	0	2
Hepatocellular vacuolation, diffuse								
- Minimal	4	6	7	4	8	9	10	8
- Slight	5	2	3	1	0	0	0	0
- Moderate	1	0	0	0	0	0	0	0
- Total	10	8	10	5	8	9	10	8
Hepatocellular single-cell degeneration/necrosis, focal/multifocal								
- Minimal	0	0	0	5	0	0	0	0
- Slight	0	0	0	1	0	0	0	0
- Total	0	0	0	6	0	0	0	0
Multinucleated hepatocytes, focal/multifocal								
- Minimal	0	0	0	4	0	0	0	0
Hepatocellular brown pigment, focal/multifocal								
- Minimal	0	0	3	4	0	0	0	0

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$
Source: Langrand-Lerche (2008)

multinucleated hepatocytes in 4/10 males and hepatocellular brown pigment in 4/10 males. At 150 ppm, minimal centrilobular hepatocellular hypertrophy was seen in 5/10 males, associated with hepatocellular brown pigment in 3/10 males (Table 23).

The cause of death in 5/12 males and 1/13 females at 500 ppm allocated to the carcinogenicity phase (18 months) of the study that died or were humanely killed before the end of the study was attributed to a haemorrhagic syndrome. These deaths were confined to the first 20 weeks of the study. In addition, dark liver was noted in 4/12 males at macroscopic examination. At 150 ppm, no treatment-related cause of death was found for animals that died or were humanely killed before the end of the study.

At the 18-month terminal kill of the carcinogenicity phase of the study, absolute and relative liver weights were increased by 26–29% in males and 25–27% in females ($P < 0.01$) at 500 ppm, when compared with controls, by 19–20% in males and 12–14% in females ($P < 0.01$) at 150 ppm, and by 8–9% in females ($P < 0.01$) at 50 ppm. Also at 500 ppm, absolute and relative adrenal gland weights were higher by 27–32% in males ($P < 0.01$) when compared with controls (Table 24).

Macroscopic examination revealed dark liver in 16/38 males and 6/37 females at 500 ppm and enlarged liver in 13/38 males and 25/37 females at 500 ppm. Microscopic examination at 500 ppm revealed a minimal to marked centrilobular hepatocellular hypertrophy and a decreased incidence of diffuse hepatocellular vacuolation in both sexes and a higher incidence of focal/multifocal hepatocellular single-cell degeneration/necrosis, multinucleated hepatocytes, hepatocellular brown pigment, hepatocellular necrotic focus and interstitial mononuclear cell infiltrate in males. At 150 ppm, minimal to moderate centrilobular hepatocellular hypertrophy was seen in 37/50 males, associated with a decreased incidence of diffuse hepatocellular vacuolation in 27/50 males, and a higher incidence of hepatocellular single-cell degeneration/necrosis and multinucleated hepatocytes was seen in 5/50 males. At 50 ppm, minimal centrilobular hepatocellular hypertrophy was seen in 2/50 males.

In the thyroid gland, a higher incidence of focal/multifocal follicular cell hyperplasia was noted in both sexes at 500 ppm and in females at 150 ppm (Table 24), whereas in the adrenal gland, a decreased incidence of cortical atrophy was noted in males at 500 ppm.

The NOAEL for carcinogenicity was 500 ppm (equal to 69.0 mg/kg bw per day for males and 85.0 mg/kg bw per day for females), the highest dose tested. The NOAEL for toxicity was 50 ppm (equal to 6.7 mg/kg bw per day for males and 8.6 mg/kg bw per day for females), based on decreased body weights and liver toxicity (single-cell degeneration/necrosis) in males and thyroid effects (follicular cell hyperplasia) in females at 150 ppm (equal to 20.4 mg/kg bw per day for males and 25.5 mg/kg bw per day for females) and above (Langrand-Lerche, 2008).

Rats

In a combined study of chronic toxicity and carcinogenicity conducted according to OECD Test Guideline 453, groups of 70 or 80 female Wistar Rj:WI (IOPS HAN) rats were given diets containing bixafen (purity 95.8%) at a concentration of 0, 50, 300 or 2000 ppm (equal to 0, 2.8, 17.4 and 117 mg/kg bw per day) for at least 24 months. After 13 weeks of exposure, 10 females allocated to the satellite groups in the controls and at the two highest dose levels were necropsied at the first scheduled interim kill and subjected to limited histopathological examination (liver, thyroid and macroscopic findings). After 52 weeks, 10 females from each group allocated to the chronic (12-month) phase were necropsied at the second scheduled interim kill. The remaining 60 females per group, allocated to the carcinogenicity (24-month) phase of the study, continued treatment until final termination after at least 104 weeks of treatment, when surviving animals were necropsied. Mortality and clinical signs were checked 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, approximately weekly from week 7 up to week 13, then every 4 weeks thereafter. Ophthalmological examinations were performed on all animals during

Table 24. Summary of selected findings in the 78-week mouse study at terminal sacrifice

	Males				Females			
	0 ppm	50 ppm	150 ppm	500 ppm	0 ppm	50 ppm	150 ppm	500 ppm
Terminal body weight (g), 18 months	28.1	28.4	27.5	27.5	23.5	23.7	23.8	23.4
Absolute liver weight (g), 18 months	1.18	1.23	1.41**	1.49**	1.18	1.29**	1.35**	1.48**
Relative liver weight (% of body weight), 18 months	4.22	4.33	5.07**	5.43**	5.03	5.43**	5.66*	6.30**
Absolute adrenal weight (mg), 18 months	4.4	4.7	4.7	5.6**	8.6	8.3	9.1	9.1
Relative adrenal weight (% of body weight, ×100), 18 months	1.56	1.65	1.71	2.05**	3.66	3.49	3.80	3.87
Liver, enlarged, 18 months	0/42	0/43	1/41	13/38	2/40	7/42	9/38	25/37
Liver, dark, 18 months	1/42	2/43	3/41	16/38	0/40	0/42	0/38	6/37
Microscopic findings at the 18-month sacrifice								
Liver								
No. of animals examined	50	50	50	50	50	50	50	50
Centrilobular hepatocellular hypertrophy; diffuse								
- Minimal	0	2	17	4	0	0	0	12
- Slight	0	0	18	8	0	0	0	3
- Moderate	0	0	2	20	0	0	0	0
- Marked	0	0	0	15	0	0	0	0
- Total	0	2	37**	47**	0	0	0	15**
Hepatocellular vacuolation, diffuse								
- Minimal	17	24	22	16	11	12	11	14
- Slight	14	9	5	0	15	22	25	6
- Moderate	0	0	0	0	11	4	2	1
- Total	31	33	27	16*	37	38	38	21**
Hepatocellular single-cell degeneration/necrosis, focal/multifocal								
- Minimal	0	0	4	23	0	1	0	0
- Slight	0	0	1	4	0	0	0	0
- Moderate	0	0	0	1	0	0	0	0
- Total	0	0	5*	28**	0	1	0	0
Multinucleated hepatocytes, focal/multifocal								
- Minimal	1	2	5	37	0	1	0	0
- Slight	0	0	0	2	0	0	0	0
- Total	1	2	5	39**	0	1	0	0

	Males				Females			
	0 ppm	50 ppm	150 ppm	500 ppm	0 ppm	50 ppm	150 ppm	500 ppm
Hepatocellular brown pigment, focal/multifocal								
- Minimal	0	0	0	14	0	0	0	0
- Slight	0	0	0	2	0	0	0	0
- Total	0	0	0	16**	0	0	0	0
Hepatocellular necrotic focus, focal/multifocal								
- Minimal	2	2	2	6	3	4	5	4
- Slight	2	0	2	3	1	0	0	0
- Moderate	0	0	0	0	1	0	1	1
- Total	4	2	4	9	5	4	6	5
Interstitial mononuclear cell infiltrate, focal/multifocal								
- Minimal	10	12	11	20	23	18	15	21
- Slight	2	2	1	3	4	5	9	4
- Moderate	1	0	0	1	0	0	1	1
- Total	13	14	12	24**	27	23	25	26
Thyroid								
No. of animals examined	48	50	50	50	48	50	50	50
Follicular cell hyperplasia, focal/multifocal								
- Minimal	4	3	5	11	11	8	15	26
- Slight	2	2	1	2	1	2	5	6
- Moderate	0	0	0	0	0	1	0	1
- Total	6	5	6	13*	12	11	20*	33**
Adrenal gland								
No. of animals examined	50	50	50	50	48	49	50	50
Cortical atrophy, focal/multifocal	42	45	35	14	0	0	1	0

ppm : parts per million ; *: $P < 0.05$; **: $P < 0.01$
Source: Langrand-Lerche (2008)

acclimatization and after approximately 12 and 24 months. Haematology and clinical chemistry determinations and urine analysis were performed during months 3/4, 6, 12, 18 and 24 on selected animals. In addition, coagulation parameters were measured in satellite groups in weeks 5 and 14. All surviving animals allocated to the chronic and carcinogenicity phases were subjected to necropsy after a minimum of 52 weeks or 104 weeks of treatment, respectively. Selected organs were weighed, and designated tissues were sampled and examined microscopically.

In the male rats originally included in the study, increased mortality (3/80, 0/70, 6/70, 9/80 and 28/80 at 0, 50, 150, 300 and 1000 ppm, respectively), elevated coagulation parameter times and haemorrhagic syndrome were observed (Tables 25 and 26). Therefore, all male animals were prematurely killed (males treated at 1000 ppm: on study day 169; males from all other groups: on study day 239) without full necropsy, and a separate study on the chronic toxicity and oncogenic potential of bixafen in male Wistar rats was conducted (Garcin, 2008; see below).

Table 25. Individual mortality in males from the initial chronic toxicity and carcinogenicity study in rats

Group	Animal no.	Day of death	Mode of death	Necropsy findings
Control	1684	104	Found dead	Stomach and/or intestines: dark contents
	1676	142	Found dead	Stomach and/or intestines: dark contents
	1711	146	Killed for humane reasons	–
150 ppm	2009	103	Found dead	Stomach and/or intestines: dark contents
	1984	104	Killed for humane reasons	–
	2010	111	Killed for humane reasons	Stomach and/or intestines: dark contents
	2032	145	Killed for humane reasons	Stomach and/or intestines: dark contents
	1979	176	Found dead	Stomach and/or intestines: dark contents
	1971	177	Found dead	–
300 ppm	2038	93	Found dead	Stomach and/or intestines: dark contents
	2047	124	Found dead	–
	2090	134	Killed for humane reasons	Stomach and/or intestines: dark contents
	2099	134	Found dead	Stomach and/or intestines: dark contents
	2059	142	Killed for humane reasons	Stomach and/or intestines: dark contents
	2053	148	Killed for humane reasons	Stomach and/or intestines: dark contents
	2087	160	Killed for humane reasons	–
	2061	165	Found dead	Stomach and/or intestines: dark contents
	2085	170	Found dead	Stomach and/or intestines: dark contents
1 000 ppm	2259	33	Found dead	–
	2197	34	Killed for humane reasons	–
	2218	44	Killed for humane reasons	Stomach and/or intestines: dark contents
	2246	75	Killed for humane reasons	–
	2235	84	Killed for humane reasons	Stomach and/or intestines: dark contents
	2244	84	Found dead	Stomach and/or intestines: dark contents
	2213	93	Found dead	Stomach and/or intestines: dark contents
	2216	93	Found dead	Stomach and/or intestines: dark contents
	2217	93	Found dead	Stomach and/or intestines: dark contents
	2226	96	Killed for humane reasons	Stomach and/or intestines: dark contents
	2273	96	Killed for humane reasons	Stomach and/or intestines: dark contents
	2263	97	Found dead	Stomach and/or intestines: dark contents
	2238	99	Found dead	Stomach and/or intestines: dark contents
	2224	107	Found dead	Stomach and/or intestines: dark contents
	2230	114	Killed for humane reasons	Stomach and/or intestines: dark contents
	2234	117	Found dead	–
	2205	118	Found dead	Stomach and/or intestines: dark contents
	2206	126	Found dead	Stomach and/or intestines: dark contents
	2267	138	Killed for humane reasons	Stomach and/or intestines: dark contents
	2269	138	Killed for humane reasons	–
	2207	139	Killed for humane reasons	Stomach and/or intestines: dark contents
	2225	140	Found dead	Stomach and/or intestines: dark contents
	2222	141	Killed for humane reasons	Stomach and/or intestines: dark contents

Group	Animal no.	Day of death	Mode of death	Necropsy findings
	2268	152	Killed for humane reasons	Stomach and/or intestines: dark contents
	2255	156	Killed for humane reasons	Stomach and/or intestines: dark contents
	2258	156	Killed for humane reasons	Stomach and/or intestines: dark contents
	2257	160	Killed for humane reasons	Stomach and/or intestines: dark contents
	2245	167	Killed for humane reasons	Stomach and/or intestines: dark contents

Source: McElligott (2008)

Table 26. Selected coagulation parameters from the initial chronic toxicity and carcinogenicity study in rats

	Males					Females			
	0 ppm	50 ppm	150 ppm	300 ppm	1 000 ppm	0 ppm	50 ppm	300 ppm	1 000 ppm
PT (s)									
- Week 4/5, satellite	19.4	ND	ND	26.7	40.2**	14.3	ND	14.6	14.0
- 3 months, main	21.0	18.8	27.1	28.5	46.1**	14.8	14.3	13.8*	14.1
- 3 months, satellite	24.1	ND	ND	33.5	45.8*	14.8	ND	13.8	14.3
- 6 months, main	17.2	17.3	17.0	17.0	ND	13.8	13.1	13.3	14.5
- 12 months, main	ND	ND	ND	ND	ND	16.3	15.6	15.9	15.5*
- 18 months, main	ND	ND	ND	ND	ND	15.7	15.2	15.5	14.4*
- 24 months, main	ND	ND	ND	ND	ND	15.6	15.6	15.2	14.9
APTT (s)									
- Week 4/5, satellite	27.9	ND	ND	38.9	51.8**	17.1	ND	17.7	20.2**
- 3 months, main	30.5	28.8	37.3	39.7	56.0**	16.2	ND	17.3	21.6**
- 3 months, satellite	32.7	ND	ND	41.5	58.8**	18.5	18.7	20.0	22.5**
- 6 months, main	18.8	18.1	18.9	19.3	ND	18.7	17.8	19.2	24.0*
- 12 months, main	ND	ND	ND	ND	ND	15.8	15.7	15.2	14.6*
- 18 months, main	ND	ND	ND	ND	ND	15.3	15.1	15.6	15.5
- 24 months, main	ND	ND	ND	ND	ND	14.6	14.4	14.5	14.4

APTT: activated partial thromboplastin time; ND: not determined; ppm: parts per million; PT: prothrombin time; *: $P < 0.05$; **: $P < 0.01$
Source: McElligott (2008)

As the mortality was unanticipated and there was evidence that the controls were also affected, albeit not to the extent of the treated animals, investigations into the reason for the mortalities commenced. One theory was that there may have been insufficient vitamin K in the diet. In fact, the vitamin K levels of the batches used in the earlier phase of this study (i.e. from 21 April 2005 to 5 October 2005) were not reported. However, analysis of the diet by the manufacturer showed that batch 50607, which was used from 6 to 12 October 2005 in the performing laboratory, contained less than 0.05 mg/kg of vitamin K_1 and less than 0.3 mg/kg of vitamin K_3. Thus, at least the vitamin K level of the diet from batch 50607 was significantly lower than the estimate of the requirement of 1 mg/kg for the Wistar rat. Discussions with the manufacturer of the diet revealed that it had moved manufacture of the diet in the middle of 2004 and had implemented new radiation and storage sterilization procedures, which temporally coincided with the deficit in vitamin K in the diet.

Based on the analyses, it was agreed with the manufacturers to supplement the diet with 15 mg/kg of the synthetic vitamin K_3 analogue menadione (batch 50720, used from 13 October 2005 to 28 December 2005, contained 15.7 mg/kg of menadione). The subsequent batches of diet used for the long-term study in female rats as well as in the supplementary long-term study in male rats (Garcin, 2008; see below) contained slightly lower amounts of menadione (7.1–10.6 mg/kg) than the target level of 15 mg/kg, but still adequate levels of the vitamin (for details, see section 2.6).

The effects of bixafen on blood coagulation parameters (prothrombin time, PT; activated partial thromboplastin time, APTT) of rats from the initial chronic toxicity and carcinogenicity study of rats receiving a vitamin K_3–deficient diet are shown in Table 26. After approximately 5–6 months of treatment, the male rats at 1000 ppm were taken from the study following significant mortality and signs of haemorrhage. After approximately 6 months of treatment, the diet was supplemented with 15 mg/kg of the synthetic vitamin K_3 analogue menadione.

In the female rats, no effect on mortality was observed at any dose level during the entire study. During the 1st year of treatment, the only treatment-related clinical sign recorded at 2000 ppm was a slightly higher incidence of hair loss compared with the control group. During the 2nd year of treatment, a slightly higher incidence of wasted appearance and genital discharge was observed at 2000 ppm in comparison with the controls; these nonspecific signs are commonly associated with ageing rats. At the ophthalmological examination at 12 months, no findings were observed at any dose. At 24 months, an increased incidence of posterior opacity of the lens was observed at all dose levels, although not in a dose-related manner, and a slightly increased incidence of abnormal colour (pale) of the retina fundus was also noted at all dose levels. In the absence of any indication of lens degeneration and in the absence of any increased retinal abnormality compared with the control group at the microscopic examination, these findings were considered not to be toxicologically relevant and not attributable to treatment (Table 27).

Treatment-related effects on body weight and body weight gain were observed at 2000 ppm throughout the study compared with the control group (Table 27). Body weight was decreased on day 8 by 3% ($P < 0.05$) and was reduced by 3%, 6%, 13%, 20% and 19% in weeks 14, 26, 54, 78 and 102, respectively; the effect was statistically significant ($P < 0.01$) for each interval. Cumulative body weight gain was also reduced at 2000 ppm throughout the study, with a reduction of 26% ($P < 0.01$) on week 1, and the overall cumulative body weight gain was reduced by 34% ($P < 0.01$) compared with the control group between day 1 and day 708. Feed consumption at 2000 ppm was reduced by 5%, 12%, 9%, 8% and 5% for the interval weeks 1–13, weeks 14–26, weeks 27–52, weeks 53–78 and weeks 79–104, respectively, in comparison with the control.

The APTT was increased at 2000 ppm for the satellite group at weeks 5 and 14 (+18% and +33%, respectively, $P < 0.01$) and for the chronic toxicity/carcinogenicity group in months 3/4 and 6 (+22% and +28%, respectively, $P < 0.01$). These changes were considered to be a consequence of the vitamin K_3 deficiency in the diet. Following the change to a vitamin K_3–adequate diet, no relevant change was observed at months 12, 18 and 24 (Table 27).

Increased total cholesterol concentrations were observed at 2000 ppm, with a decreasing magnitude from months 3/4 to 18 (from 68% to 36%, statistically significant for each time point), together with higher triglyceride concentrations at months 3/4 and 6 only (+74%, $P < 0.01$, and +27%, $P < 0.05$, respectively), whereas at 300 ppm, a significantly higher total cholesterol concentration (24% and 21%, respectively) was observed at months 3/4 and 6 only. Lower total bilirubin concentrations were noted at months 12, 18 and 24 at all dietary levels. The variations observed at months 3/4 and 6 were considered to be minimal in view of their low magnitude and the absence of a dose–effect relationship (Table 27). The slightly lower enzyme activities (aspartate aminotransferase, alanine aminotransferase and/or alkaline phosphatase) observed at 2000 and 300 ppm throughout the sampling periods were considered not to be adverse effects of the test substance.

Table 27. Selected findings in the 24-month rat study (females only)

	0 ppm	50 ppm	300 ppm	2 000 ppm
Mortality (no.)				
- Chronic toxicity and carcinogenicity phase, 12 months	2/80	3/70	1/80	2/80
- Carcinogenicity phase, 24 months	33/60	22/60	30/60	26/60
Hair loss (no.), chronic toxicity and carcinogenicity phase, 12 months	7/80	3/70	6/80	14/80
Posterior opacity of lens, 24 months	11/30	34/40	18/33	31/40
Retinal fundus abnormal colour (pale), 24 months	3/30	6/40	6/33	10/40
Body weight (g)				
- Day 1	195	195	194	194
- Day 8	214	216	211	208*
- Day 92	289	291	289	279**
- Day 176	321	321	316	301**
- Day 372	374	376	359	324**
- Day 540	425	419	399	342**
- Day 708	444	429	412	360**
Overall body weight gain (g), days 1–708	250	234	220	166**
Feed consumption (g/animal per day)				
- Weeks 1–13	19.2	19.9	20.1	18.3
- Weeks 14–26	18.7	18.9	18.3	16.4
- Weeks 27–52	19.1	19.3	19.2	17.4
- Weeks 53–78	20.1	19.9	20.0	18.4
- Weeks 79–104	22.2	21.6	22.6	21.1
APTT (s)				
- Week 5, satellite group	17.1	ND	17.7	20.2**
- Week 14, satellite group	16.2	ND	17.3	21.6**
- Month 3/4	18.5	18.7	20.0	22.5**
- Month 6	18.7	17.8	19.2	24.0**
- Month 12	15.8	15.7	15.2	14.6*
- Month 18	15.3	15.1	15.6	15.5
- Month 24	14.6	14.4	14.5	14.4
Cholesterol (mmol/L)				
- Month 3/4	1.75	1.80	2.17*	2.94**
- Month 6	1.92	1.96	2.33**	3.09**
- Month 12	2.16	2.31	2.43	3.19**
- Month 18	2.05	2.23	2.24	2.79*
- Month 24	2.33	2.27	2.52	2.60
Total bilirubin (µmol/L)				
- Month 3/4	4.0	3.4**	3.1**	3.6*
- Month 6	4.3	3.5**	3.6**	3.9*
- Month 12	2.6	1.9	1.1**	0.8**
- Month 18	2.8	1.9	1.2**	1.0**
- Month 24	1.7	1.2	1.0	0.7**

APTT: activated partial thromboplastin time; ND: not determined; ppm: parts per million: *: $P < 0.05$; **: $P < 0.01$
Source: McElligott (2008)

At the interim sacrifice of the 3-month satellite group, at 2000 ppm, absolute and relative liver weights were statistically significantly increased by 41–51% when compared with control animals. Macroscopic examination revealed a higher incidence of enlarged liver and/or dark liver compared with the controls, and at the microscopic examination, treatment-related effects were observed in the two organs examined (i.e. the liver and the thyroid gland). In the liver, a higher incidence and severity of diffuse centrilobular to panlobular hepatocellular hypertrophy were observed compared with the controls, and in the thyroid gland, minimal follicular cell hypertrophy was observed in some females (Table 28).

At the end of the chronic toxicity phase (12 months), at 2000 ppm, terminal body weight was lower (−8%, not statistically significant) in treated females compared with the controls. Absolute and relative liver weights were higher (by 31–42%, $P < 0.01$) when compared with controls. The macroscopic evaluation at 2000 ppm revealed enlarged liver and dark thyroid gland. At the microscopic examination, treatment-related effects were observed at 2000 ppm in the liver (minimal to moderate centrilobular to panlobular hepatocellular hypertrophy) and in the thyroid gland (higher incidence and severity of minimal to slight diffuse follicular cell hypertrophy and colloid alteration) (Table 28).

At the end of the carcinogenicity phase (24 months), terminal body weight was reduced at 2000 ppm by 18% ($P < 0.01$) and at 300 ppm by 7% (not statistically significant) when compared with controls (Table 29). Absolute and relative liver weights were increased by 17–41% ($P < 0.01$) at 2000 ppm, and relative liver weight was increased by 12% ($P < 0.01$) at 300 ppm. Also at 2000 ppm, relative thyroid weight was significantly higher (+41%; $P < 0.01$) compared with controls. At the macroscopic evaluation, higher incidences of enlarged and dark liver, dark thyroid and dark kidneys were observed at 2000 ppm compared with the controls.

Microscopic examination revealed treatment-related non-neoplastic changes in the liver and the thyroid gland at 300 and 2000 ppm and in the kidney at 2000 ppm. In the liver, minimal to slight centrilobular to panlobular hepatocellular hypertrophy was observed, and a higher incidence and severity of hepatocellular brown pigments and multinucleated hepatocytes were noted. In the thyroid gland, minimal to slight follicular cell hyperplasia was observed. A higher incidence and/or severity of follicular cell hypertrophy and colloid alteration were observed together with a higher incidence of brown pigments in follicular cells. In the kidney, a higher incidence and severity of intratubular golden/brown pigments were observed. In the absence of any cellular alteration in the kidney, this finding was considered to be treatment related but not adverse (Table 29).

In the thyroid gland, two follicular cell carcinomas were observed at each of 50 and 2000 ppm. Also, benign follicular cell adenomas were observed in two decedent females at 2000 ppm, but not in any of the final kill animals at the end of the 24-month treatment period. The incidence in the current study was within the control range of the Registry of Industrial Toxicology Animals (RITA) database (version from 21 September 2007), based on the incidence data from 98 studies with 4823 female rats examined in total: thyroid follicular cell adenoma was observed in 84 animals (1.7%; range 0–10%), whereas thyroid follicular cell adenocarcinoma was observed in 38 animals (0.8%; range 0–6%).

The NOAEL for carcinogenicity was 2000 ppm (equal to 117 mg/kg bw per day for females), the highest dose tested. The NOAEL for toxicity was 50 ppm (equal to 2.8 mg/kg bw per day for females), based on liver effects (increased cholesterol, higher incidence and/or severity of hepatocellular brown pigments and multinucleated hepatocytes) and thyroid effects (higher incidence and/or severity of follicular cell hypertrophy and colloid alteration) at 300 ppm (equal to 17.4 mg/kg bw per day for females) and above (McElligott, 2008).

Table 28. Selected findings at the interim sacrifices in the 24-month rat study (females only)

	0 ppm	50 ppm	300 ppm	2 000 ppm
3-month interim sacrifice				
Terminal body weight (g)	278.1	ND	276.3	269.5
Absolute liver weight (g)	6.17	ND	7.02	8.72**
Relative liver weight (% of body weight)	2.22	ND	2.54*	3.23**
Liver: large	0/10	ND	1/10	8/10
Liver: dark	2/10	ND	2/10	7/10
Liver: prominent lobulation	0/10	ND	7/10	7/10
Liver: centrilobular to panlobular hepatocellular hypertrophy				
- Minimal	1/10	ND	0/10	6/10
- Slight	0/10	ND	0/10	2/10
- Moderate	0/10	ND	0/10	1/10
- Total	1/10	ND	0/10	9/10
Thyroid: follicular cell hypertrophy, diffuse; minimal	0/10	ND	0/10	3/10
12-month interim sacrifice				
Terminal body weight (g)	344.7	330.7	343.7	317.1
Absolute liver weight (g)	7.72	7.72	8.51	10.09**
Relative liver weight (% of body weight)	2.24	2.33	2.48*	3.19**
No. of animals examined macroscopically	10	10	10	9
Liver: obviously large	0	0	3	8
Thyroid: dark	0	0	0	3
No. of animals examined microscopically	10	10	10	10
Liver: centrilobular to panlobular hepatocellular hypertrophy				
- Minimal	0	0	0	2
- Slight	0	0	0	5
- Moderate	0	0	0	2
- Total	0	0	0	9
Thyroid: follicular cell hypertrophy, diffuse				
- Minimal	1	1	1	5
- Slight	0	0	0	3
- Total	1	1	1	8
Thyroid: colloid alteration				
- Minimal	1	1	2	3
- Slight	0	1	0	2
- Total	1	2	2	5

ND: not determined; *: $P < 0.05$; **: $P < 0.01$
Source: McElligott (2008)

Table 29. Selected findings at the terminal sacrifice in the 24-month rat study (females only)

	0 ppm	50 ppm	300 ppm	2 000 ppm
Terminal body weight (g)	411.7	398.0	383.1	338.9**
Absolute liver weight (g)	9.89	9.80	10.39	11.57**
Relative liver weight (% of body weight)	2.43	2.46	2.73**	3.42**
Absolute thyroid weight (mg)	25.3	26.1	27.2	28.1
Relative thyroid weight (% of body weight, ×1 000)	6.00	6.54	7.11	8.43**
No. of animals examined macroscopically	27	38	30	34
Liver: obviously large	6	7	9	25
Liver: dark	1	0	1	23
Thyroid: dark	0	0	0	5
Kidney: dark	4	4	4	11
Liver				
No. of animals examined microscopically	60	60	59	60
Centrilobular to panlobular hepatocellular hypertrophy				
- Minimal	0	0	11	23
- Slight	0	0	1	19
- Total	0	0	12**	42**
Hepatocellular brown pigments, focal/multifocal				
- Minimal	1	0	5	14
- Slight	0	0	0	5
- Total	1	0	5	19**
Multinucleated hepatocytes, focal/multifocal				
- Minimal	5	4	8	15
- Slight	0	0	0	3
- Total	5	4	8	18*
Thyroid				
No. of animals examined microscopically	57	60	58	59
Follicular cell hyperplasia, focal/multifocal				
- Minimal	0	0	0	5
- Slight	0	0	0	3
- Total	0	0	0	8**
Follicular cell hypertrophy, diffuse				
- Minimal	1	2	9	24
- Slight	0	0	0	10
- Total	1	2	9*	34**
Colloid alteration				
- Minimal	11	14	15	7
- Slight	3	3	17	30
- Moderate	0	0	2	14
- Total	14	17	34**	51**
Brown pigments, follicular cells; minimal	2	2	3	7
Follicular cell adenoma	0	0	0	2

	0 ppm	50 ppm	300 ppm	2 000 ppm
Follicular cell carcinoma	0	2	0	2
Follicular cell adenoma + carcinoma	0	2	0	4
Kidney				
No. of animals examined microscopically	60	60	60	60
Intratubular golden/brown pigments, focal/multifocal				
- Minimal	20	16	20	34
- Slight	0	2	0	5
- Total	20	18	20	39**

*: $P < 0.05$; **: $P < 0.01$
Source: McElligott (2008)

In a complementary combined study of chronic toxicity and carcinogenicity conducted according to OECD Test Guideline 453, groups of 70 male Wistar Rj:WI (IOPS HAN) rats were given diets containing bixafen (purity 95.8%) at a concentration of 0, 50, 300 or 2000 ppm (equal to 0, 2.0, 12.1 and 80.5 mg/kg bw per day, respectively) for at least 24 months. After 54 weeks, the surviving 10 males from each group allocated to the chronic toxicity phase (12 months) were necropsied at the first scheduled interim kill. The remaining 60 animals of each sex per group, allocated to the carcinogenicity phase (24 months) of the study, continued treatment until final termination of the study after at least 104 weeks of treatment, when surviving animals were necropsied. Mortality and clinical signs were checked 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, approximately weekly from week 7 up to week 13, then every 4 weeks thereafter. Ophthalmological examinations were performed on all animals during acclimatization and after approximately 12 and 24 months. Haematology and clinical chemistry determinations and urine analysis were performed during months 3, 6, 12–13, 18 and 24 on selected animals. At the scheduled chronic toxicity and carcinogenicity phase kill, selected organs were weighed, and designated tissues were sampled and examined microscopically.

The overall incidence and percentage of mortality were similar between the control and treated groups and showed no evidence of a treatment-related increase throughout the study. There were no treatment-related clinical signs observed during treatment. Up to and including the highest dose level tested (2000 ppm), there was no significant effect on feed consumption, except for a minimal decrease observed at 2000 ppm only during the 1st week of the study. No treatment-related changes were observed at ophthalmoscopy or at urine analysis. At the end of the 24-month treatment period, there was no evidence of treatment-related neoplastic findings.

Body weight gain was reduced at 2000 ppm compared with the controls on days 8 and 15 of the study, by 8% and 5%, respectively ($P < 0.01$ and $P < 0.05$, respectively). Following the first 2 weeks of treatment, body weight gain was essentially comparable to that of the controls for the remainder of the 24-month treatment period (Table 30).

Increased cholesterol concentrations were observed during the 1st year of treatment at 2000 ppm (20–27%; statistically significant for each time point) and at 300 ppm (17–25%; statistically significant at months 6 and 12/13). Also, slightly higher urea and inorganic phosphorus concentrations were observed until month 18 at 2000 ppm, and lower total bilirubin concentrations were observed throughout the sampling periods at 2000 and 300 ppm (Table 30). The slightly lower enzyme activities (aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase) observed at 2000 ppm throughout the sampling periods and at 300 ppm at month 24 were considered not to be adverse effects of the test substance.

Table 30. Selected findings in the 24-month rat study (males only)

	0 ppm	50 ppm	300 ppm	2 000 ppm
Mortality (no.)				
- Chronic toxicity and carcinogenicity phase, 12 months	2/70	5/70	8/70	4/70
- Carcinogenicity phase, 24 months	35/60	40/60	43/60	38/60
Body weight (g)				
- Day 1	226	227	226	226
- Day 8	285	285	282	279
- Day 92	542	538	543	534
- Day 176	623	624	627	609
- Day 372	711	717	707	699
- Day 540	752	745	726	717
- Day 708	685	691	645	646
Body weight gain (g)				
- Days 1–8	59	59	57	54**
- Days 1–15	109	108	109	104*
Overall body weight gain (g), days 1–708	459	468	422	420
PT (s)				
- Month 3	17.3	16.3	16.2*	15.6**
- Month 6	17.0	16.9	16.1**	15.9**
- Month 12/13	16.2	16.3	16.1	15.0**
- Month 18	18.7	18.0	17.9	17.4**
- Month 24	18.9	17.7	17.7	16.7**
APTT (s)				
- Month 3	18.3	19.3	18.8	18.3
- Month 6	17.4	18.9**	18.2	18.1
- Month 12/13	17.9	18.0	18.1	18.2
- Month 18	15.7	15.3	15.4	14.5
- Month 24	14.4	14.1	14.0	13.5
Cholesterol (mmol/L)				
- Month 3	1.38	1.49	1.62	1.75*
- Month 6	1.69	1.86	2.08**	2.03*
- Month 12/13	1.90	2.07	2.37**	2.35**
- Month 18	2.48	2.55	2.68	2.65
- Month 24	2.22	3.15	2.59	3.14
Total bilirubin (µmol/L)				
- Month 3	1.0	0.6	0.5**	0.4**
- Month 6	1.8	1.3**	0.9**	0.8**
- Month 12/13	1.9	1.3*	1.0**	0.9**
- Month 18	2.2	1.6	1.2**	1.1**
- Month 24	1.6	1.5	1.0	0.8**

APTT: activated partial thromboplastin time; ppm: parts per million; PT: prothrombin time: *: $P < 0.05$; **: $P < 0.01$

Source: Garcin (2008)

At the end of the chronic toxicity phase (12 months), absolute and relative liver weights were increased by 34–37% at 2000 ppm and by 17–21% at 300 ppm, whereas absolute and relative thyroid weights were increased by 49–52% at 2000 ppm compared with the control group (Table 31). At the macroscopic examination, enlarged livers were observed in some animals at 2000 and 300 ppm, whereas dark thyroid glands were observed in 3/8 animals at 2000 ppm. These findings were correlated with microscopic changes. In the liver, a higher incidence of minimal to slight centrilobular to panlobular hepatocellular hypertrophy was observed at 2000 and 300 ppm, along with a higher incidence of eosinophilic and tigroid foci of hepatocellular alteration at 2000 ppm. In the thyroid gland, a higher incidence and severity of minimal to slight follicular cell hypertrophy and colloid alteration were observed at 2000 and 300 ppm.

Table 31. Selected findings at the 12-month interim sacrifice in the 24-month rat study (males only)

	0 ppm	50 ppm	300 ppm	2 000 ppm
Terminal body weight (g)	650.6	703.6	668.6	664.2
Absolute liver weight (g)	11.90	13.75*	14.30**	16.28**
Relative body weight (% of body weight)	1.83	1.95	2.14**	2.45**
Absolute thyroid weight (mg)	22.8	26.8	25.4	34.7**
Relative thyroid weight (% of body weight ×1 000)	3.52	3.80	3.80	5.25**
No. of animals examined macroscopically	9	8	8	8
Liver: enlarged	0	0	2	7
Thyroid: dark	0	0	0	3
No. of animals examined microscopically	9	8	8	8
Liver				
Centrilobular to panlobular hepatocellular hypertrophy				
- Minimal	0	0	7	3
- Slight	0	0	0	5
- Total	0	0	7	8
Eosinophilic foci of hepatocellular alteration	4	4	3	7
Tigroid foci of hepatocellular alteration	3	2	3	7
Thyroid				
Follicular cell hypertrophy, diffuse				
- Minimal	0	0	2	5
- Slight	0	0	0	1
- Total	0	0	2	6
Colloid alteration				
- Minimal	3	3	4	6
- Slight	0	0	2	2
- Total	3	3	6	8

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$
Source: Garcin (2008)

At the end of the carcinogenicity phase (24 months), absolute and relative liver weights were increased by 22–32% at 2000 ppm compared with controls, and at macroscopic examination, a higher incidence of enlarged and dark livers with a higher incidence of white foci was observed at 2000 ppm (Table 32). At the microscopic examination, a higher incidence of minimal to moderate centrilobular

Table 32. Selected findings at the terminal sacrifice in the 24-month rat study (males only)

	0 ppm	50 ppm	300 ppm	2 000 ppm
Terminal body weight (g)	642.7	639.0	598.3	597.4
Absolute liver weight (g)	12.72	12.76	12.91	15.62**
Relative liver weight (% of body weight)	1.99	1.99	2.16	2.63**
Absolute thyroid weight (mg)	51.6	41.2	35.5	41.1
Relative thyroid weight (% of body weight, ×1 000)	8.32	6.41	5.97	7.12
No. of animals examined macroscopically	25	20	17	22
Liver: enlarged	2	0	0	5
Liver: dark	0	0	0	9
Liver: foci, white	4	5	8	9
Liver				
No. of animals examined microscopically	60	60	60	60
Centrilobular to panlobular hepatocellular hypertrophy				
- Minimal	0	0	13	20
- Slight	0	0	0	20
- Moderate	0	0	0	1
- Total	0	0	13**	41**
Hepatocellular brown pigments, focal/multifocal; minimal	0	0	1	9**
Eosinophilic foci of hepatocellular alteration				
- Minimal	41	39	35	39
- Slight	6	1	6	11
- Moderate	0	1	1	2
- Total	47	41	42	52*
Cystic degeneration, focal/multifocal				
- Minimal	1	5	0	6
- Slight	1	0	0	4
- Total	2	5	0	10*
Thyroid				
No. of animals examined microscopically	60	60	60	59
Follicular cell hyperplasia, focal/multifocal				
- Minimal	0	1	0	4
- Slight	1	0	1	1
- Total	1	1	1	5
Follicular cell hypertrophy, diffuse; minimal	1	0	1	5
Colloid alteration				
- Minimal	17	26	20	14
- Slight	7	8	14	33
- Moderate	0	0	2	5
- Total	24	34*#	36**	52**
Brown pigments, follicular cells; minimal	4	2	4	15**
Follicular cell adenoma	0	0	0	0
Follicular cell carcinoma	1	0	0	0

	0 ppm	50 ppm	300 ppm	2 000 ppm
Epididymides				
No. of animals examined microscopically	59	60	60	60
Oligospermia				
- Bilateral	5	7	12	11
- Unilateral	18	13	10	13
Testes				
No. of animals examined microscopically	59	60	60	60
Tubular degeneration, diffuse				
- Bilateral	4	7	9	10
- Unilateral	18	10	11	13

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$; #: not statistically significant using a two-sided test
Source: Garcin (2008)

to panlobular hepatocellular hypertrophy was noted at 2000 and 300 ppm, whereas higher incidences of minimal hepatocellular brown pigments, of eosinophilic foci of hepatocellular alteration and of cystic degeneration were observed at 2000 ppm.

In the thyroid gland, higher incidences of focal/multifocal follicular cell hyperplasia, diffuse follicular cell hypertrophy and brown pigments in follicular cells were observed at 2000 ppm (Table 32). Also, a statistically significant higher incidence and/or severity of colloid alteration were observed at 50 ppm and above when applying a one-sided statistical test. However, the one-sided statistical analysis may be considered overly conservative when applied to non-neoplastic changes that could indeed be either increased or decreased by the treatment and are occurring with a high frequency in the control group. In such cases, a two-sided test would be more appropriate than a one-sided test. When applying the two-sided statistical analysis, the incidence of colloid alteration in the 50 ppm dose group was not statistically significant compared with the control group.

The alteration of colloid (stippled, granular or clumped colloid with variable staining characteristics) was considered not to be adverse at 50 ppm for the following reasons:

1) This finding is seen in the thyroid gland of control rats and reflects a normal age-related physiological process associated with the rapid turnover of colloid.
2) The female thyroid gland appeared to be more sensitive to bixafen, as evidenced by the occurrence of diffuse follicular cell hypertrophy at 300 ppm (9/58, $P < 0.05$) and 2000 ppm (34/59, $P < 0.01$) and of follicular cell hyperplasia at 2000 ppm (8/59, $P < 0.01$), but no difference in colloid alteration was observed in females at 50 ppm (McElligott, 2008).
3) The incidence was only slightly above the highest incidence seen in historical controls provided for five studies (colloid alteration was observed in 91/282 animals [32.3%], with a range from 11/50 to 28/58 [22–48%]), and there was no difference in severity when compared with controls.
4) Colloid alteration was not associated with any other findings indicative of stimulation (follicular cell hypertrophy or hyperplasia).

Slight trends towards increased incidences of bilateral tubular degeneration in the testis and of bilateral oligospermia in the epididymis observed at all dose levels were considered to be incidental and not related to treatment, as these findings also occurred in the control group, and the incidence of both findings in the treatment groups was within the range of historical control data provided for eight studies: bilateral diffuse tubular degeneration in the testis was observed in 83/464 animals (17.9%), with a range from 3/50 to 20/60 (6–33%), whereas bilateral oligospermia in the epididymis was observed in 101/464 animals (21.8%), with a range from 5/50 to 22/60 (10–37%).

At the end of the 24-month treatment period, there was no evidence of a test compound–related carcinogenic potential. All the neoplastic findings observed in treated animals were those commonly observed in this strain of rats kept under monitored environmental conditions and were considered to be incidental in origin.

The NOAEL for carcinogenicity was 2000 ppm (equal to 80.5 mg/kg bw per day for males), the highest dose tested. The NOAEL for toxicity was 50 ppm (equal to 2.0 mg/kg bw per day for males), based on liver effects (increased cholesterol levels, increased liver weights) and thyroid effects (higher incidence and/or severity of colloid alteration) at 300 ppm (equal to 12.1 mg/kg bw per day for males) and above (Garcin, 2008).

2.4 Genotoxicity

The results of the genotoxicity studies with bixafen are summarized in Table 33.

Table 33. Summary of genotoxicity studies with bixafen

End-point	Test object	Concentration or dose	Purity (%)	Result	Reference
In vitro					
Reverse mutation	*Salmonella typhimurium* (TA98, TA100, TA102, TA1535, TA1537)	±S9 mix: 0–5 000 µg/plate	95.8	Negative	Herbold (2005b)
Gene mutation (induced forward), *HPRT* locus	Chinese hamster V79 lung cells / HPRT	±S9 mix: 0–288 µg/mL	95.8	Negative	Herbold (2006b)
Chromosomal aberration	Chinese hamster V79 lung cells	±S9 mix: 0–240 µg/mL	95.8	Negative	Herbold (2006a)
In vivo					
Micronucleus induction	Male mice (Hsd/Win:NMRI) bone marrow erythrocytes	0, 125, 250 and 500 mg/kg bw; twice (24 h apart); intraperitoneal injection	95.8	Negative	Herbold (2005a)

HPRT: hypoxanthine–guanine phosphoribosyltransferase; S9: 9000 × *g* supernatant fraction from rat liver homogenate

In a reverse gene mutation assay in bacteria conducted according to OECD Test Guideline 471, *Salmonella typhimurium* (strains TA98, TA100, TA102, TA1535 and TA1537) was exposed to bixafen (purity 95.8%) diluted in DMSO in the presence and absence of S9 metabolic activation, using the plate incorporation and preincubation methods. Doses were selected based on the results of a range-finding study. For the plate incorporation assay, doses of up to 5000 µg/plate were used, with three plates for each strain, condition and dose. The independent repeat was performed as a preincubation test using doses of up to 1581 µg/tube. Vehicle and controls (positive and negative) were included in each experiment. A reproducible and dose-related increase in mutant colonies of at least one strain was considered to be positive. In the main assay, bixafen did not influence the growth of any strain tested at dose levels of up to 5000 µg/plate. There was no indication of a bacteriotoxic effect of bixafen up to 5000 µg/plate. Total bacterial counts were either comparable with those of negative controls or differed only insignificantly. No inhibition of growth was noted. None of the strains, in both assays, showed a dose-related and biologically relevant increase in mutant colonies over those of the negative controls. Under the conditions of this study, bixafen did not induce gene mutations in any of the strains of *S. typhimurium* used (Herbold, 2005b).

In an in vitro mammalian cell gene mutation test conducted according to OECD Test Guideline 476, bixafen (purity 95.8%) was tested for its ability to induce forward mutations at the X-chromosome-linked hypoxanthine–guanine phosphoribosyltransferase (*HPRT*) locus in Chinese hamster V79 lung (CHL) cells. Two independent sets of experiments were conducted in the presence and absence of S9 metabolic activation. Both negative and positive controls were used. After 5 hours of treatment with bixafen, cells were rinsed, trypsinized and transferred into growth media and further incubated for 6 days. CHL cells were exposed to bixafen concentrations of up to and including 288 µg/mL, in the absence and in the presence of S9 mix. At 144 µg/mL and above, the formulation in the vehicle began to form a precipitate. The means of the absolute cloning efficiency for the vehicle controls in the mutation experiments were 51.1% and 58.6% in the experiment without activation and 52.2% and 59.9% in the experiments with activation. In the mutation assay without metabolic activation, the mutant frequencies of the negative controls and of the vehicle controls were all within the normal range. The positive control ethyl methane sulfonate induced clear mutagenic and statistically significant effects in all trials. For bixafen-treated cultures, decreases in relative population growth were observed at 288 µg/mL. No relevant increases in mutant frequencies could be found in trials using bixafen. Furthermore, bixafen was evaluated as non-mutagenic in the non-activation trial. Similarly, in the case of the mutation assay with metabolic activation, the mutant frequencies of the negative controls were all within the normal range. The positive control 7,12-dimethylbenz[*a*]anthracene induced clear mutagenic and statistically significant effects in both trials. Bixafen-treated cultures showed no concentration-related decreases in relative survival or in relative population growth. However, precipitation was observed in the medium for the two highest tested concentrations. Relevant bixafen-induced increases in mutant frequencies could not be found. In addition, statistical analysis revealed no statistically significant increases. Therefore, bixafen was evaluated as non-mutagenic in the activation trial. Based on the results of the study, bixafen is considered not to be mutagenic in the V79/HPRT forward mutation assay (Herbold, 2006b).

In an in vitro mammalian chromosomal aberration test conducted according to OECD Test Guideline 473, the clastogenic potential of bixafen (purity 95.8%) was tested in CHL cells. Cultures were exposed in a pretest to bixafen at concentrations ranging from 1 to 600 µg/mL with or without S9 mix for 4 hours and harvested at 18 hours. In parallel, another group of cells was exposed from 1 to 200 µg/mL for 18 hours. Mitotic index was determined for all cultures. Concentrations that resulted in approximately 50% cytotoxicity were selected as the maximum concentrations for the main study. Osmolality and pH were unaffected by bixafen at concentrations up to 600 µg/mL. Based on the results of the pretests, the following concentrations were tested in the main study: 15–240 µg/mL for an exposure period of 4 hours and a harvest time after 18 hours; 60–240 µg/mL for an exposure period of 4 hours and a harvest time after 30 hours; and 1–16 µg/mL without S9 mix only for an exposure period of 18 hours and a harvest time after 18 hours. After 4 hours of treatment of CHL cells with bixafen, concentrations of 15, 30 and 60 µg/mL were used without S9 mix for assessment of the clastogenic potential of bixafen. With S9 mix, concentrations of 30, 60 and 120 µg/mL were employed. In addition, after 18 hours of treatment with bixafen, concentrations of 1, 4 and 8 µg/mL were selected for reading without S9 mix. None of the cultures treated with bixafen in the presence or absence of S9 mix showed statistically significant or biologically relevant increases in numbers of metaphases with aberrations. The positive controls mitomycin C and cyclophosphamide induced clear clastogenic effects and demonstrated the sensitivity of the test system. Based on the results of the study, bixafen is not considered to be clastogenic for mammalian cells in vitro (Herbold, 2006a).

In a mammalian erythrocyte micronucleus test conducted according to OECD Test Guideline 474, bixafen (purity 95.8%) was administered (by intraperitoneal injection) on two occasions, separated by 24 hours, to five male mice (Hsd/Win:NMRI) at a dose of 125, 250 or 500 mg/kg bw. Clinical signs were recorded for up to at least 24 hours after the second injection, and 4/10 mice in the 500 mg/kg bw group died. Both positive and negative control groups were used. The negative control group was treated with two intraperitoneal injections of 0.5% aqueous Cremophor emulsion, whereas the positive control group received only one intraperitoneal injection of cyclophosphamide at 20 mg/kg bw. Five additional animals were treated at 500 mg/kg bw of bixafen in case of mortality in the

initial group or a need for replacement slides due to pathological altered ratio of polychromatic erythrocytes (PCEs) to normochromatic erythrocytes (NCEs). Bone marrow from at least one femur from each animal was sampled 24 hours after the last intraperitoneal injection. Slides of bone marrow cells were prepared and scored for the occurrence of micronucleated PCEs, the occurrence of micronucleated NCEs and PCE/total erythrocyte ratios. Two thousand PCEs were counted per animal. Four out of 10 treated mice died during the period, due to the acute intraperitoneal toxicity of 500 mg/kg bw of bixafen. Animals treated at 125, 250 and 500 mg/kg bw showed the following symptoms until their termination: apathy, staggering gait, weight loss, sternal recumbency, spasm, twitching, periodic stretching of body and difficulty breathing. There were no statistically significant changes in the mean PCE/total erythrocyte ratio observed for any of the bixafen-treated groups or the control group. There were no statistically or biologically significant variations in the incidence of micronucleated PCEs between the control and the bixafen-treated groups. No statistically significant variations in the number of micronucleated NCEs were observed between the negative control and bixafen-treated groups. The positive control, cyclophosphamide, caused a clear increase in the number of PCEs with micronuclei, but did not alter the ratio of PCEs to NCEs. Thus, from the study, there was no indication of a clastogenic effect of intraperitoneally administered bixafen in the micronucleus test in male mice (Herbold, 2005a).

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

In a dose range–finding reproductive toxicity study, groups of 10 male and 10 female Wistar Crl: WI(Glx/BRL/Han)IGS BR rats were given diets containing bixafen (purity 95.8%) at a concentration of 0, 500, 1500 or 4500 ppm for 10 weeks before mating and during the mating period. Additionally, satellite groups of five males each were assigned to the control and 4500 ppm dose levels for additional blood collection for the evaluation of PT and APTT on day 29 of premating. The substance intakes at 500, 1500 and 4500 ppm during the premating period were equal to 36.2, 110 and 326 mg/kg bw per day for males and 41.3, 125 and 368 mg/kg bw per day for females, respectively. During lactation, the concentration of the test substance in the feed for the females was adjusted by 50%; the corresponding substance intakes were equal to 41.5, 123 and 371 mg/kg bw per day, respectively.

Body weight and feed consumption determinations and detailed clinical examinations of each animal were conducted weekly throughout the study. Blood samples were collected from adult females prior to termination and from adult males during week 13 (PT and APTT determinations). Reproductive parameters, such as mating, fertility and gestation indices, were evaluated. Litter parameters, such as litter size, sex ratio, pup weight, pup viability, body weight gain and clinical signs, were studied. All adult animals received a gross examination at necropsy. Selected organs (adrenals, brain, epididymides, kidney, liver, spleen, thyroid, ovaries, pituitary, prostate, seminal vesicles, testes, thymus and uterus) were weighed in adult rats. Offspring were subjected to a gross examination, and selected organs (brain, thymus, spleen and uterus) were removed and weighed. No microscopic examinations were performed in this study.

There were no compound-related mortalities or clinical observations observed during the course of this study at any dietary level tested.

P generation. A slight decrease in body weight at 4500 ppm was observed during premating in males and females, with decreases in body weight gain more pronounced in both sexes. A significant body weight decrease in females at 4500 ppm continued throughout gestation and lactation (Table 34). Also, terminal body weights at 4500 ppm were decreased in both males and females. At necropsy in males, liver and thyroid weights were increased at all dose levels, whereas thymus weight was decreased at 4500 ppm. In females, liver weight was increased at 1500 ppm and above, whereas reduced thymus, ovary and uterine weights were observed at 4500 ppm (Table 34).

Table 34. Summary of selected findings in parental animals of the range-finding reproductive toxicity study in rats

	Males				Females			
	0 ppm	500 ppm	1 500 ppm	4 500 ppm	0 ppm	500 ppm	1 500 ppm	4 500 ppm
Premating								
Body weight (g), week 10	409	441	411	392	244	244	238	232
Body weight gain (g), weeks 1–10	156	174	146	133	66	63	60	57
Feed consumption (g/animal per day), weeks 1–10	24.4	26.1	25.0	23.7	18.4	17.5	17.1	16.6
Gestation								
Body weight gain (g), days 0–20	ND	ND	ND	ND	105	109	98	72**
Feed consumption (g/animal per day), days 0–20	ND	ND	ND	ND	19.0	19.8	18.4	17.5
Lactation								
Body weight (g), day 21	ND	ND	ND	ND	292	299	295	248**
Feed consumption (g/animal per day), days 0–21	ND	ND	ND	ND	46.0	47.8	45.7	40.6
Terminal sacrifice								
Body weight (g)	449	485	455	419	292	294	296	248*
Absolute liver weight (g)	15.1	17.6*	17.9*	18.6*	14.4	14.5	16.8*	15.7
Relative liver weight (% of body weight)	3.36	3.63*	3.92*	4.43*	4.94	4.91	5.68*	6.34*
Absolute thyroid weight (mg)	28	33	35*	34	24	22	26	24
Relative thyroid weight (% of body weight ×1 000)	6.3	6.9	7.7*	8.1*	8.3	7.6	8.8	9.7
Absolute thymus weight (mg)	490	467	420	367*	265	250	250	139*
Relative thymus weight (% of body weight ×1 000)	109	96	92	87	91	86	85	55*
Absolute ovary weight (mg)	ND	ND	ND	ND	124	128	104	79*
Relative ovary weight (% of body weight ×1 000)	ND	ND	ND	ND	42	44	35	32*

ND: not determined; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$
Source: Young (2006)

The clinical chemistry determinations in control and 4500 ppm satellite males showed no compound-related effects on PT or APTT. In males bled after 13 weeks of exposure, there was no effect on PT, whereas the increased mean APTT level at 4500 ppm was within the range of historical control values. In females, no compound-related findings on PT or APTT were observed at any dietary level tested.

There were no treatment-related effects on reproductive performance (Table 35).

F_1 offspring. Pup body weight was significantly decreased by day 4, with increasing declines continuing throughout lactation, resulting in a significantly decreased pup body weight gain throughout lactation (Table 35). Also, absolute brain, thymus, spleen and uterine weights were decreased, with concomitant increased relative (organ to body weight ratio) brain and uterine weights and decreased relative spleen weight.

Table 35. Summary of selected findings on reproduction and offspring in the range-finding reproductive toxicity study in rats

	0 ppm	500 ppm	1 500 ppm	4 500 ppm
Mating index (%)	100	90	100	100
Fertility index (%)	100	100	100	100
Gestation index (%)	100	100	100	100
Days to insemination	2.4	2.3	2.7	1.9
Gestation length (days)	21.7	21.8	22.1	21.6
Mean no. of implantations per litter	12.6	11.9	11.6	10.7
Number born live	110	102	105	98
Number born dead	11	1	4	2
Birth index	95.7	96.4	93.1	93.5
Live birth index	88.8	99.1	95.7	98.2
Viability index	90.0	99.0	96.1	99.0
Lactation index	100	97.2	98.8	98.8
Offspring body weight (g)				
- Lactation day 0	5.5	5.9	6.0	5.5
- Lactation day 4 (before culling)	9.3	9.5	9.4	7.9*
- Lactation day 4 (after culling)	9.3	9.6	9.4	7.8*
- Lactation day 7	14.9	15.2	14.2	11.4**
- Lactation day 14	31.0	32.2	30.0	21.7**
- Lactation day 21	47.2	48.6	46.7	32.6**
Body weight gain (g), lactation days 0–21	41.6	42.8	40.7	27.1**

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$
Source: Young (2006)

The NOAEL for reproductive toxicity was 4500 ppm (equal to 326 mg/kg bw per day for males and 368 mg/kg bw per day for females), the highest dose tested.

A NOAEL for parental toxicity could not be established, as increased liver and thyroid weights in males were observed at all dose levels tested. The lowest-observed-adverse-effect level (LOAEL) in males was 500 ppm (equal to 36.2 mg/kg bw per day).

The NOAEL for offspring toxicity was 1500 ppm (equal to 123 mg/kg bw per day), based on reduced body weight and body weight gain at 4500 ppm (equal to 371 mg/kg bw per day) (Young, 2006).

In a two-generation reproductive toxicity study conducted according to OECD Test Guideline 416, groups of 30 male and 30 female Wistar Han Crl: WI (HAN) rats were given diets containing bixafen (purity 95.8%) at a concentration of 0, 50, 400 or 2500 ppm (equal to 0, 3.4, 27.3 and 169.2 mg/kg bw per day for P generation males [premating period] and 0, 3.9, 30.8 and 193.7 mg/kg bw per day for P generation females [premating period]) for 10 weeks prior to mating through to weaning of the F_1 offspring. Groups of 30 male and 30 female F_1 generation offspring were then similarly treated; the mean substance intakes at 0, 50, 400 and 2500 ppm were equal to 0, 3.3, 26.4 and 177.6 mg/kg bw per day for F_1 generation males (premating period) and 0, 4.0, 31.7 and 198.4 mg/kg bw per day for F_1 generation females (premating period). During the lactation period, the concentration of the test substance in the feed for the females was reduced by 50%; the corresponding substance intakes at 0, 25, 200 and 1250 ppm were equal to 0, 4.3, 33.3 and 207.5 mg/kg bw per day for P generation females and 0, 4.3, 35.9 and 226.0 mg/kg bw per day for F_1 generation females.

After a gestation period of about 22 days, litters were born, and 4 days after birth, the F_1 litters were reduced to eight pups (of equal sex ratio where possible). Pups found in a moribund state on day 4 were excluded from lactation. This was done to investigate possible malformations and to prevent cannibalism during the further rearing period. The selected F_1 pups were reared to an age of 4 weeks. At weaning, one male and one female per litter were chosen for further treatment and to produce the F_2 generation. Weanlings not chosen, as well as P generation females, were necropsied. P generation males were necropsied after the mating period, partly in the course of spermatological investigations. The weaned F_1 offspring were treated further with the test substance for at least 10 weeks (including a 3-week period for estrous cycle determination) and then co-housed for mating. The F_1 parental animals were killed as scheduled after their F_2 litters had been weaned at about day 28 postpartum.

Body weights, feed consumption and clinical signs were monitored regularly. Reproductive parameters, such as mating performance, fertility, duration of pregnancy, estrous cycling and sperm analyses, were examined in P generation and F_1 generation rats. Litter parameters, such as litter size, sex ratio, pup weight, pup viability, body weight gain and clinical signs, were studied in F_1 and F_2 offspring. Developmental milestones were evaluated in F_1 weanlings.

All parental animals were subjected to a gross examination at necropsy. Selected organs (brain, liver, kidneys, spleen, thyroid, thymus, adrenals, pituitary, testes, prostate, epididymides, seminal vesicles, coagulating glands, ovaries and uterus with cervix and oviducts) from adult rats were removed and weighed. For F_1 and F_2 pups, the brain, spleen, thymus and uterus from one male and/or one female pup per litter on day 21 were weighed. Microscopic examinations were performed on tissues from the control and 2500 ppm adult animals (brain, cervix, epididymides, gross lesions, kidney, liver, ovaries, pituitary, prostate, testes, seminal vesicles, coagulating glands, spleen, uterus with oviducts and vagina). Liver was also microscopically examined in the 50 and 400 ppm dose groups of the P generation and F_1 adults. Selected tissues (cervix, an epididymis, gross lesions, ovary, oviduct, prostate, seminal vesicles, coagulating glands, testes, uterus and/or vagina) from control and 2500 ppm 21-day F_1 and F_2 pups were examined microscopically. In F_1 females, ovarian follicle staging was evaluated in 10 randomized animals that had viable litters from the control and 2500 ppm groups.

There were no treatment-related mortalities or clinical signs during the course of the study in either generation.

The P generation males did not exhibit any test substance–related effects on body weight or body weight gain at any dietary level tested after 14 weeks of exposure to the test substance. In the F_1 generation males, absolute body weight was decreased by 6.2% at 2500 ppm, whereas body weight gain was unaffected after 14 weeks of treatment (Table 36). The lower body weight was considered to be due to lower pup weight in this group. There was a decrease in feed consumption in the 2500 ppm group P generation males in the 1st week of the study, thought to be due to taste aversion. Thereafter, feed consumption was unaffected by treatment at any dose level. In the F_1 generation males, feed consumption increased by 8.1% from week 5 of treatment in the 2500 ppm group.

In the P generation females at 2500 ppm, slight but significant decreases in body weight were seen during the premating period from weeks 7 through 10 (mean decline of 4.8%). Overall, the body weight gain in this group was reduced by 21.1%. Also at 2500 ppm, significant body weight declines were noted throughout gestation, with a reduction of body weight gain by 14.5%, as well as throughout the lactation period, with a significant reduction of body weight by 5.7%. Body weight effects were not observed at any other dietary level (Table 36).

F_1 generation females at 2500 ppm showed reduced body weights through the premating period (decline of 7.6%), with an overall reduction in body weight gain of 13.3%. The lower body weight was considered to be due to lower pup weight in this group. Also at 2500 ppm, significant body weight declines were noted throughout gestation, with a reduction of body weight gain by 18.2%, as well as throughout the lactation period, with a significant reduction of body weight by 8.3%. Body weight effects were not observed at any other dietary level (Table 36).

Table 36. Summary of selected findings in parental animals of the reproductive toxicity study in rats

	Males				Females			
	0 ppm	50 ppm	400 ppm	2 500 ppm	0 ppm	50 ppm	400 ppm	2 500 ppm
P generation								
Premating								
Body weight (g), weeks 14/10 (M/F)	448	448	453	447	244	243	242	232**
Body weight gain (g), weeks 1–14/1–10 (M/F)	198	199	198	194	60.2	58.1	56.5	47.5
Feed consumption (g/animal per day), weeks 1–10	23.5	23.5	24.0	23.5	17.4	17.1	16.9	16.6
Gestation								
Body weight (g), day 20	ND	ND	ND	ND	347	345	341	321**
Body weight gain (g), days 0–20	ND	ND	ND	ND	101.0	95.4	95.6	86.4**
Feed consumption (g/animal per day), days 0–20	ND	ND	ND	ND	19.7	19.2	19.0	18.6
Lactation								
Body weight (g), day 21	ND	ND	ND	ND	294	296	293	281*
Feed consumption (g/animal per day), days 0–21	ND	ND	ND	ND	45.0	44.5	47.5	44.0
Postmortem								
Body weight (g)	454	456	457	452	292	294	294	281*
Absolute liver weight (g)	15.9	16.5	17.2*	20.0*	14.4	14.8	15.3	18.0*
Relative liver weight (% of body weight)	3.51	3.61	3.77*	4.42*	4.92	5.03	5.21	6.39*
Liver: centrilobular hypertrophy	0/30	0/30	0/30	30*/30	0/30	0/30	0/30	28*/30
F_1 generation								
Premating								
Body weight (g), weeks 14/10 (M/F)	450	449	454	422*	245	245	245	226**
Body weight gain (g), weeks 1–14/1–10 (M/F)	159	161	157	154	54.8	57.0	54.2	47.5
Feed consumption (g/animal per day), weeks 1–10	24.5	24.2	24.4	23.8	17.2	17.4	17.2	16.0
Gestation								
Body weight (g), day 20	ND	ND	ND	ND	336	335	334	299**
Body weight gain (g), days 0–20	ND	ND	ND	ND	92.5	88.9	94.8	75.7**
Feed consumption (g/animal per day), days 0–20	ND	ND	ND	ND	17.8	18.2	18.6	16.7
Lactation								
Body weight (g), day 21	ND	ND	ND	ND	283	288	286	268*
Feed consumption (g/animal per day), days 0–21	ND	ND	ND	ND	44.2	44.7	43.6	40.5
Postmortem								
Body weight (g)	453	457	458	428	282	287	287	266*
Absolute liver weight (g)	15.6	16.2	16.7	18.5*	12.7	13.8*	14.2*	16.3*
Relative liver weight (% of body weight)	3.44	3.54	3.64*	4.31*	4.51	4.80*	4.95*	6.11*
Liver: centrilobular hypertrophy	0/30	0/30	2/30	28*/30	0/30	0/30	4/30	26*/30

F: female; F_1: first filial; M: male; ND: not determined; P: parental; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$
Source: Young (2007)

In P generation females, initial declines in feed consumption in the 1st week of the study were seen in both the 400 and 2500 ppm groups, considered to be a result of palatability of the test material. In the F_1 generation females, no effects on feed consumption were seen in any dose group.

At necropsy, treatment-related organ weight changes for the P generation were limited to kidney (increased at 2500 ppm in males), liver (increased at and above 400 ppm in males and at 2500 ppm in females), spleen (increased at 2500 ppm in males) and thymus (decreased at 2500 ppm in females). For the F_1 generation, treatment-related organ weight changes were limited to the increased liver weights in males at and above 400 ppm and in females at and above 50 ppm (Table 36). Treatment-related histopathological findings were limited to the liver. Minimal to slight, primarily centrilobular and/or diffuse hypertrophy of the liver was present in males and females of the P generation at 2500 ppm and in males and females of the F_1 generation at 400 and 2500 ppm (Table 36). This liver hypertrophy, coded as "centrilobular hypertrophy", was characterized by an enlarged cell, primarily involving the cytoplasm. Often the basophilic staining cytoplasmic organelles appeared to be compressed towards the outer edges of the cell. In general, the centrilobular hepatocytes were affected, with occasional involvement of the midzonal hepatocytes. The hypertrophy changes of the liver at 400 and/or 2500 ppm correlated well with the statistically significant liver weight increases present at these dietary levels.

Overall reproductive performance was not affected for any parameter (e.g. mating, fertility or gestation indices, days to insemination, gestation length or the median number of implantations) in either generation at any dietary level (Table 37).

Table 37. Summary of reproductive performance data in the reproductive toxicity study in rats

	0 ppm	50 ppm	400 ppm	2 500 ppm
P generation				
Co-housed females	30	30	30	30
Matings until day 0 post-coitum	30	29	29	30
Mating index	100	96.7	96.7	100
Fertility index	93.3	96.6	100	100
Gestation index	100	100	96.6	100
Gestation length (days)	22.0	21.9	21.9	21.9
Number of animals delivered	28	28	28	30
F_1 generation				
Co-housed females	30	30	30	30
Matings until day 0 post-coitum	30	30	30	30
Mating index	100	100	100	100
Fertility index	93.3	100	90.0	93.3
Gestation index	100	93.3	96.3	100
Gestation length (days)	21.9	21.9	21.8	21.8
Number of animals delivered	28	28	26	28

F_1: first filial; P: parental; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$
Source: Young (2007)

Results from the evaluation of vaginal smears in both the P and F_1 generation females did not indicate any test substance–related findings on estrous cycle length or periodicity at any dietary level tested.

There were no test substance–related effects observed on any sperm parameter evaluated at any dietary level tested for either generation.

The number of implantation sites, the live birth, viability and lactation indices, the number of pups born, the sex distribution, the litter sizes during lactation as well as prenatal losses were not altered by the treatment up to 2500 ppm.

A trend of an increase (non-statistically significant) in the incidence of early stillbirths (lungs did not float) occurred at 2500 ppm in the F_1 and F_2 generations (Table 38). Litters evaluated for early stillbirths (lungs did not float) were also non-statistically significantly elevated above controls at 2500 ppm in the F_1 and F_2 generations. These non-statistically significant increases in stillbirths in the F_1 and F_2 generations were considered to be secondary to the toxicity observed in dams at this dietary level and were within the range of historical control data for this finding. Historical control values were provided for eight one-generation studies conducted from 1998 to 2005 and for nine two-generation studies conducted from 1999 to 2005. In the one-generation studies, which included 7–10 litters, the number of stillborn pups ranged from 0/118 to 11/121 (0–9.1%) in 0/10 to 2/8 litters (0–25%). The control group with 11/121 stillborn pups was from the range-finding study for reproductive toxicity of bixafen (Young, 2006), including one dam delivering eight stillborn pups (out of nine pups in total). In the two-generation studies, which included 19–29 litters, the number of stillborn pups ranged from 0/306 to 6/291 (0–2.1%) in 0/28 to 2/24 litters (0–8.3%).

Table 38. Summary of selected litter data in the reproductive toxicity study in rats

	0 ppm	50 ppm	400 ppm	2 500 ppm
F_1 generation				
No. of litters	28	28	28	30
No. of implantations, total/per litter	342/12.2	318/11.4	332/11.9	338/11.3
No. of pups born	320	294	305	318
No. of pups missing/found dead/cannibalized	1/2/2	0/0/1	0/1/0	5/2/0
No. of stillborn pups	4	0	0	13
No. of pups born live	314	294	305	292
Litter size	11.4	10.5	10.9	10.6
No. of deaths, days 0–4/days 4–21	5/0	0/1	1/0	6/1
Pup weight (g), viable pups				
- Birth	5.9	6.0	6.0	5.8
- Day 4, pre-culling	9.6	9.9	9.8	9.2
- Day 7	15.4	15.9	15.5	14.1*
- Day 14	32.1	32.4	31.5	28.2**
- Day 21	48.8	49.5	48.0	43.2**
Pup weight gain (g), days 0–21	42.9	43.5	42.0	37.4**
Birth index[a]	93.1	92.1	92.2	93.4
Live birth index[b]	97.8	100	100	96.3
Viability index[c]	98.6	100	99.6	97.7
Lactation index[d]	100	99.6	100	99.5
F_2 generation				
No. of litters	28	28	26	28
No. of implantations, total/per litter	303/10.8	312/10.4	292/10.8	275/9.8
No. of pups born	290	296	276	265
No. of pups missing/found dead/cannibalized	4/0/0	0/3/0	4/1/6	3/5/0
No. of stillborn pups	1	6	2	6
No. of pups born live	289	290	274	259

	0 ppm	50 ppm	400 ppm	2 500 ppm
Litter size	10.4	10.6	10.6	9.5
No. of deaths, days 0–4/days 4–21	3/1	3/0	11/0	4/4
Pup weight, viable pups (g)				
- Birth	6.0	5.9	5.9	5.9
- Day 4, pre-culling	10.2	10.4	10.0	9.8
- Day 7	16.0	16.1	15.8	15.0
- Day 14	32.4	32.2	31.4	29.5**
- Day 21	48.4	49.1	47.3	44.5**
Pup weight gain (g), days 0–21	42.5	43.2	41.4	38.6**
Birth index[a]	95.3	89.0	91.6	96.1
Live birth index[b]	99.6	98.0	97.2	97.9
Viability index[c]	99.1	99.1	97.4	98.5
Lactation index[d]	99.6	100	100	98.2

F_1: first filial; F_2: second filial; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$
[a] Birth index = Total no. of pups born per litter/Total no. of implantation sites per dam × 100.
[b] Live birth index = No. of live pups born per litter/Total no. of pups per litter × 100.
[c] Viability index = No. of live pups on day 4 pre-culling per litter/ No. of live pups born per litter × 100.
[d] Lactation index = No. of live pups on day 21 per litter / No. of live pups on day 4 post-culling per litter × 100.
Source: Young (2007)

There was no treatment-related effect on mean litter size or pup viability (survival) during lactation.

Offspring birth weights were not affected by treatment at any dose level, but there was a treatment-related decrease in offspring body weight and body weight gain during lactation for the F_1 and F_2 pups at 2500 ppm. This decrease in weight gain was apparent by day 4 postpartum and continued to intensify during lactation (Table 38).

The balanopreputial separation and vaginal opening in F_1 post-weanlings were unaffected up to 2500 ppm.

Offspring organ weights in the F_1 or F_2 pups at 2500 ppm that were statistically significantly different from those of controls included brain, spleen and thymus. As all these organ weight differences were associated with statistically significant deceases in body weights at day 21 of the lactation period, they were considered to be secondary to body weight changes in this group. No other dose groups exhibited any changes in organ weights compared with controls. There were no remarkable incidences of macroscopic or microscopic findings at necropsy in the F_1 or F_2 pups.

The NOAEL for reproductive toxicity was 2500 ppm (equal to 169.2 mg/kg bw per day for males and 193.7 mg/kg bw per day for females), the highest dose tested.

The NOAEL for parental toxicity was 400 ppm (equal to 26.4 mg/kg bw per day for males and 30.8 mg/kg bw per day for females), based on a reduction in body weight and liver effects (liver weight increase > 20%) at 2500 ppm (equal to 169.2 mg/kg bw per day for males and 193.7 mg/kg bw per day for females).

The NOAEL for offspring toxicity was 400 ppm (equal to 26.4 mg/kg bw per day for males and 31.7 mg/kg bw per day for females), based on a slight elevation in stillbirths and reduced pup weight/weight gain during lactation at 2500 ppm (equal to 177.6 mg/kg bw per day for males and 198.4 mg/kg bw per day for females) (Young, 2007).

(b) Developmental toxicity

Rats

In a prenatal developmental toxicity study conducted according to OECD Test Guideline 414, groups of 23 mated female Sprague-Dawley Crj:CD(SD) rats were administered bixafen (purity 95.8%) in 0.5% aqueous methyl cellulose at a dosing volume of 10 mL/kg bw by oral gavage at a dose of 0, 20, 75 or 250 mg/kg bw per day from gestation day (GD) 6 to GD 20. Clinical observations were recorded daily. Maternal body weights and feed consumption were recorded for all females at appropriate intervals from GD 0 to GD 21. At scheduled termination, on GD 21, the gravid uterine weight was recorded, and the dams were evaluated for number of corpora lutea and number and status of implantations (resorptions, dead and live fetuses). Live fetuses were removed from the uteri, counted, weighed, sexed and examined externally. Approximately half of the live fetuses from each litter were fixed in Bouin's solution and subsequently dissected for internal examination. The remaining half were eviscerated, fixed in absolute ethanol and stained for skeletal examination of bone and cartilage.

At 250 mg/kg bw per day, one female was found dead on GD 20. Clinical signs for this female consisted of soiling around the nose and mouth on GD 18 and soiling of the fur in the head region on GD 19. This female lost 29 g in body weight between GD 6 and GD 8, which was associated with very low feed consumption. Body weight gain and feed consumption for this female were also reduced between GD 14 and GD 18, compared with the controls. Treatment-related clinical signs in surviving females at this dose level consisted of one female with soiling around the nose on GD 19, one female with soiling around the nose, mouth and abdomen on GD 21 and one female with piloerection on GDs 14 and 15. No premature deaths or treatment-related clinical signs were observed in dams at 75 or 20 mg/kg bw per day.

There was a marked loss in maternal body weight of 21.9 g on GDs 6–8 at 250 mg/kg bw per day, compared with a 5.7 g body weight gain in the controls over the corresponding period. On GDs 18–21, body weight gain was reduced by 21%. Overall, on GDs 6–21, maternal body weight gain was reduced by 17%, compared with the controls, at 250 mg/kg bw per day, and maternal corrected body weight change was 42% less than in the controls. At 75 mg/kg bw per day, there was a slight maternal body weight loss of 1.8 g on GDs 6–8, compared with a 5.7 g body weight gain in the control group. Thereafter, body weight gain was unaffected by treatment at 75 mg/kg bw per day, and maternal corrected body weight change was comparable with that of the control group (Table 39).

Mean maternal feed consumption at 250 mg/kg bw per day was reduced by 58% on GDs 6–8, 16% on GDs 8–10 and 11% on GDs 18–21, compared with the controls, the effect being statistically significant at each interval. At 75 mg/kg bw per day, maternal feed consumption was reduced by 21% on GDs 6–8, compared with the controls, the effect being statistically significant, but was comparable thereafter (Table 39).

No treatment-related maternal findings were observed at 20 mg/kg bw per day. No treatment-related findings were observed in dams at necropsy at any dose level.

There was no treatment-related effect on pregnancy rate. Pregnancy rate was 83% at 250 mg/kg bw per day, 96% at 75 mg/kg bw per day and 87% at 20 mg/kg bw per day and in the control group (Table 40).

Litter parameters, including number of live fetuses, early or late resorptions and dead fetuses, were unaffected by treatment. Mean fetal weight was reduced for both the combined and separate sexes by 6% at 250 mg/kg bw per day and by 2% at 75 mg/kg bw per day. There was no effect on mean fetal weight at 20 mg/kg bw per day (Table 40).

At least 18 litters with live fetuses were present in each group, and fetal evaluations were performed through external, visceral and skeletal examinations. No treatment-related external fetal observations were observed. The number of runt fetuses observed was very low, with no indication of a treatment-related effect (Table 41).

Table 39. Summary of selected maternal findings in a prenatal developmental toxicity study in rats

	0 mg/kg bw per day	20 mg/kg bw per day	75 mg/kg bw per day	250 mg/kg bw per day
Body weight (g)				
- GD 0	269.0	273.2	269.0	268.2
- GD 6	302.7	306.8	299.8	300.4
- GD 21	430.6	436.7	427.7	408.2
Body weight gain (g)				
- GDs 0–6	33.8	33.7	30.8	32.2
- GDs 6–8	5.7	4.6	−1.8**	−21.9**
- GDs 6–14	32.9	32.6	28.1	20.5**
- GDs 6–21	127.9	129.9	127.9	106.3*
Corrected body weight gain (g), GDs 0–21	60.4	59.1	56.3	34.9**
Feed consumption (g/day)				
- GDs 1–6	24.9	25.1	24.2	24.9
- GDs 6–8	24.9	24.0	19.7**	10.4**
- GDs 8–10	24.9	25.4	22.7	20.8*
- GDs 18–21	27.4	28.3	27.9	24.4*

bw: body weight; GD: gestation day; *: $P < 0.05$; **: $P < 0.01$
Source: Wason (2006)

Table 40. Summary of selected reproduction data in a prenatal developmental toxicity study in rats

	0 mg/kg bw per day	20 mg/kg bw per day	75 mg/kg bw per day	250 mg/kg bw per day
No. of animals mated	23	23	23	23
No. of animals pregnant	20	20	22	19
No. of animals with viable young at term	19	20	21	18
No. of animals with total resorption	1	0	1	0
No. of intercurrent deaths (with evidence of pregnancy)	0	0	0	1
No. of animals not pregnant	3	3	1	4
No. of females with implantations	20	20	22	19
No. of corpora lutea per dam	17.1	17.4	17.3	17.5
No. of implantations per dam	15.3	15.1	15.6	15.8
Preimplantation loss per dam (%)	10.0	14.4	8.9	8.6
No. of early/late resorptions per litter	0.5/0.1	0.7/0.1	1.0/0.0	0.9/0.2
No. of live fetuses per litter	14.7	14.3	14.6	14.7
No. of live fetuses	279	285	306	264
No. of litters with live fetuses	19	20	21	18
No. of dead fetuses/litters with dead fetuses	0/0	0/0	0/0	1/1
Postimplantation loss (% loss per female)	3.9	4.9	6.5	6.9
Male fetuses (%)	52.0	54.1	47.3	49.1
Male fetal weight (g)	5.72	5.65	5.58**	5.39**
Female fetal weight (g)	5.41	5.33	5.31*	5.09**

bw: body weight; *: $P < 0.05$; **: $P < 0.01$
Source: Wason (2006)

Table 41. Summary of selected fetal findings in the prenatal developmental toxicity study in rats

	0 mg/kg bw per day	20 mg/kg bw per day	75 mg/kg bw per day	250 mg/kg bw per day
External findings				
No. of fetuses/litters examined	279/19	285/20	306/21	264/18
No. of runts	1/1	0/0	0/0	2/2
Visceral findings				
No. of fetuses/litters examined	135/19	137/20	148/21	127/18
Thymus misshapen	4/1	7/5	5/5	5/3
Ureter dilated	10/7	14/8	11/5	19/10
Renal pelvis dilated	4/3	4/3	5/4	9/4
Skeletal findings				
No. of fetuses/litters examined	144/19	148/20	158/21	137/18
Thoracic centrum: bipartite or dumbbell	1/1	1/1	3/3	3/3
5th sternebra: incomplete ossification, normal cartilage	20/9	21/13	25/13	36**/12
5th sternebra: unossified, normal cartilage	1/1	1/1	3/3	7*/3
14th thoracic rib(s), unilateral or bilateral: short	3/3	6/4	4/3	8/5
10th or 11th or 13th costal cartilage: discontinuous	3/2	3/2	4/2	6/5

bw: body weight; *: $P < 0.05$; **: $P < 0.01$
Source: Wason (2006)

At the visceral examination, one fetus at 250 mg/kg bw per day was observed with the malformation diaphragmatic hernia. In isolation, this finding was considered to have occurred by chance. There was one fetus at 75 mg/kg bw per day and one fetus in the control group with the malformation renal papilla absent. In isolation and with one of the cases occurring in the control group, this malformation was considered to be fortuitous. No other malformations were noted at visceral examination. The incidence of the following variant findings was higher at both the fetal and litter levels than in the control group: misshapen thymus at all three dose levels, although not in a dose-related manner; and dilated ureter and dilated renal pelvis at 250 mg/kg bw per day (Table 41). However, as all three parameters were well within the in-house historical control range at both the fetal and litter levels, they were considered to be fortuitous findings.

At the skeletal examination, no malformations were observed. The incidence of the anomaly thoracic centrum: bipartite or dumbbell/dumbbell cartilage was higher at 250 mg/kg bw per day in terms of mean percentage of fetuses affected and percentage of litters affected and was outside the in-house historical control range for both parameters. To further evaluate this finding, statistical analysis was performed, using both the fetus and the litter as the statistical unit. The slightly higher incidence of this finding was found to be non-statistically significant at both the fetal and litter levels and was therefore considered to be a chance finding (Table 41).

The incidence of the following three variant findings was higher than in the control group at the specified dosage and was outside the in-house historical control range: 1) 5th sternebra: incomplete ossification/normal cartilage, at the fetal level, at 250 mg/kg bw per day; 2) 5th sternebra: unossified/normal cartilage, at both the fetal and litter levels at 250 mg/kg bw per day and at the litter level only at 75 mg/kg bw per day; and 3) 14th thoracic ribs (unilateral or bilateral): short, at both the fetal and litter levels at 250 and 20 mg/kg bw per day and at the fetal level only at 75 mg/kg bw per day. For the following variant finding, no in-house historical data exist, as this was the first study in which this combination of effects has been scored: 10th or 11th or 13th costal cartilage (unilateral or bilateral): discontinuous, higher at both the fetal and litter levels at 250 mg/kg bw per day compared with the controls. To further evaluate the relevance of these findings, statistical analysis was performed on the data using both the fetus and the litter as the statistical unit. The only findings where

a statistically significant effect was observed were 5th sternebra: incomplete ossification, normal cartilage and 5th sternebra: unossified, normal cartilage, at the fetal level only, at 250 mg/kg bw per day (Table 41). The two other findings were considered to have occurred by chance.

The NOAEL for maternal toxicity was 20 mg/kg bw per day, based on decreased body weight gain and feed consumption in the first days of treatment (i.e. GDs 6–8) at 75 mg/kg bw per day and above.

The NOAEL for embryo and fetal toxicity was 20 mg/kg bw per day, based on decreased fetal weights at 75 mg/kg bw per day and above (Wason, 2006).

Rabbits

In a prenatal developmental toxicity study conducted according to OECD Test Guideline 414, groups of 23 mated female New Zealand White rabbits were administered bixafen (purity 95.8%) in 0.5% aqueous methyl cellulose at a dosing volume of 4 mL/kg bw by oral gavage at a dose of 0, 25, 100 or 400 mg/kg bw per day from GD 6 to GD 28. Maternal body weights and feed consumption were recorded for all surviving females at suitable intervals from GD 3 to GD 29. Clinical observations were recorded daily. At scheduled sacrifice, on GD 29, the gravid uterine weight and the number of ribs were recorded, and the dams were evaluated for number of corpora lutea and number and status of implantations (resorptions, dead and live fetuses). The liver was weighed for all pregnant females and retained from all females. Live fetuses were removed from the uteri, counted, weighed and examined externally. The heads of fetuses from approximately half of each litter were immersed in Bouin's fluid, and the internal structures were examined after fixation. The bodies of all fetuses were dissected for soft tissue anomalies and sexed. Fetuses were eviscerated, skinned and fixed in absolute ethanol before staining, and a subsequent skeletal examination was performed.

At the highest dose level tested (400 mg/kg bw per day), eight females were humanely killed before the end of the study (Table 42). Three females were humanely killed after spontaneously aborting, one on GD 18 and two on GD 26. On the day of termination, the females had red traces and/or fetal/placental tissue on the tray. Two females had generalized hair loss with scab-like lesions on the skin of the neck area. The three females had a body weight loss of between 0.32 and 0.77 kg from GD 6 to termination, which was associated with very poor feed consumption several days before termination.

Five females at 400 mg/kg bw per day were humanely killed on GDs 11, 15, 16 and 22 (two females) without spontaneously aborting. Principal clinical signs observed in these females included no/few faeces, no urine, scabs/skin lesions and generalized hair loss on one or more occasions. The five females lost between 0.34 and 0.72 kg in body weight from GD 6 to the day of termination, which was associated with poor feed consumption. At necropsy, four of the eight females had white foci on the liver, and three showed prominent lobulation of the liver.

Treatment-related clinical signs in surviving females at 400 mg/kg bw per day consisted of no/few faeces noted for 9/15 females on one or more occasions, no urine in 4/15 females, scabs/skin lesions in 5/15 females and hair loss in 11/15 females, on two or more occasions. At 100 mg/kg bw per day, treatment-related signs were confined to 8/23 females with hair loss and with no or few faeces on one or more occasions, 2/23 with no urine on GDs 18–19 and 2/23 with a red vaginal discharge on GDs 28–29 (Table 42). At 25 mg/kg bw per day, vaginal discharge with red traces on the tray on GDs 26–27 was seen in 1/23 females, and 1/23 females had red traces on GD 28.

At 400 mg/kg bw per day, there was a body weight loss of 0.08 kg between GD 6 and GD 8 and a loss of 0.02 kg between GDs 10 and 14, compared with a body weight gain of 0.02 kg and 0.07 kg, respectively, over the corresponding period in the control group. Thereafter, body weight gain tended to be less at the high dose compared with the controls, resulting in an overall reduction in body weight gain of 59% by GD 29, the effect being statistically significant for periods of GDs 6–8 and GDs 18–22. Body weight change at 100 mg/kg bw per day was slightly less than that of controls over several periods, with a resulting overall reduction of 29% between GDs 6 and 28. At 25 mg/kg bw per

day, body weight gain was comparable with that of the controls. Maternal corrected body weight change was less in all three dose groups (−0.29, −0.25 and −0.23 kg, respectively, for the 400, 100 and 25 mg/kg bw per day groups) compared with the controls (−0.15 kg), but the effect was not statistically significant (Table 42).

Table 42. Summary of selected maternal findings in the prenatal developmental toxicity study in rabbits

	0 mg/kg bw per day	25 mg/kg bw per day	100 mg/kg bw per day	400 mg/kg bw per day
No. of animals/no. of intercurrent deaths	23/0	23/0	23/0	23/8
No. of animals with hair loss	3	1	8	18
No. of animals with no faeces/few faeces	0/1	0/1	2/8	5/17
No. of animals with no urine	0	0	2	10
Body weight (kg)				
- GD 6	3.47	3.47	3.45	3.44
- GD 14	3.57	3.57	3.55	3.36
- GD 22	3.72	3.67	3.64	3.42
- GD 29	3.81	3.75	3.69	3.62
Body weight change (kg)				
- GDs 6–8	0.02	−0.01	−0.01	−0.08**
- GDs 6–14	0.10	0.10	0.10	−0.07*
- GDs 6–18	0.18	0.17	0.16	−0.05**
- GDs 6–26	0.31	0.25	0.21	0.08**
Corrected body weight change (kg), GDs 0–29	−0.15	−0.23	−0.25	−0.29
Feed consumption (g/day)				
- GDs 3–6	155	153	154	162
- GDs 6–8	165	161	163	120
- GDs 10–14	155	164	161	105
- GDs 14–18	155	158	134	85**
- GDs 18–22	163	154	143	108*
- GDs 22–26	130	119	102*	100*
Liver weight (g), pregnant females	92.8	91.2	102.1*	116.2**

bw: body weight; GD: gestation day; *: $P < 0.05$; **: $P < 0.01$
Source: Wason (2007)

Feed consumption at 400 mg/kg bw per day was reduced by 23–45% at each interval compared with the controls. The effect was most pronounced and statistically significant between GDs 14 and 18, GDs 18 and 22 and GDs 22 and 26. Feed consumption at 100 mg/kg bw per day was reduced at each interval between GD 14 and GD 26 by between 12% and 21%, but the effect was statistically significant only between GDs 22 and 26. Feed consumption was similar to that of controls at all intervals at 25 mg/kg bw per day.

At necropsy, liver weight was increased by 25% and 10% at 400 and 100 mg/kg bw per day, respectively, compared with controls (Table 42). In females at 400 mg/kg bw per day surviving to scheduled termination, white foci on the liver were observed in 4/15 females, with 1/15 females also having prominent lobulation and a depression in the median lobe of the liver. Enlarged urinary bladder containing purulent-like urine was noted for 1/25 females.

At 400 mg/kg bw per day, the percentage of dead fetuses was 8.1%, compared with 3.6% in controls. Also at 400 mg/kg bw per day, postimplantation loss was slightly higher (11.9%) than in the control group (8.4%), and combined fetal weights were decreased by 6%, which was statistically significantly lower than in controls. At 100 mg/kg bw per day, the only litter finding was a statistically significant lowering of male fetal weights by 5%, whereas female fetal weights were not different from those of the controls. No treatment-related litter findings occurred at 25 mg/kg bw per day (Table 43).

Table 43. Summary of selected reproduction data in the prenatal developmental toxicity study in rabbits

	0 mg/kg bw per day	25 mg/kg bw per day	100 mg/kg bw per day	400 mg/kg bw per day
No. of animals mated	23	23	23	23
No. of animals pregnant	22	22	21	22
No. of animals reaching term with viable young	22	22	21	14
No. of animals reaching term with total resorption	0	0	0	1
No. of animals aborted	0	0	0	3
No. of intercurrent deaths with evidence of pregnancy	0	0	0	4
No. of animals reaching term/not pregnant	1	1	2	0
No. of intercurrent deaths with no evidence of pregnancy	0	0	0	1
No. of corpora lutea per dam	11.0	11.7	11.3	11.4
No. of implantation sites per dam	9.6	10.0	9.7	9.9
Preimplantation loss per dam (%)	12.6	14.7	14.8	12.0
No. of early resorptions per litter	0.4	0.5	0.3	0.4
No. of late resorptions per litter	0.1	0.0	0.0	0.1
No. of live fetuses per litter	8.8	9.4	9.0	8.6
No. of live fetuses	193	206	189	121
No. of litters with live fetuses	22	22	21	14
No. of dead fetuses	8	2*	8	12*
No. of litters with dead fetuses	4	2	4	5
Percentage of dead fetuses per litter	3.6	0.9	3.6	8.1
Postimplantation loss per litter (%)	8.4	5.7	6.1	11.9
Male fetuses (%)	53.8	56.3	49.7	57.7
Fetal weight (g)	37.6	37.6	36.3	35.2*
Male fetal weight (g)	38.6	37.8	36.5*	35.9
Female fetal weight (g)	36.4	37.4	36.0	34.4

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

At caesarean section, there were 22, 22, 21 and 14 females with live fetuses in the control, 25, 100 and 400 mg/kg bw per day groups, respectively.

At the external examination, there were no treatment-related malformations at any dose level. The number of runt fetuses (body weight < 28 g) was increased at 400 mg/kg bw per day, where the mean percentage of fetuses classified as runts was 20% and the percentage of litters affected was 50%, compared with 6.3% and 27.3%, respectively, in the control group (Table 44).

Table 44. Summary of selected fetal findings in the prenatal developmental toxicity study in rabbits.

	0 mg/kg bw per day	25 mg/kg bw per day	100 mg/kg bw per day	400 mg/kg bw per day
External findings				
No. of fetuses/litters examined	193/22	206/22	189/21	121/14
No. of runts	15/6	10/6	17/5	22/7
Visceral findings				
No. of fetuses/litters examined	193/22	206/22	189/21	121/14
Innominate artery: short	1/1	0/0	3/3	2/2
Subclavian artery (right): retro-oesophageal	1/1	2/2	3/2	6/4
Ureter (unilateral): convoluted/dilated	0/0	0/0	2/2	2/2
Renal pelvis (unilateral): dilated	0/0	0/0	0/0	3/2
Kidney: enlarged	0/0	0/0	0/0	3/3
Skeletal findings				
No. of fetuses/litters examined	193/22	206/22	189/21	121/14
Internasal or interfrontal suture: extra ossification site	0/0	0/0	0/0	3/2
Nasal split (unilateral)	1/1	0/0	1/1	2/2
At least one sternebra (except 5th or 6th sternebra) incompletely ossified	0/0	2/2	1/1	3/3
6th sternebra: unossified	6/4	4/2	8/6	11/6
1st metacarpal: unossified	9/5	4/2	3/2	10/8
1st proximal phalanx of the forepaw: unossified	0/0	0/0	1/1	2/2
Insertion point of pelvic girdle: unossified	1/1	2/1	1/1	6/3
Pubis (unilateral or bilateral): incompletely ossified	2/2	0/0	1/1	5/4
Pubis (bilateral): unossified	1/1	0/0	0/0	2/2
Astragalus (unilateral or bilateral): unossified	2/1	0/0	0/0	2/2

bw: body weight
Source: Wason (2007)

At the visceral examination, there were no treatment-related malformations at any dose level. At 400 mg/kg bw per day, a number of visceral findings occurred, including 1) innominate artery: short, 2) subclavian artery (right): retro-oesophageal; 3) ureter (unilateral): convoluted and dilated; 4) renal pelvis (unilateral): dilated (not severe); and 5) enlarged kidney (unilateral or bilateral). The small sample size in the high-dose group precluded a robust comparison (Table 44).

At 100 mg/kg bw per day, the only visceral anomaly that was increased over that of controls was ureter (unilateral): convoluted and dilated. However, statistical analysis found that this was not statistically significant at either the fetal or litter level, and therefore it was considered not to be treatment related. Also at 100 mg/kg bw per day, the variation innominate artery: short was increased over controls. However, the fetal incidence for this finding was within the in-house historical control range and only marginally outside the historical control range for litters, neither being statistically significant. Therefore, this variant finding was concluded to be unrelated to treatment.

At the skeletal examination, no malformations were observed. Several skeletal variations at 400 mg/kg bw per day were seen to be elevated in comparison with controls and historical control ranges. All of these variants related to ossification changes consistent with a delay in development. The findings included 1) internasal or interfrontal suture: extra ossification site, 2) nasal split, 3) at least one sternebra (except 5th or 6th sternebrae) incompletely ossified, 4) 6th sternebra unossified, 5) 1st metacarpal unossified, 6) 1st proximal phalanx of the forepaw unossified, 7) insertion point of

pelvic girdle unossified, 8) pubis unossified or incompletely ossified and 9) astragalus unossified. There were no treatment-related skeletal variations seen at 25 or 100 mg/kg bw per day (Table 44).

In conclusion, the numerous variants and anomalies observed at 400 mg/kg bw per day were considered to result from the severe maternal toxicity produced (32% mortality, from dramatic reductions in feed intake and body weight gain), which were clear signs that the maximum tolerated dose had been exceeded in pregnant rabbits.

The NOAEL for maternal toxicity was 25 mg/kg bw per day, based on clinical signs (hair loss, no or reduced excreta) and a reduction in body weight gain and feed consumption at 100 mg/kg bw per day and above.

The NOAEL for embryo and fetal toxicity was 25 mg/kg bw per day, based on reduced fetal weight at 100 mg/kg bw per day and above (Wason, 2007).

2.6 Special studies

(a) Analyses of diet for vitamin K_3 content

In the initial chronic toxicity and carcinogenicity study in rats, there was a significantly increased incidence of mortalities in the male rats at a dose level of 1000 ppm, with deaths occurring from study day 33 (McElligott, 2008). The analysis of the diet used in this study (batch 50607) showed that the level of vitamin K was very significantly lower than the estimate of the requirement of 1 mg/kg for the Wistar rat, and discussions with the manufacturer of the diet revealed that it had moved manufacture of the diet from Villemoisson to Augy in the middle of 2004 and had implemented new radiation and storage sterilization procedures, which temporally coincided with the deficit in vitamin K in AO4C-10P1 diet. Based on the analyses, it was agreed with the manufacturers to supplement the diet with 15 mg/kg of the synthetic vitamin K_3 analogue menadione. The analysed levels of vitamin K_3 are presented in Table 45. The batches of diet used for the 28-day and 90-day rat and mouse studies were not retrospectively analysed. For reference, analysis of the Purina diet, which was used for the multigeneration studies, showed the level of vitamin K_1 to be 0.65 mg/kg and that of vitamin K_3 0.8 mg/kg, indicating that there was sufficient vitamin K in the diet used for the multigeneration studies. These studies were performed in a laboratory in the United States of America (USA), and the diet supplier was different.

The developmental toxicity studies were performed using pelleted diet, and these were not retrospectively analysed, as no specific problems were encountered. This may be due to several factors. For the rabbit, the vitamin K requirements may be different from those of the rat, and the diet supplied to them, although from the same supplier (SAFE), was different from the diet supplied for the rat. In addition, the duration of treatment was shorter. For the rat, the treatment (which was shorter) was only in females, which are less susceptible to vitamin K problems; in addition, the strain was the Sprague-Dawley and not the Wistar.

(b) Mechanistic studies on blood clotting parameters

In a mechanistic study, the effects of bixafen on blood coagulation parameters of male rats receiving either a vitamin K_3–deficient diet or a diet supplemented with vitamin K_3 in excess were investigated. Forty male Wistar rats were taken from the initial chronic toxicity and carcinogenicity study of bixafen in rats (McElligott, 2008) after approximately 6 months on treatment with 1000 ppm bixafen in the diet. These rats all exhibited prolonged PT and APTT and were randomly placed into one of two groups of 20: group 1 with the existing diet (1000 ppm bixafen; vitamin K_3 concentration < 0.3 ppm), and group 2 given the same diet (1000 ppm bixafen; with 16 ppm vitamin K_3 added). The rats were placed on these regimens for 28 days. The mean achieved doses at the dietary concentration of 1000 ppm bixafen were 41.0 and 41.5 mg/kg bw per day for group 1 and group 2, respectively. Animals were observed daily for mortality and clinical signs. Physical examinations were performed weekly. Body weight and feed consumption were recorded once weekly. Haematology parameters were determined on study days 1 (before the change in diet) and 15 and at the end of the study. All

Table 45. Analyses of diet for vitamin K$_3$ content

Study (reference)	In-life dates	Diet	Batches of diet	When used	Vitamin K$_3$ concentration (mg/kg)
Mouse, 28 day (Steiblen, 2004)	02/2004–04/2004	A04C-10P1	Diet not retrospectively analysed for vitamin K content		
Mouse, 90 day (Steiblen, 2005a)	06/2004–09/2004	A04C-10P1	Diet not retrospectively analysed for vitamin K content		
Rat, 28 day (Langrand-Lerche, 2004)	12/2003–01/2004	A04C-10P1	Diet not retrospectively analysed for vitamin K content		
Rat, 90 day (Steiblen, 2005b)	04/2004–08/2004	A04C-10P1	Diet not retrospectively analysed for vitamin K content		
Mouse, carcinogenicity (Langrand-Lerche, 2008)	05/2005–12/2006	A04C-10P1	50107	30/05/05–24/07/05	Not analysed
			50322	25/07/05–18/09/05	Not analysed
			50607	19/09/05–13/11/05	< 0.3
			50720	14/11/05–08/01/06	15.7
			51027	09/01/06–05/03/06	8.2
			60118	06/03/06–20/08/06	9.6
			60323	21/08/06–12/12/06	7.1
Rat, chronic toxicity and carcinogenicity (McElligott, 2008)	03/2005–04/2007	A04C-10P1	41108/41123	21/04/05–01/06/05	Not analysed
			50107/50322	02/06/05–24/08/05	Not analysed
			50510	25/08/05–05/10/05	Not analysed
			50607	06/10/05–12/10/05	< 0.3
			50720	13/10/05–28/12/05	15.7
			51027	29/12/05–03/05/06	8.2
			60118	04/05/06–18/10/06	9.6
			60323	19/10/06–07/02/07	7.1
			60816	08/02/07–04/04/07	10.6
Rat, chronic toxicity and carcinogenicity (Garcin, 2008)	11/2005–12/2007	A04C-10P1	51027	08/12/05–01/03/06	8.2
			60118	02/03/06–27/09/06	9.6
			60323	28/09/06–14/12/07	7.1
			60816	15/12/07–01/08/07	10.6
Rat, reproductive toxicity (Young, 2006)	07/2005–11/2005	Purina Mills Rodent Lab CH Chow 5002 Meal	–	–	0.8
Rat, two-generation reproductive toxicity (Young, 2007)	01/2006–10/2006	Purina Mills Rodent Lab CH Chow 5002 Meal	–	–	0.8
Rat, mechanistic study with vitamin K$_3$–deficient and vitamin K$_3$–supplemented diet	10/2005–11/2005	A04C-10P1	50607	Batch used for the whole study	< 0.3
			50720	Batch used for the whole study	15.7

Study (reference)	In-life dates	Diet	Batches of diet	When used	Vitamin K_3 concentration (mg/kg)
(Steiblen, 2006)					
Rat, 28-day mechanistic study (Blanck, 2006)	10/2005–11/2005	A04C-10P1	50720	Batch used for the whole study	15.7

animals were necropsied, selected organs were weighed and a range of tissues was sampled and fixed, but not examined.

Nine animals fed with 1000 ppm bixafen in the vitamin K_3–deficient diet died before the end of the study. Eight animals were killed for humane reasons between study days 2 and 15, and one animal was found dead on day 15. During the study, 7/9 decedent animals had swollen eyelids, and 3/9 had protruding eyes on one or more occasions. In addition, half-closed right eye, ocular discharge, damaged eye(s) during the blood sampling, white area on the right eye, wasted appearance, focal swelling of the head, uncoordinated movements, soiled fur around the anogenital region or around the nose, cold to touch, reduced motor activity, general pallor and hunched posture were noted in one or two males on one or more occasions. Most of these animals had a loss of body weight on day 15 when compared with day 8, which was considered to be a consequence of the blood sampling session (at day 15). At necropsy, some animals were noted to have a pale appearance and soiled fur. Dark content in the stomach and/or in the intestines, haemorrhage(s) in the eye and haematoma in the Harderian gland were noted in some animals, whereas multifocal haemorrhage(s) in the submaxillary gland, exorbital gland, skeletal muscle, subcutis, blood clot at the surface of the brain and spinal cord, and red foci in the prostate or thymus were observed in one or two male animals. Haemorrhagic syndrome was considered to be the most probable cause of death or premature sacrifice. All these findings were considered to be related to the vitamin K_3 deficiency of this diet.

Two male animals fed with 1000 ppm bixafen in the vitamin K_3–supplemented diet died before the end of the study. One animal was found dead at the beginning of the study (day 2). Prior to necropsy, this animal had right eyelid swollen and half-closed eye on day 1. At necropsy, this animal was noted with a red focus in the kidney, mottled red thymus, dark content in the stomach and damaged right eye due to blood sampling. This death probably occurred due to the previous exposure to the vitamin K_3–deficient diet, as this animal died at the beginning of the study. Another animal was killed for humane reasons on day 15, prior to necropsy; this animal had protruding eyes, swollen eyelids, focal swelling of the head, white area on the left eye and damaged eyes due to blood sampling on day 15. In addition, this animal was noted to have a body weight loss on day 15 when compared with day 8, which was considered to be a consequence of the blood sampling session (at day 15). At necropsy, damaged eyes due to blood sampling and haematoma in the Harderian gland were noted. This last finding is likely to be a consequence of the blood sampling procedure and was therefore considered to be incidental.

Most of the clinical signs described in the decedent animals were also found in the surviving animals. Some surviving animals fed with vitamin K_3–deficient diet were noted to have ocular discharge and swollen right eyelid on one or two occasions. In addition, one male was noted to have a protruding eye on one occasion. Some surviving animals fed with vitamin K_3–adequate diet had right eye half-closed, ocular discharge of the right eye and swollen right eyelid on one or two occasions. In this study, all the clinical observations described in the two groups were considered to be a consequence of the repeated blood sampling conducted in the present study and the previous study (McElligott, 2008). Most of the surviving animals of the two groups had a body weight loss on day 15 when compared with day 8, which was considered to be a consequence of the blood sampling session (at day 15).

On day 1 (before the change of diet), similar values were noted for PT and APTT between the two groups. In both groups, the mean values of the two parameters were markedly increased when

compared with the normal physiological range in this species kept under similar conditions. On days 15 and 29, after feeding with vitamin K_3–adequate diet, mean PT and APTT values were back to normal values for rats of this strain and age, whereas they remained significantly elevated in the group receiving the vitamin K_3–deficient diet (Table 46).

Table 46. Selected findings in the 28-day rat study (males only) with vitamin K_3–deficient diet or vitamin K_3–supplemented diet

	Group 1 (1 000 ppm) (< 0.3 ppm vitamin K_3)	Group 2 (1 000 ppm) (16 ppm vitamin K_3)
Premature deaths	9/19	2/17
PT (s)		
- Day 1	43.0	45.1
- Day 15	37.8	15.3**
- Day 29	34.0	16.0**
- Historical control data: mean (range)	14.48 (9.2–19.1)	
APTT (s)		
- Day 1	65.4	68.1
- Day 15	67.6	17.8**
- Day 29	57.4	18.7**
- Historical control data: mean (range)	23.16 (15.7–32.4)	

APTT: activated partial thromboplastin time; ppm: parts per million; PT: prothrombin time; *: $P < 0.05$; **: $P < 0.01$

Source: Steiblen (2006)

In conclusion, the deficiency of vitamin K_3 in the diet in combination with the administration of bixafen was considered to be the cause of mortalities, elevated coagulation parameter times and haemorrhagic syndrome observed in male rats from the chronic toxicity and carcinogenicity study, as reported above (Steiblen, 2006).

A further mechanistic study was conducted to determine if bixafen dietary exposure in male rats results in changes to blood clotting parameters or significantly affects coagulation factors when the vitamin K_3 level in feed is 16 ppm. Groups of 10 male Wistar Rj: WI (IOPS HAN) rats were fed diets containing bixafen (purity 95.8%) at a concentration of 0, 2000, 4500 or 10 000 ppm (equal to 0, 162, 375 and 828 mg/kg bw per day) for 28 days. Animals were observed daily for clinical signs and twice daily for mortality. Physical examinations were performed weekly. Body weight and feed consumption were recorded at least weekly. Haematology and clinical chemistry parameters (including coagulation parameters) were determined at the end of the study. All animals were necropsied, selected organs were weighed and a range of tissues was taken and fixed. Histological examinations were not performed.

There were no mortalities during the study. Clinical signs consisted of nasal discharge on day 2 only for 3/10 animals at 10 000 ppm and for 2/10 animals at 4500 ppm. No treatment-related clinical signs were noted at 2000 ppm.

Body weights at 10 000 ppm were reduced by 13% throughout the study, and the body weight gain was reduced by 31%. At 4500 ppm, body weights were reduced by 8–9%, with body weight gain reduced by 19%. At 2000 ppm, body weights were reduced by 2–3%, with body weight gain reduced by 8% (Table 47).

Feed consumption was reduced by 9–26% at 10 000 ppm during the course of the study and by 20% at 4500 ppm for the 1st week of treatment.

The haematological examinations revealed no toxicologically significant variations for the parameters assayed. At 10 000 and 4500 ppm, lower total leukocyte counts (−21% and −24%, respectively) were noted, with lower lymphocyte counts (−24% and −23%, respectively). However, no clear dose–effect relationship was observed; therefore, these slight changes were considered not to be toxicologically relevant. Concerning the coagulation parameters, there was no evidence for an increase in PT. The slight statistically significant decreases observed at 4500 and 10 000 ppm were not dose related and not believed to be toxicologically meaningful. The statistically significant increases of very low amplitude of factors IX and XII, observed at 2000, 4500 and 10 000 ppm, carry no known toxicological consequence (Table 47).

Table 47. Selected findings in the 28-day rat study (males only) with vitamin K_3–supplemented diet

	0 ppm	2 000 ppm	4 500 ppm	10 000 ppm
Body weight (g)				
- Day 1	211	213	212	214
- Day 8	264	256	240**	230**
- Day 15	310	304	284**	273**
- Day 22	352	343	323**	306**
- Day 28	381	370	349**	331**
Body weight gain (g)				
- Days 1–8	53	43*	28**	16**
- Days 1–15	99	91	72**	59**
- Days 1–22	141	130	111**	92**
- Days 1–28	170	157	137**	118**
Feed consumption (g/animal per day)				
- Day 8	25.3	23.1	20.3**	18.6**
- Day 15	27.5	25.5	25.9	24.2*
- Day 22	28.9	26.5	26.3	24.9**
- Day 28	28.3	26.6	26.7	25.8
White blood cell count (10^9/L)	16.8	14.0	12.8**	13.2**
Lymphocyte count (10^9/L)	13.4	10.9	10.3**	10.2**
PT (s)	14.70	13.85	13.34*	13.67*
Factor IX (s)	34.55	36.19**	36.69**	36.58**
Factor XII (s)	25.35	26.31*	26.51**	26.64**
Glucose (mmol/L)	5.93	5.02	4.47**	4.50**
Absolute liver weight (g)	10.70	13.09**	13.07**	13.39**
Relative liver weight (% of body weight)	3.06	3.82	4.04**	4.32**
Absolute thyroid weight (mg)	19.7	22.1	23.8	24.3
Relative thyroid weight (% of body weight, ×1 000)	5.65	6.50	7.37*	7.85**
Liver: enlarged	0/10	6/10	9/10	10/10
Liver: dark	0/10	4/10	7/10	3/10

ppm: parts per million; PT: prothrombin time; *: $P < 0.05$; **: $P < 0.01$
Source: Blanck (2006)

At necropsy at 10 000 ppm, liver weights were increased by 25–41%, and relative thyroid weight was increased by 39%. Enlarged livers were seen in all 10 animals, and dark livers in 3/10

animals. At 4500 ppm, liver weights were increased by 22–32%, with relative thyroid weight increased by 30%. Enlarged and dark livers were found in 9/10 and 7/10 animals, respectively. At 2000 ppm, absolute and relative liver weights were increased by 22% and 25%, respectively, and relative thyroid weight was increased by 15%. Dark and enlarged livers were seen in 4/10 and 6/10 animals, respectively (Table 47).

A NOAEL could not be identified, as decreased body weight gain and liver toxicity (increased [> 20%] liver weight, enlarged liver, dark liver) were observed at all doses. The LOAEL was 2000 ppm (equal to 162 mg/kg bw per day). The NOAEL for blood coagulation effects in male rats fed a vitamin K_3–supplemented diet was 10 000 ppm (equal to 828 mg/kg bw per day), the highest dose tested (Blanck, 2006).

(c) Mechanistic study on thyroid hormone levels

A mechanistic study was conducted to investigate the thyroid changes induced by bixafen in the rat by measuring plasma thyroid hormone levels (TSH, T_3 and T_4) and liver enzyme induction following continuous oral administration (gavage) for up to 14 days. Groups of 15 male and 15 female Wistar Rj: WI (IOPS HAN) rats were treated orally by gavage for 1, 3, 7 or 14 days with vehicle (aqueous 0.5% methyl cellulose 400) or bixafen (purity 95.8%) at a dose of 150 mg/kg bw per day. Clinical observations were performed daily, and body weight and feed intake were measured weekly for animals treated for 7 or 14 days only. A detailed physical examination was performed weekly during the treatment period, commencing on day 1. Animals were killed 24 hours after 1, 3, 7 or 14 daily doses. On each day of sacrifice, blood samples were taken for analysis of thyroid hormone (TSH, T_3 and T_4) concentrations in the plasma. On the last day (day 15), all animals were killed, the livers were sampled and weighed, and the livers from six animals of each sex per group were used to prepare microsomes for the assessment of hepatic cytochrome P450 content and P450 isoenzyme and uridine diphosphate–glucuronosyltransferase (UDPGT) activities. Terminal body weights were also recorded.

There were no mortalities, treatment-related clinical signs or effects on body weight in either sex. During the 1st week of treatment, feed consumption was reduced by 19% in females.

Hormonal investigations showed that treatment increased plasma TSH levels in females on days 3 (+70%), 7 (+93%) and 14 (+151%) and in males on day 14 (+81%). In addition, a slight transient reduction in T_3 levels was seen in females on days 3 (−23%) and 7 (−10%), and a tendency towards lower mean T_4 levels was seen in males on day 14 (−12%) (Table 48).

After 14 days of exposure, a dose of 150 mg/kg bw per day increased liver weight by 22–24% in both sexes. Total hepatic P450 content was increased by 17–20% in both sexes, EROD activity was increased by 30% in males and PROD activity was increased by 73–123% in both sexes. A considerable increase in BROD activity was seen in males (562%) and in females (451%) when compared with controls. A slight increase was also observed in mean UDPGT activity (55–60%) in both sexes (Table 48).

In summary, these data indicate that bixafen after 14 days at 150 mg/kg bw per day induces phase I and II hepatic enzymes (Rascle, 2008).

3. Observations in humans

There were no reports of adverse health effects in manufacturing plant personnel. Also, there were no reports of poisonings with bixafen.

Table 48. Selected findings in the 14-day mechanistic study in rats

	Males		Females	
	0 mg/kg bw per day	150 mg/kg bw per day	0 mg/kg bw per day	150 mg/kg bw per day
T_3 (nmol/L)				
- Day 1	0.895	0.894	0.884	0.845
- Day 3	0.829	0.779	0.946	0.724**
- Day 7	1.107	1.033	1.143	1.031
- Day 14	1.052	1.005	1.027	1.043
T_4 (nmol/L)				
- Day 1	55.5	53.4	35.4	33.2
- Day 3	45.8	45.4	34.4	31.1
- Day 7	50.7	45.4	36.5	32.7
- Day 14	48.9	43.1*	31.8	32.3
TSH (ng/mL)				
- Day 1	2.55	2.89	1.00	0.87
- Day 3	3.46	4.19	1.17	1.99**
- Day 7	3.81	4.64	1.31	2.53**
- Day 14	3.65	6.61**	1.46	3.67**
Absolute liver weight (g)	14.81	18.10**	8.79	10.91**
Relative liver weight (% of body weight)	3.46	4.11**	3.40	4.28**
Total P450 content (nmol/mg protein)	1.15	1.38*	1.11	1.30
BROD (pmol/min per milligram protein)	8.28	54.79**	2.70	14.87**
EROD (pmol/min per milligram protein)	26.32	34.37*	32.68	34.46
PROD (pmol/min per milligram protein)	5.65	12.62*	2.89	5.01*
UDPGT (nmol/min per milligram protein)	11.28	17.44**	7.12	11.40**

BROD: benzyloxyresorufin *O*-dealkylase; bw: body weight; EROD: ethoxyresorufin *O*-deethylase; PROD: pentoxyresorufin *O*-depentylase; T_3: triiodothyronine; T_4: thyroxine; TSH: thyroid stimulating hormone; UDPGT: uridine diphosphate–glucuronosyltransferase; *: $P < 0.05$; **: $P < 0.01$

Source: Rascle (2008)

Comments

Biochemical aspects

In rats given [dichlorophenyl-U-^{14}C]bixafen orally by gavage, absorption was rapid and accounted for at least 83% of the total administered radioactivity after a single low dose (2 mg/kg bw). The maximum plasma concentrations of radioactivity were reached approximately 2–4 and 8 hours after administration of the low and high doses (2 and 50 mg/kg bw), respectively. Radioactivity was widely distributed throughout the body. Elimination of the radioactivity was mainly via faeces (> 91%), whereas elimination via urine accounted for 1–3% of the administered dose. In bile duct–cannulated rats, extensive biliary excretion (up to 83%) was demonstrated. Elimination of the radioactivity from the body was rapid, with a half-life in plasma of 8–9 hours and a mean residence time of 13–19 hours (for the low dose). Residues in tissues at 72 hours after a single oral dose as well as after repeated oral dosing accounted for 0.1–3% of the administered radioactivity, with liver and kidneys containing the highest concentrations of residues.

Metabolism of bixafen in rats was extensive, and more than 30 metabolites were identified. The main metabolic routes included demethylation, hydroxylation of the parent and bixafen-

desmethyl, and conjugation with glucuronic acid or glutathione. A minor metabolic reaction was the cleavage of the amide bridge of bixafen.

Toxicological data

The LD_{50} in rats treated orally or dermally with bixafen was greater than 2000 mg/kg bw, and the inhalation LC_{50} in rats was greater than 5.38 mg/L. Bixafen was not a skin irritant in rabbits, was not irritating to the eye of rabbits and was not a skin sensitizer in the local lymph node assay in mice.

Following repeated administration of bixafen, the liver was the primary target organ in mice, rats and dogs. Increased liver weights and hepatocellular hypertrophy were observed in all species tested and were considered to reflect hepatic microsomal enzyme induction. Also, in several studies, there was evidence for liver toxicity based on clinical chemistry changes (increased serum alkaline phosphatase and cholesterol, decreased serum albumin) and histopathological changes (hepatocellular pigmentation, degeneration and necrosis). In mice and rats, the thyroid was an additional target, which was considered to be secondary to the enhanced hepatic clearance of thyroid hormones. This suggestion was supported by a 14-day mechanistic study in rats in which a marked induction of phase I and II hepatic enzymes, a slight reduction of thyroid hormone (T_3, T_4) levels and a significant increase of TSH levels were observed at 150 mg/kg bw per day, the only dose tested.

In a 4-week study in mice using dietary concentrations of 0, 100, 500 and 2500 ppm (equal to 0, 17, 81 and 305 mg/kg bw per day for males and 0, 21, 103 and 424 mg/kg bw per day for females, respectively), the NOAEL was 100 ppm (equal to 17 mg/kg bw per day), based on liver toxicity (increased liver weight, clinical chemistry changes, focal coagulative necrosis) at 500 ppm (equal to 81 mg/kg bw per day) and above. In a 13-week study in mice using dietary concentrations of 0, 50, 200 and 500 ppm (equal to 0, 8.5, 34.3 and 88 mg/kg bw per day for males and 0, 10.4, 42.9 and 110 mg/kg bw per day for females, respectively), the NOAEL was 50 ppm (equal to 8.5 mg/kg bw per day), based on liver toxicity in males (increased liver weight, clinical chemistry changes, diffuse hepatocellular vacuolation) and focal/multifocal squamous cell hyperplasia of the stomach in both sexes at 200 ppm (equal to 34.3 mg/kg bw per day) and above.

In a 4-week study in rats using dietary concentrations of 0, 50, 350 and 2000 ppm (equal to 0, 3.5, 25 and 137 mg/kg bw per day for males and 0, 4.1, 28 and 138 mg/kg bw per day for females, respectively), the NOAEL was 350 ppm (equal to 25 mg/kg bw per day), based on reduced body weight gain, reduced feed consumption, liver toxicity (increased liver weight, increased cholesterol level) and thyroid effects (hypertrophy of follicular cells) at 2000 ppm (equal to 137 mg/kg bw per day). In a 13-week study in rats using dietary concentrations of 0, 50, 200, 800 and 2000 ppm (equal to 0, 3.2, 12.9, 50.4 and 130 mg/kg bw per day for males and 0, 3.9, 15.0, 59.2 and 153 mg/kg bw per day for females, respectively), the NOAEL was 200 ppm (equal to 12.9 mg/kg bw per day), based on liver effects (enlarged liver, increased liver weight) and thyroid effects (hypertrophy of follicular cells) at 800 ppm (equal to 50.4 mg/kg bw per day) and above.

In a 13-week study in dogs testing dose levels of 0, 100, 300 and 1000 mg/kg bw per day by oral gavage, the NOAEL was 100 mg/kg bw per day, based on an increase (> 20%) in absolute and relative liver weights of females at 300 mg/kg bw per day and above. In a 1-year study in dogs testing dose levels of 0, 10, 100 and 1000 mg/kg bw per day by oral gavage, the NOAEL was 10 mg/kg bw per day, based on haematological effects (decrease in red blood cell count, haemoglobin and haematocrit) in males and liver toxicity (increased liver weight, increased alkaline phosphatase and cholesterol levels) in females at 100 mg/kg bw per day and above.

Long-term studies of toxicity and carcinogenicity were conducted in mice and rats. As a result of technical problems in the production of the feed, the vitamin K_3 level of the diet (< 0.3 ppm) used in the first 5–6 months of the studies was significantly lower than the estimated requirement for mice and rats (approximately 1 ppm), with the consequence of a haemorrhagic syndrome and increased mortality, especially in the male animals from the high-dose group of mice and the mid-dose and high-dose groups of rats. After approximately 6 months of treatment, the diet was supplemented with 7–16 ppm of the synthetic vitamin K analogue menadione, and the studies were completed as

scheduled, with the exception of the male rats, which were prematurely terminated after approximately 6–8 months of treatment. An additional study of chronic toxicity and carcinogenicity was therefore conducted in male rats (see below).

In the 78-week study of carcinogenicity in mice using dietary concentrations of 0, 50, 150 and 500 ppm (equal to 0, 6.7, 20.4 and 69.0 mg/kg bw per day for males and 0, 8.6, 25.5 and 85.0 mg/kg bw per day for females, respectively), there was no evidence for carcinogenicity up to the highest dose tested (500 ppm, equal to 69.0 mg/kg bw per day). The NOAEL for toxicity was 50 ppm (equal to 6.7 mg/kg bw per day), based on thyroid effects (follicular cell hyperplasia) in females and decreased body weights and liver toxicity (single-cell degeneration/necrosis) in males at 150 ppm (equal to 20.4 mg/kg bw per day) and above.

In the initial 24-month study of toxicity and carcinogenicity in rats, which was completed as planned for females only, dietary concentrations of 0, 50, 300 and 2000 ppm (equal to 0, 2.8, 17.4 and 117 mg/kg bw per day, respectively) were tested. There was no evidence for carcinogenicity up to the highest dose tested (2000 ppm, equal to 117 mg/kg bw per day). The NOAEL for toxicity was 50 ppm (equal to 2.8 mg/kg bw per day), based on liver effects (increased cholesterol, higher incidence and/or severity of hepatocellular brown pigments and multinucleated hepatocytes) and thyroid effects (higher incidence and/or severity of follicular cell hypertrophy and colloid alteration) at 300 ppm (equal to 17.4 mg/kg bw per day) and above. In the complementary 24-month study of toxicity and carcinogenicity in male rats using a vitamin K_3–supplemented diet (7–11 ppm) and dietary concentrations of bixafen of 0, 50, 300 and 2000 ppm (equal to 0, 2.0, 12.1 and 80.5 mg/kg bw per day, respectively), there was no evidence for carcinogenicity up to the highest dose tested (2000 ppm, equal to 80.5 mg/kg bw per day). The NOAEL for toxicity was 50 ppm (equal to 2.0 mg/kg bw per day), based on liver effects (increased cholesterol levels, increased liver weights) and thyroid effects (higher incidence and/or severity of colloid alteration) at 300 ppm (equal to 12.1 mg/kg bw per day) and above.

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

Bixafen was tested for genotoxicity in vitro and in vivo in an adequate range of assays. There was no evidence of genotoxicity.

The Meeting concluded that bixafen is unlikely to be genotoxic.

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

In a two-generation study of reproductive toxicity in rats using dietary concentrations of 0, 50, 400 and 2500 ppm (equal to 0, 3.3, 26.4 and 169.2 mg/kg bw per day for males and 0, 3.9, 30.8 and 193.7 mg/kg bw per day for females, respectively), the NOAEL for reproductive toxicity was 2500 ppm (equal to 169.2 mg/kg bw per day), the highest dose tested. The NOAEL for parental toxicity was 400 ppm (equal to 26.4 mg/kg bw per day), based on a reduction in body weight and liver effects (liver weight increased > 20%) at 2500 ppm (equal to 169.2 mg/kg bw per day). The NOAEL for offspring toxicity was 400 ppm (equal to 26.4 mg/kg bw per day), based on a slight elevation in stillbirths and reduced pup weight/weight gain during lactation at 2500 ppm (equal to 177.6 mg/kg bw per day).

In a developmental toxicity study in rats testing dose levels of 0, 20, 75 and 250 mg/kg bw per day, the NOAEL for maternal toxicity was 20 mg/kg bw per day, based on decreased body weight gain and feed consumption at 75 mg/kg bw per day and above in the first days of treatment (i.e. GDs 6–8). The NOAEL for embryo and fetal toxicity was 20 mg/kg bw per day, based on decreased fetal weights at 75 mg/kg bw per day and above.

In a developmental toxicity study in rabbits testing dose levels of 0, 25, 100 and 400 mg/kg bw per day, the NOAEL for maternal toxicity was 25 mg/kg bw per day, based on clinical signs (hair loss, no or reduced excreta) and a reduction in body weight gain and feed consumption at 100 mg/kg bw per day and above. The NOAEL for embryo and fetal toxicity was 25 mg/kg bw per day, based on reduced fetal weight at 100 mg/kg bw per day and above.

The Meeting concluded that bixafen is not teratogenic in rats or rabbits.

A study using high-dose male rats from the initial 24-month study of toxicity and carcinogenicity provided evidence that the low vitamin K_3 level of the diet (< 0.3 ppm) was the cause of the haemorrhagic syndrome, as the prolonged blood coagulation time could be reversed by a vitamin K_3–supplemented diet (16 ppm). This conclusion was supported by the fact that no adverse effects on blood coagulation were observed in the multigeneration studies using diets with an adequate level of vitamin K (vitamin K_1: 0.65 ppm; vitamin K_3: 0.8 ppm).

In a 28-day study in male rats using a vitamin K_3–supplemented diet (16 ppm) and dietary concentrations of bixafen of 0, 2000, 4500 and 10 000 ppm (equal to 0, 162, 375 and 828 mg/kg bw per day, respectively), the NOAEL for effects of bixafen on blood coagulation parameters was 10 000 ppm (equal to 828 mg/kg bw per day), the highest dose tested.

Human data

In reports on manufacturing plant personnel, no adverse health effects were noted. Also, there were no reports of poisonings with bixafen.

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

Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) for bixafen of 0–0.02 mg/kg bw, based on the NOAEL of 2.0 mg/kg bw per day for liver and thyroid effects observed at 12.1 mg/kg bw per day in the 24-month study of toxicity and carcinogenicity in male rats. A safety factor of 100 was applied.

The Meeting established an acute reference dose (ARfD) for bixafen of 0.2 mg/kg bw, based on the NOAEL of 20 mg/kg bw for decreased body weight gain and feed consumption observed in the first days of treatment at 75 mg/kg bw in a developmental toxicity study in rats. A safety factor of 100 was applied.

As the estimated exposures to M18, M20, M44, M45 and M47 are below the respective acute and chronic thresholds of toxicological concern for Cramer class III compounds, there is no concern for these metabolites. Bixafen-desmethyl has been tested in rodents through its formation from the parent compound and is therefore covered by the ADI for bixafen. For M25 and M26, their structural similarity to bixafen-desmethyl is such that the Meeting concluded that they would also be covered by the ADI for bixafen.

Levels relevant to risk assessment of bixafen

Species	Study	Effect	NOAEL	LOAEL
Mouse	Eighteen-month study of toxicity and carcinogenicity[a]	Toxicity	50 ppm, equal to 6.7 mg/kg bw per day	150 ppm, equal to 20.4 mg/kg bw per day
		Carcinogenicity	500 ppm, equal to 69.0 mg/kg bw per day[b]	–
Rat	Two-year studies of toxicity and carcinogenicity[a,c]	Toxicity	50 ppm, equal to 2.0 mg/kg bw per day	300 ppm, equal to 12.1 mg/kg bw per day
		Carcinogenicity	2 000 ppm, equal to 80.5 mg/kg bw per day[b]	–

Species	Study	Effect	NOAEL	LOAEL
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	2 500 ppm, equal to 169.2 mg/kg bw per day[b]	–
		Parental toxicity	400 ppm, equal to 26.4 mg/kg bw per day	2 500 ppm, equal to 169.2 mg/kg bw per day
		Offspring toxicity	400 ppm, equal to 26.4 mg/kg bw per day	2 500 ppm, equal to 177.6 mg/kg bw per day
	Developmental toxicity study[d]	Maternal toxicity	20 mg/kg bw per day	75 mg/kg bw per day
		Embryo and fetal toxicity	20 mg/kg bw per day	75 mg/kg bw per day
Rabbit	Developmental toxicity study[d]	Maternal toxicity	25 mg/kg bw per day	100 mg/kg bw per day
		Embryo and fetal toxicity	25 mg/kg bw per day	100 mg/kg bw per day
Dog	One-year study of toxicity[d]	Toxicity	10 mg/kg bw per day	100 mg/kg bw per day

bw: body weight; LOAEL: lowest-observed-adverse-effect level; NOAEL: no-observed-adverse-effect level; ppm: parts per million

[a] Dietary administration.

[b] Highest dose tested.

[c] Two studies combined.

[d] Gavage administration.

Estimate of acceptable daily intake

0–0.02 mg/kg bw

Estimate of acute reference dose

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 bixafen

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid; ≥ 83%
Dermal absorption	No data
Distribution	Widely distributed; highest concentrations in liver and kidneys
Potential for accumulation	None
Rate and extent of excretion	> 93% within 72 h (> 91% in faeces, including up to 83% in bile; 1–3% in urine)

Metabolism in animals	Extensive (> 30 metabolites identified); demethylation, hydroxylation of parent and bixafen-desmethyl; conjugation with glucuronic acid and glutathione; cleavage of the amide bridge of bixafen as a minor metabolic reaction
Toxicologically significant compounds in animals, plants and the environment	Bixafen
Acute toxicity	
Rat, LD_{50}, oral	> 2 000 mg/kg bw
Rat, LD_{50}, dermal	> 2 000 mg/kg bw
Rat, LC_{50}, inhalation	> 5.38 mg/L (4 h, nose-only exposure)
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	Liver in mice, rats and dogs; thyroid in rats
Lowest relevant oral NOAEL	8.5 mg/kg bw per day (mouse)
Lowest relevant dermal NOAEL	No data
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Liver and thyroid in mice and rats
Lowest relevant NOAEL	2.0 mg/kg bw per day (rat)
Carcinogenicity	Not carcinogenic
Genotoxicity	
	Not genotoxic
Reproductive toxicity	
Target/critical effect	No reproductive toxicity
Lowest relevant parental NOAEL	26.4 mg/kg bw per day
Lowest relevant offspring NOAEL	26.4 mg/kg bw per day
Lowest relevant reproductive NOAEL	169.2 mg/kg bw per day, the highest dose tested
Developmental toxicity	
Target/critical effect	Reduced fetal weights, visceral or skeletal variations at maternally toxic dose (rats and rabbits)
Lowest relevant maternal NOAEL	20 mg/kg bw per day (rat)
Lowest relevant embryo/fetal NOAEL	20 mg/kg bw per day (rat)
Neurotoxicity	
Acute and subchronic neurotoxicity	No specific data, but no indications from repeated-dose studies
Other toxicological studies	
Study on blood coagulation	Vitamin K_3–deficient diet contributed to prolonged blood coagulation times and haemorrhagic effects in male rats
Mechanistic study on thyroid effects	Induction of phase I and II hepatic enzymes was likely the cause of the observed thyroid hormone changes
Medical data	
	No adverse health effects reported in manufacturing plant personnel

bw: body weight; LC_{50}: median lethal concentration; LD_{50}: median lethal dose; NOAEC: no-observed-adverse-effect concentration; NOAEL: no-observed-adverse-effect level

Summary

	Value	Study	Safety factor
ADI	0–0.02 mg/kg bw	Two-year study of toxicity and carcinogenicity in rats	100
ARfD	0.2 mg/kg bw	Developmental toxicity study in rats (maternal toxicity)	100

ADI: acceptable daily intake; ARfD: acute reference dose; bw: body weight

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CHLORFENAPYR (addendum)

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Explanation

Chlorfenapyr is the International Organization for Standardization (ISO)–approved name for 4-bromo-2-(4-chlorophenyl)-1-ethoxymethyl-5-trifluoromethyl-1*H*-pyrrole-3-carbonitrile (International Union of Pure and Applied Chemistry [IUPAC]), with Chemical Abstracts Service (CAS) No. 122453-73-0. Chlorfenapyr is a contact and stomach pyrrole insecticide that acts, following metabolic activation, as an uncoupler of oxidative phosphorylation in mitochondria. It has limited systemic activity.

Chlorfenapyr was evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 2012, when an acceptable daily intake (ADI) of 0–0.03 mg/kg body weight (bw) was established, based on a no-observed-adverse-effect level (NOAEL) of 2.8 mg/kg bw per day for decreases in body weight gain and vacuolation of the white matter of the brain at 16.6 mg/kg bw per day in an 18-month mouse study and a NOAEL of 2.9 mg/kg bw per day for reduced body weight and body weight gain and increased liver weight associated with hepatocellular enlargement at 15 mg/kg bw per day in a 2-year rat study. The 2012 Meeting also established an acute reference dose (ARfD) for chlorfenapyr of 0.03 mg/kg bw, based on the NOAEL of 3 mg/kg bw for depression of grooming and reactivity and decreased spontaneous motor activity observed at 10 mg/kg bw in a pharmacological study in mice. At that meeting, it was noted that the oral median lethal dose (LD_{50}) in the rat of the livestock metabolite tralopyril, the ISO-approved name for 4-bromo-2-(4-chlorophenyl)-5-(trifluoromethyl)-1*H*-pyrrole-3-carbonitrile (IUPAC, CAS No. 122454-29-9, also known as AC 303,268), was an order of magnitude lower than that of the parent.

Although the sponsor had not conducted any repeated-dose studies of the toxicity of this metabolite, it became apparent during the 2012 Meeting that such information had been produced by another company to support registration of tralopyril as a biocide (molluscicide). Studies with the metabolite tralopyril on its subchronic oral and inhalational toxicity and developmental toxicity and further studies on its genotoxicity have now been provided and reviewed, allowing completion of the

assessment of chlorfenapyr. Relevant parts of the most recent assessment of chlorfenapyr have been incorporated into this toxicological evaluation.

All critical studies contained certificates of compliance with good laboratory practice (GLP).

Evaluation for acceptable daily intake

1. Studies on metabolites

1.1 Acute toxicity

The oral LD_{50} of tralopyril in rats is 27 mg/kg bw for males and 29 mg/kg bw for females (Bradley, 1994).

In comparison, the oral LD_{50} of chlorfenapyr in rats is 441 mg/kg bw for males and 1152 mg/kg bw for females (Lowe, 1993).

1.2 Short-term studies of toxicity

(a) Oral exposure

Tralopyril

In a 3-month study, tralopyril (purity 94.6%) was administered to Sprague-Dawley rats (10 of each sex per group) in the diet at a concentration of 0, 80, 250 or 750 parts per million (ppm). These concentrations corresponded to intakes of 0, 5.2, 16.2 and 51.9 mg/kg bw per day for males and 0, 6.3, 20.9 and 62.0 mg/kg bw per day for females, respectively. Diets were assessed for homogeneity, concentration and stability. Additional groups of 10 rats of each sex per dose were treated for 3 months at the same dose levels; the first 5 animals were killed after 3 months, and the second 5 animals were killed after a further 4-week recovery period. Feed consumption and body weight were determined weekly. The animals were examined for clinical signs of toxicity or mortality at least once per day. Clinicochemical and haematological examinations were performed towards the end of the administration period and after the recovery period. All animals of the main groups were assessed by gross pathology followed by histopathological examination. This study was GLP compliant and performed according to test guidelines for European Commission Directive 2001/59/EC, Organisation for Economic Co-operation and Development (OECD) Test Guideline 408 and United States Environmental Protection Agency (USEPA) Health Effects Test Guideline OPPTS 870-3100.

The analytical data indicated that the mixing procedure was adequate and that the variance between nominal and actual dosages to the study animals was acceptable (94.9–96.6% of the nominal concentrations). Significant effects at the 750 ppm dose level included reduced feed consumption, feed efficiency and body weights in both sexes. Increased mean corpuscular haemoglobin concentration and alkaline phosphatase, inorganic phosphate and urea levels were observed in both sexes. Additional clinical chemistry changes included increased mean corpuscular haemoglobin, platelets, alanine aminotransferase and creatinine in males and increased globulin, cholesterol and magnesium levels in females. Decreased prothrombin times and glucose level were observed in both sexes, accompanied by decreased triglycerides in males and albumin in females. Liver weights (absolute and relative to body weight) were significantly increased in females, and the increases were considered treatment related, as concomitant indications of mild functional impairment of the liver were noted (increased alkaline phosphatase levels and shortened prothrombin times). An increase in brain weight relative to body weight was observed in both sexes, but was attributed to the decreased body weights in these animals. In the pathology evaluation, changes observed in both sexes included minimal to slight (simple) mucosal hyperplasia of the duodenum, multifocal vacuolation of the white matter of the central nervous system and multifocal intramyelinic vacuolation of the proximal peripheral nervous system (root fibres and proximal sciatic nerve). At the end of the recovery period, feed consumption achieved values similar to control values, but body weight remained reduced in treated animals. Additionally, multifocal vacuolation of white matter in the central nervous system persisted in both sexes at the end of the recovery period.

At the 250 ppm dose level, there were reduced body weight values in males and a significant reduction in body weight gain in males from day 28 onward. Clinical chemistry changes included increased globulin, increased mean corpuscular haemoglobin concentration, increased cholesterol and decreased glucose levels in females. Increased absolute and relative liver weights in females were not considered toxicologically significant, as no accompanying functional or morphological changes were noted. In the pathological examination, there was minimal to slight (simple) mucosal hyperplasia in the duodenum in both sexes, multifocal vacuolation of the white matter in the central nervous system and multifocal intramyelinic vacuolation of the proximal peripheral nervous system (root fibres), with histological ranking of minimal to slight in males and minimal to moderate in females. During recovery, the multifocal vacuolation of the white matter in the central nervous system persisted, with a minimal grading in males and minimal to moderate grading in females.

At 80 ppm, multifocal vacuolation of the white matter in the central nervous system was identified in all treated females (minimal to slight), and multifocal intramyelinic vacuolation of the proximal peripheral nervous system (root fibres) was observed in 1/5 females (minimal). This multifocal vacuolation of the white matter in the central nervous system persisted after recovery in 2/5 females (minimal). Increased absolute and relative liver weights in females were not considered toxicologically significant, as no accompanying functional or morphological changes were noted (Mellert, 2004).

A peer review pathology assessment was performed on selected tissues from male and female rats. The purpose of the peer review assessment was to examine, by light microscopy, the brain and lumbar spinal cord for all main and recovery study animals. In particular, the peer reviewer compared the effects observed in samples prepared using two different techniques: immersion and perfusion fixation. The following tissues were examined by light microscopy: brain (frontal and parietal lobes), cerebellum, cervical dorsal root ganglia, lumbar spinal cord, lumbar dorsal root ganglia, lumbar ventral root ganglia, midbrain and pons. There were no differences in opinion between the study and peer review pathologist that impacted the interpretation of the study (Radovsky, 2005).

Regarding the findings of multifocal intramyelinic vacuolation (Table 1), vacuolation was symmetrical, was specific for white matter, typically showed a dose–response relationship and was not evident in controls. The vacuolation was therefore considered treatment related by the peer review pathologist. Multifocal vacuolation was noted primarily in the white matter of the external capsule, corpus callosum and internal capsule of the rostral hippocampus. Vacuolation of white matter at the base of the midbrain (brachium pontis) was localized to the more posterior sections, and vacuolation in the cerebellum was observed primarily in the white matter supporting the cerebellar folia. In the lumbar spinal cord, vacuolation was observed in the cauda equina.

In conclusion, tralopyril at a concentration of 750 ppm (equal to 51.9 mg/kg bw per day for males and 62.0 mg/kg bw per day for females) caused severe effects on feed consumption and body weight. The marked decrease in body weight change is indicative of a dose level exceeding the maximum tolerated dose. Moreover, at this dose level, adverse effects on red blood cells and platelets as well as functional impairment of the liver and kidneys occurred in the absence of morphological changes. These findings (kidney and liver dysfunction) were reversible after cessation of treatment. Without evidence of corresponding microscopic changes in the liver or kidneys, these changes may not be toxicologically significant. It was not possible to identify a NOAEL in this study, as vacuolation of the central nervous system and peripheral nervous system was evident at 80 ppm in females, the lowest dose tested. Therefore, the LOAEL for this study was 80 ppm (equal to 6.3 mg/kg bw per day). The study authors identified a NOAEL in male animals of 80 ppm (equal to 5.2 mg/kg bw per day), based on vacuolation of white matter in animals at the next highest dose level. The absence of neuronal degeneration or neuronal necrosis in both the main and recovery groups suggests that full morphological reversibility can be expected after a longer recovery period. This is supported by the partial recovery of vacuolation in the brain and lumbar cords of recovery animals observed by the study (Mellert, 2004) and review (Radovsky, 2005) pathologists.

Table 1. Multifocal vacuolation of the white matter in rats (n = 10 per group) after oral administration of tralopyril

Vacuolation of the white matter, multifocal[a,b]	Treatment period test groups (ppm)								Recovery period test groups (ppm)							
	Control		80		250		750		Control		80		250		750	
	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
Brain																
- No evidence	10	10	10	5	9	9	0	1	5	5	5	5	5	4	2	1
- Grade 1	0	0	0	5	1	1	7	3	0	0	0	0	0	1	3	3
- Grade 2	0	0	0	0	0	0	1	3	0	0	0	0	0	0	0	1
- Grade 3	0	0	0	0	0	0	2	3	0	0	0	0	0	0	0	0
Thoracic cord																
- No evidence	10	10	10	10	10	10	10	8	5	5	5	5	5	5	5	5
- Grade 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
- Grade 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
- Grade 3	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
- Grade 4	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Lumbar cord																
- No evidence	10	10	10	5	2	1	0	0	5	5	5	3	3	0	0	0
- Grade 1	0	0	0	3	6	3	0	0	0	0	0	2	2	2	0	1
- Grade 2	0	0	0	2	2	4	2	0	0	0	0	0	0	2	3	2
- Grade 3	0	0	0	0	0	2	6	3	0	0	0	0	0	1	2	2
- Grade 4	0	0	0	0	0	0	2	7	0	0	0	0	0	0	0	0

[a] There was no evidence of multifocal vacuolation of the white matter in the cervical cord in any of the treatment or recovery animals (not shown).
[b] A peer review pathology examination was performed with slightly different scoring of multifocal vacuolation lesion grading. These differences did not significantly impact the study results and are not shown here.
Source: Mellert (2004)

Previously reviewed chlorfenapyr study

Chlorfenapyr technical (purity 93.6%; lot no. AC 7171-141A) was fed to six groups of 20 male and 20 female albino rats for 13 weeks at a dietary concentration of 0 (control), 150, 300, 600, 900 or 1200 ppm (equal to 0, 10.9, 22.0, 44.9, 69.5 and 92.2 mg/kg bw per day for males and 0, 12.5, 26.1, 51.8, 75.4 and 102.8 mg/kg bw per day for females, respectively). The protocol was in compliance with test method B.7 of Directive 92/69/EEC and OECD Test Guideline 408.

No treatment-related mortality was noted at any dose level. Low incidences of clinical signs, including ataxia (3/20), decreased activity (3/20), dark material around nose (4/20) and anorexia (5/20), were observed in a few males of the 1200 ppm group. An increased incidence of brown material around the nose was also observed in males at 1200 ppm (9/20).

Body weights and body weight gains were lower than control values for both sexes at 1200 and 900 ppm and for males only at 600 ppm. Total body weight gains were decreased in males and females by 37% and 24%, respectively, at 1200 ppm, by 25% and 21%, respectively, at 900 ppm and by 14% and 8%, respectively, at 600 ppm.

Red blood cell parameters (haematocrit, erythrocyte count and haemoglobin) were reduced for both sexes at 1200 ppm and for females at 900 ppm; in females, a significant reduction of haemoglobin was also evident at the 600 ppm dose level. Haematocrit and erythrocyte count were reduced in this group, but not significantly. Platelet counts were elevated in both sexes at 1200 and 900 ppm, but the changes were statistically significant only in males. Serum alkaline phosphatase

level tended to be higher after 6 weeks and was clearly elevated in both sexes at 1200 and 900 ppm after 13 weeks of treatment. Gamma-glutamyl transpeptidase levels in both sexes and alanine aminotransferase levels in males were elevated at 1200 ppm after 6 weeks, but not 13 weeks. Blood urea nitrogen was elevated at 1200 ppm in both sexes after 6 weeks, but only in males after 13 weeks. Also after 13 weeks, total serum protein was elevated in females at 1200 ppm and in both sexes at 900 ppm. Albumin was reduced after 6 and 13 weeks in males at 1200 ppm.

Increased absolute and relative liver and spleen weights (correlated with decreased red cell parameters) were observed at termination in the 1200 and 900 ppm groups. Other organ weight changes at these treatment levels were secondary to body weight decreases.

Hepatic parenchymal cell hypertrophy was observed in one male at 1200 ppm and one male at 900 ppm. Spongiform myelopathy (vacuolation) of the brain and spinal cord was seen in two males at 1200 ppm, two males at 900 ppm and one male at 600 ppm. In the two males at 1200 ppm with myelopathies of the brain and spinal cord, one also exhibited this lesion in the sciatic nerve and the other in the optic nerve. Testicular atrophy was observed in two males at 1200 ppm and three males at 900 ppm.

The NOAEL for chlorfenapyr in the rat was 300 ppm (equal to 22.0 mg/kg bw per day for males and 26.1 mg/kg bw per day for females), based on increases in relative liver weight, alkaline phosphatase and blood urea nitrogen and, in females, changes in red cell parameters seen at 600 ppm (equal to 44.9 mg/kg bw per day for males and 51.8 mg/kg bw per day for females) (Fischer, 1993).

(b) Inhalation exposure

Tralopyril

In a 90-day combined nose-only inhalation toxicity and neurotoxicity study, tralopyril (purity 92.4%) was administered for 6 hours/day, 7 days/week, for 90 days to Crl:CD(SD) rats in three groups in the general toxicity phase (Phase 1: 10 rats of each sex per group) and three groups in the neurotoxicity phase (Phase II: 12 rats of each sex per group). The target exposure levels were 20, 40 and 80 mg/m^3. A concurrent control group for each phase was exposed to filtered air. 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 and feed consumption were recorded weekly. Functional observational battery and locomotor activity data were recorded for all animals in Phase II prior to the initiation of dose administration and during study weeks 3, 7 and 12. Ophthalmic examinations were performed for all animals in Phase I during study weeks −1 and 13. Clinical pathology evaluations (haematology, serum chemistry and urine analysis) were performed on all animals in Phase I at the scheduled necropsy (study week 13). Complete necropsies were conducted on all animals in Phase I, and selected organs were weighed at the scheduled necropsy. Selected tissues were examined microscopically from all animals in the control and 80 mg/m^3 groups in Phase I. For all animals in Phase II, brain weights (excluding olfactory bulbs) and size (length and width) were recorded at the scheduled euthanasia. In addition, a neuropathology evaluation was performed on six animals of each sex per group in the control and 80 mg/m^3 groups. This study was GLP compliant and fulfilled requirements for USEPA OPPTS 870.3465 and 870.6200 and OECD Test Guidelines 413 and 424.

At 80 mg/m^3, there were two unscheduled deaths in males assigned to Phase II on days 20 and 85, which were attributable to exposure to the test article. In addition, there were unscheduled deaths of one male and one female in the Phase I group after test article exposure on day 0. Because of the number of deaths in the 80 mg/m^3 group and the death of only one female in the control group, the male deaths in the 80 mg/m^3 group were considered test article related. Body weights, feed consumption and feed efficiency were decreased in males and females. Clinical observations of brown staining on various body surfaces in both sexes of both test phases were considered non-adverse. In measures of activity, there were increased grooming counts in open-field evaluations of treated males during study weeks 3, 7 and 12, but these were considered attributable to the soiled and discoloured fur. Additionally, there was a decrease in total locomotor and ambulatory activity in males during study week 3, possibly attributable to reduced activity at all subsession intervals. Higher alkaline

phosphatase levels were observed in males and females. Increased lung weights (absolute and relative to body weight) were observed in females, with no correlative histopathological changes.

At 40 mg/m^3, body weight and feed consumption were decreased in both sexes. Clinical observations of brown staining on various body surfaces in both sexes (both phases) were not considered adverse. Lower final body weights were observed in all males and females, and there was lower feed efficiency in males from study weeks 0–1 through 6–7 and in females from weeks 4 to 5. An observed decrease in total locomotor activity in males during study week 3 was considered the result of reduced activity at all subsession intervals. The only significant clinical chemistry change was higher alkaline phosphatase levels in females, and the only organ weight change was higher relative (to body weight) lung weights in females, but no correlative histopathological changes were observed.

At 20 mg/m^3, changes observed were lower body weights and feed consumption in males, clinical observations of brown staining on various body surfaces in animals of both sexes of both phases, lower final body weight in males, lower feed efficiency in males from study weeks 0–1 through 6–7 and higher relative (to body weight) lung weights in females, which occurred in the absence of correlative histopathological changes. In the absence of test article–related changes in clinical pathology parameters or histomorphology, and given that the changes in body weight were limited to one sex and one phase within that sex, the test article–related changes in body weights, body weight gains and feed consumption noted at this concentration were not considered adverse.

Histopathological alterations were noted in males and females in the 40 and 80 mg/m^3 groups. These were limited to the nose and tended to be located in dorsal regions. Ulcerations and erosions were typically accompanied by inflammation and were localized to rostral levels in the mid or dorsal septum and sometimes on the medial aspects of the dorsal turbinates. Inflammation was not separately diagnosed unless there was extension into non-ulcerated/eroded areas. The margins of ulcerated/ eroded areas often exhibited hyperplasia of the epithelium as a response to the injury, but this also was not separately diagnosed unless it occurred in other locations besides the margins of injury. In more posterior sections, alterations were primarily metaplasia or degeneration in the epithelium of the dorsal arches. When the dorsal arches were affected by either metaplasia or degeneration, there often was loss of the submucosal glands, which was diagnosed as atrophy. Degeneration of the respiratory or olfactory epithelium was a disorganization of the architecture, such as loss of the polarity of nuclei. Degeneration of olfactory epithelium also included decreased thickness of the cell layers. Other than these effects in the nose, histological effects were not considered test article related.

The no-observed-adverse-effect concentration (NOAEC) for systemic toxicity in males and females was 20 mg/m^3. The NOAEC for neurotoxicity was 20 mg/m^3 in males and 80 mg/m^3 (the highest concentration tested) in females (Beck, 2006).

Previously reviewed chlorofenapyr study

Fifteen male and 15 female Wistar rats per test group were exposed (nose only) to dynamic inhalation containing dust aerosol atmospheres of chlorfenapyr (purity 97.8%; batch no. 2181H88HV) for 6 hours per working day, 5 days/week, for approximately 90 days (65 exposures). From the 15 animals of each sex per group, 10 were designated as main group animals and 5 as recovery group animals. The main group animals were terminated 1 day after the exposure period; the recovery group animals were terminated after a recovery period of 28 days. The doses were 0 mg/m^3 (control air) (group 0 dose / 01 recovery), 5 mg/m^3 (group 1 dose / 11 recovery), 20 mg/m^3 (group 2 dose / 21 recovery), 40 mg/m^3 (group 4 dose / 41 recovery) and 80 mg/m^3 (group 3 dose / 31 recovery).

Inhalation of a chlorfenapyr dust aerosol led to the premature death of male animals at a concentration of 80 mg/m^3 within the first 3 exposure days (7/15 male animals: 4/10 in the main group and 3/5 in the recovery group). Because of this high lethality, surviving animals of this group were terminated prematurely. Substitute groups were exposed to 40 mg/m^3. However, one male animal was found dead after each of study day 35 and study day 80.

In addition to lethality, a slightly increased respiration rate was observed in animals exposed to 40 mg/m^3. This finding is considered to be substance related. Alopecia or injuries that were observed in individual animals were considered to be incidental.

There were no statistically significant changes in the mean body weight or mean body weight gain in animals exposed to 5 or 20 mg/m^3. Statistically significant differences were noted only in the substitute animals (groups 4 and 41). These changes in body weight and body weight gain were also considered to be incidental, as they were not concentration or time related.

A functional observational battery was carried out on assigned animals before exposure, during exposure (on study day 84), at the end of exposure (on study day 92) and at the end of the recovery period (study day 119). The functional observational battery started with passive observations, without disturbing the animals, followed by removal from their cage and open-field observations. Thereafter, sensorimotor tests and reflex tests were conducted. No substance-related effects were observed in home cage observations. The grip strength (forelimbs) was statistically significantly increased in males of the 40 mg/m^3 group at the end of recovery. This change was considered to be incidental and not of biological relevance. Motor activity was measured on the same day and with the same animals on which the functional observational battery was performed. The measurement was performed in the dark using the Multi-Varimex System with four infrared beams per cage. The numbers of beam interrupts were counted over 12 intervals, each lasting 5 minutes. Measurements ended exactly 60 minutes thereafter. There were some variations of motor activity (mostly increases and some decreases) seen at some intervals in both sexes. These changes were considered to be incidental, because there was no concentration–response relationship and the finding was not consistently observed among intervals.

Regarding clinical pathology findings, inhalation of the test compound at 40 mg/m^3 caused slight increases in white blood cells and lymphocytes at the end of the exposure period. These findings were assessed as being treatment related. At the end of the recovery period, leukocytes and lymphocytes were decreased when compared with the respective control values. The prolonged prothrombin times in the animals of both sexes exposed to 40 mg/m^3 were also considered treatment related and are indicative of slight disturbance of coagulation.

Regarding pathology, the two animals dosed with 40 mg/m^3 that did not survive to termination of the study were examined by histopathology and showed autolytic changes to a different degree and marked pulmonary congestion in both cases, with one animal showing additional macroscopic lung changes (all lung lobes with diffuse discoloration, dark red). This is considered to be related to the mode of death due to asphyxiation and/or agonal respiratory distress. Considering the lethality that occurred at 80 mg/m^3, the two deaths at 40 mg/m^3 are considered to be substance related.

There were no major organ weight changes in either sex in the treated groups, and those observed were not dose related and exhibited no macroscopic or microscopic pathology. All further macroscopic or histopathological findings were regarded to be incidental in origin and not related to treatment.

No treatment-related effects were observed in animals exposed to the 5 or 20 mg/m^3 concentrations of the test compound. Thus, the NOAEC was 20 m g/m^3, based on lethality, haematological alterations and prolonged prothrombin times seen at 40 mg/m^3 (Ma-Hock et al., 2005).

1.3 Genotoxicity

The genotoxic potential of tralopyril was investigated in an in vitro Chinese hamster ovary *Hprt* forward mutation assay and an in vivo mouse micronucleus assay (Table 2). Tralopyril showed no evidence of mutagenic, clastogenic or aneugenic activity in these assays.

Table 2. Results of genotoxicity studies on tralopyril

End-point	Test object	Concentration	Purity (%)	Results	Reference
In vitro					
Gene mutation[a,b]	Chinese hamster ovary (CHO-K1); *Hprt* locus	−S9: 0–50 µg/mL +S9: 0–40 µg/mL	94.6	Negative (±S9)	Engelhardt (2004a)[c]
In vivo					
Micronucleus[d,e]	Mouse (Crl:NMRI)	3, 6, 12 mg/kg bw single administration; killed 24 or 48 h after dosing	94.6	Negative	Engelhardt (2004b)[f]

Hprt: hypoxanthine–guanine phosphoribosyltransferase; S9: 9000 × *g* supernatant fraction of rat liver homogenate

[a] Test in duplicate; positive and negative controls included; hepatic S9 fraction of Aroclor 1254–induced rat; GLP and quality assurance statements included.

[b] Cytotoxicity (small colonies and/or reduction in cell density) seen in first test at 5 µg/plate –S9 and at 10 µg/plate +S9.

[c] Performed in accordance with OECD Test Guideline 476.

[d] Five male mice per treatment group; positive and negative controls included; GLP and quality assurance statements included.

[e] In an oral toxicity pretest, deaths were observed at a dose of 15 mg/kg bw. A dose of 12 mg/kg bw was survived by all animals, but 4/9 exhibited squatting posture in clinical examination.

[f] Performed in accordance with OECD Test Guideline 474.

1.4 Developmental toxicity

Tralopyril

In a prenatal developmental toxicity study, tralopyril (purity 94.6%) was administered by oral gavage in olive oil to 25 presumed pregnant rats per group at a dose of 5, 10 or 20 mg/kg bw on gestation days (GDs) 6–19. The control group (25 pregnant rats) was dosed with the vehicle (olive oil) only. Treatment solutions were analysed for homogeneity, concentration and stability and were within 90.8–100.2% of nominal concentrations. At termination (GD 20), 23–25 females per group had implantation sites. Feed consumption and body weights of the animals were recorded regularly throughout the study period. All surviving females were assessed by gross pathology. Measured parameters included weight determinations of unopened uterus and placenta, corpora lutea counts, and number and distribution of implantation sites (resorptions, live and dead fetuses). The fetuses were removed from the uterus, sexed, weighed and further investigated for any external anomalies. Half of the fetuses of each litter were examined for soft tissue findings, and the remainder were examined for skeletal findings. This study was GLP compliant and fulfilled the requirements for European Commission Directive 87/302/EEC, OECD Test Guideline 414 and USEPA Health Effects Test Guideline OPPTS 870.3700.

At 20 mg/kg bw per day, deaths of 2/25 rats occurred on GD 6. In these animals, partly reddish salivation was observed immediately after dosing and also in the afternoon shortly before they were found dead. The reddish salivation was considered test article related. One of the animals found dead exhibited tremor and convulsions preterminally. Transient salivation in 23/25 rats was observed immediately after gavage on GDs 6–19. Dams exhibited significantly reduced feed consumption on GDs 6–8 and absolute body weight gain on GDs 19–20. This was accompanied by a lower net weight change, but this change did not attain statistical significance. In the postmortem assessment, there were significantly decreased uterine weights, a statistically higher number of resorptions, significantly reduced placental weights, significantly reduced fetal weights and a significantly increased incidence of some skeletal variations, such as disturbances and delays of ossification (non/incomplete/dumbbell/bipartite) of various skeletal structures (skull, sternebrae, vertebrae) as well as

supernumerary ribs (14th; Table 3). In addition, there was a slightly increased rate of affected fetuses per litter for notched manubrium.

Table 3. Skeletal variations in rat fetuses following oral gavage administration of tralopyril to pregnant dams

Parameter	Incidence of skeletal variation[a]			
	0 mg/kg bw per day	5 mg/kg bw per day	10 mg/kg bw per day	20 mg/kg bw per day
Incomplete ossification of interparietal: unchanged cartilage	46 (19)	46 (20)	35 (19)	52 (19)*
Incomplete ossification of nasal: unchanged cartilage	4 (3)	43 (15)**	57 (16)**	101 (21)**
Incomplete ossification of thoracic centrum: unchanged cartilage	22 (10)	37 (14)	25 (13)	68 (19)**
Unossified thoracic centrum: unchanged cartilage	0 (0)	4 (4)*	4 (2)	8 (6)**
Unilateral ossification of thoracic centrum: unchanged cartilage	0 (0)	0 (0)	1 (1)	4 (3)*
Unossified sternebra: unchanged cartilage	82 (21)	102 (23)	94 (22)	89 (19)*
Bipartite ossification of sternebra: unchanged cartilage	8 (7)	12 (11)	12 (9)	15 (11)*
Supernumerary rib (14th): cartilage present	3 (2)	10 (8)*	4 (4)	11 (8)**
Supernumerary rib (14th): cartilage not present	34 (15)	40 (16)	48 (20)	54 (16)*

*: $P < 0.5$; **: $P < 0.01$
[a] Fetus (litter).
Source: Schneider (2004)

At 10 m g/kg bw per day, transient salivation in 16/25 rats was noted immediately after gavage on GDs 9–19, and there were statistically significantly reduced fetal weights. At 5 mg/kg bw per day, no substance-related adverse effects were observed in dams, in fetuses or on gestational parameters.

The NOAEL for maternal and embryo/fetal toxicity was 5 mg/kg bw per day. The LOAEL for maternal and embryo/fetal toxicity was 10 mg/kg bw per day, based on maternal salivation (16/25 rats on GDs 9–19) and slightly decreased mean fetal weights compared with controls. At a dose of 20 mg/kg bw per day, noticeable signs of maternal and embryo/fetal toxicity were observed. Slight but noticeable embryo/fetal toxicity on various skeletal structures as well as supernumerary ribs were seen at a dose at which clear maternal toxicity was present, suggesting that maternal toxicity rather than test article exposure was responsible for the observed effects (Schneider, 2004).

Previously reviewed study on chlorfenapyr for comparison

An analogous study was performed using chlorfenapyr. Four groups of 25 presumed pregnant Sprague-Dawley rats were used in this study. Females were presumed pregnant on the basis of observations of sperm in smears of vaginal contents and/or copulatory plugs. Chlorfenapyr technical (purity 94.5%; lot no. AC 7504-59A) was administered by gavage in an aqueous suspension with 0.5% carboxymethyl cellulose at a dose level of 0 (control), 25, 75 or 225 mg/kg bw per day on day 6 through day 15 of gestation. These dose levels were chosen on the basis of results from a pilot study in which a dose of 160 mg/kg bw per day caused slightly reduced body weight gain and feed consumption in pregnant females and 270 mg/kg bw per day for 7 days caused body weight loss, reduced feed consumption and increased liver to body weight ratios in non-pregnant females.

There was no mortality or signs of toxicity. A dose level of 225 mg/kg bw per day resulted in significantly reduced maternal body weight gains, feed consumption and water consumption during intervals of the treatment period. These parameters were also reduced during some intervals of the treatment period at 75 mg/kg bw per day, but the reductions were less severe than those at the higher dose level, and only those for feed consumption reached statistical significance. Maternal body weight gain, feed consumption and water consumption were not reduced at 25 mg/kg bw per day.

Ovarian, uterine and fetal observations were unaffected at all dose levels. No external, soft tissue or skeletal malformations or variations were attributable to treatment.

Based on these results, the NOAEL for maternal toxicity was 75 mg/kg bw per day, based on decreased body weight gain at 225 mg/kg bw per day. The NOAEL for developmental toxicity was 225 mg/kg bw per day, the highest dose tested. Chlorfenapyr is neither a developmental toxicant nor a teratogenic agent in the rat (Martin, 1993).

1.5 Special studies

(a) Subchronic neurotoxicity

Previously reviewed chlorfenapyr study

Chlorfenapyr (purity 94.5%; lot no. AC 7504-59A) was fed to four groups of 25 male and 25 female Sprague-Dawley rats for 52 weeks at a dietary concentration of 0 (control), 60, 300 or 600 ppm. Achieved test article intakes were 0, 2.6, 13.6 and 28.2 mg/kg bw per day for males and 0, 3.4, 18.0 and 37.4 mg/kg bw per day for females, respectively. The test substance was stable in rodent feed over a 14-day period. Following 13 weeks of treatment, five rats of each sex per group were anaesthetized and then perfused in situ with formalin. After 52 weeks of treatment, 10 rats of each sex from the control and 600 ppm groups and 5 rats of each sex from the 60 and 300 ppm groups were selected for perfusion. The remaining animals were fed control diet for a 16-week recovery period. At the end of the recovery period, all surviving male rats from the control and 600 ppm groups, along with 5 and 10 randomly selected male rats from the 60 and 300 ppm groups, respectively, and 5 female rats per group were selected for perfusion. All surviving animals not selected for perfusion were terminated by carbon dioxide asphyxiation.

No treatment-related mortality occurred, and the test substance had no biologically significant effects on the parameters of the functional observational battery, body temperature or clinical observations. Motor activity was not affected by treatment with chlorfenapyr. There were no necropsy observations attributable to the test substance, and alterations observed in the microscopic examination (excluding neurohistological examination, see below) of tissues from female rats were considered unrelated to chlorfenapyr.

Body weights, body weight gains and feed efficiency were statistically significantly reduced for animals in the 300 and 600 ppm groups either at intervals during treatment or at the end of the treatment period. These findings were more prevalent in males than in females. During the 16-week recovery period, overall body weight gains for animals in the 300 and 600 ppm groups were higher than during the treatment period. Relative mean feed consumption values (g/kg bw per day) were reduced for animals in the 600 ppm groups during the first 2 weeks of the treatment period and tended to be increased at other intervals during the exposure period. For the entire treatment period (day 1 through day 365), relative feed consumption values were increased for both sexes in the 600 ppm group and for males in the 300 ppm group. During the recovery period, little increase in relative feed consumption values was noted for the 300 and 600 ppm groups when compared with control values.

Neurohistological examination of males in the 600 ppm group terminated after 13 weeks of exposure revealed swelling of the myelin sheath in the spinal roots. A more generalized myelinopathic process, consisting of vacuolar myelinopathy, vacuolation and/or myelin sheath swelling of the brain and spinal cord, was present in male rats in the 300 and 600 ppm groups terminated after 52 weeks of exposure. This process was not associated with any evidence of myelin or axon degeneration and was not evident in the male rats terminated after the recovery period.

No adverse effects of treatment were noted at the 60 ppm dietary level. Thus, the NOAEL for a dietary exposure to chlorfenapyr was 60 ppm (equal to 2.6 mg/kg bw per day for males and 3.4 mg/kg bw per day for females), based on body weight data and neurohistopathological findings at 300 ppm (equal to 13.6 mg/kg bw per day for males and 18.0 mg/kg bw per day for females) (Foss, 1994).

The toxicities of chlorfenapyr and tralopyril are compared in Table 4.

Table 4. Comparison of toxicities of chlorfenapyr and tralopyril

End-point	Chlorfenapyr		Tralopyril	
	NOAEL	LOAEL	NOAEL	LOAEL
LD_{50} (rat)	–	M: 441 mg/kg bw[a] F: 1 152 mg/k bw[a]	–	M: 27 mg/kg bw[a] F: 29 mg/kg bw[a]
Ninety-day oral (rat)	M: 22 mg/kg bw per day F: 26.1 mg/kg bw per day	M: 44.9 mg/kg bw per day F: 51.8 mg/kg bw per day (increased relative liver weight, AP, BUN and changes in RBCs [females only])	M: 5.2 mg/kg bw per day F: –	M: 16.2 mg/kg bw per day F: 6.3 mg/kg bw per day[b] (multifocal vacuolation of white matter in CNS and PNS)
Ninety-day inhalation (rat)	M & F: 20 mg/m^3 (5 days/week)	M & F: 40 mg/m^3 (lethality, haematological alterations and prolonged prothrombin times)	M & F: 20 mg/m^3 (7 days/week)	40 mg/m^3 (lower body weight, feed consumption, feed efficiency in M & F; decreased total locomotor activity in M)
Developmental toxicity (rat) Maternal toxicity (rat)	75 mg/kg bw per day	225 mg/kg bw per day (decreased body weight gain)	5 mg/kg bw per day	10 mg/kg bw per day (salivation)
Embryo and fetal toxicity (rat)	225 mg/kg bw per day[c]	–	5 mg/kg bw per day	10 mg/kg bw per day (reduced fetal weight)

AP: alkaline phosphatase; BUN: blood urea nitrogen; CNS: central nervous system; F: females; LD_{50}: median lethal dose; LOAEL: lowest-observed-adverse-effect level; M: males; NOAEL: no-observed-adverse-effect level; PNS: peripheral nervous system; RBC: red blood cell

[a] Values are LD_{50}s, not LOAELs.

[b] Lowest dose tested.

[c] Highest dose tested.

Comments

Biochemical aspects

Although tralopyril is a metabolite of chlorfenapyr in the rat, the systemic exposure to this compound cannot be quantified. This is because systemic exposure estimates are based on metabolites that could potentially have arisen from tralopyril, but which could also have arisen from an alternative pathway in which the initial step is debromination rather than dealkylation. In the absence of information on relative rates of conversion of chlorfenapyr by these two pathways, it is not possible to reach any conclusions on exposure of test species to tralopyril.

Toxicological data

Tralopyril was of high acute oral toxicity in rats (LD_{50} = 27 mg/kg bw). In contrast, the oral LD_{50} of chlorfenapyr in rats is 441 mg/kg bw.

In a 90-day study of toxicity in rats, tralopyril was administered in the diet at concentrations of 0, 80, 250 and 750 ppm (equal to 0, 5.2, 16.2 and 51.9 mg/kg bw per day for males and 0, 6.3, 20.9 and 62.0 mg/kg bw per day for females, respectively). Administration of tralopyril resulted in dose-dependent, multifocal vacuolation of the white matter in the central nervous system and multifocal intramyelinic vacuolation of the peripheral nervous system. At high doses, a variety of effects were observed, including a reduction in body weight and body weight gain and changes in a number of clinical chemistry parameters. It was not possible to identify a NOAEL in this study, as vacuolation of the central nervous system and peripheral nervous system was evident at 80 ppm in females, the lowest dose tested. Therefore, the LOAEL for this study was 80 ppm (equal to 6.3 mg/kg bw per day). The absence of neuronal degeneration or neuronal necrosis suggests that morphological reversibility can be expected, and there is some evidence for this in a recovery group.

For comparison, the subchronic toxicity of chlorfenapyr was examined in a 90-day oral toxicity study in rats administered a dietary concentration of 0, 150, 300, 600, 900 or 1200 ppm (equal to 0, 10.9, 22.0, 44.9, 69.5 and 92.2 mg/kg bw per day for males and 0, 12.5, 26.1, 51.8, 75.4 and 102.8 mg/kg bw per day for females, respectively) and in a 13-week interim kill of males only within a 1-year oral neurotoxicity study at 0, 60, 300 and 600 ppm (equal to 0, 2.6, 13.6 and 28.2 mg/kg bw per day for males, respectively). In the 90-day study, chlorfenapyr produced vacuolation of the white matter in rats in one male at 600 ppm and in two males at 900 ppm and produced effects on relative liver weights, alkaline phosphatase, blood urea nitrogen and red blood cells at 600 ppm (equal to 44.9 mg/kg bw per day for males and 51.8 mg/kg bw per day for females). In the 13-week interim kill, males exhibited swelling of the myelin sheath in the spinal roots at 600 ppm (equal to 28.2 mg/kg bw per day). By the terminal kill at 52 weeks, chlorfenapyr produced vacuolar myelinopathy, vacuolation and/or myelin sheath swelling of the brain and spinal cord at 300 ppm (equal to 13.6 and 18 mg/kg bw per day for males and females, respectively). As seen in the 90-day tralopyril study, the myelinopathic process was not associated with any evidence of myelin or axon degeneration and was not evident in the male rats terminated after the recovery period. Thus, it appears that chlorfenapyr can produce effects on white matter similar to those of tralopyril, but is neurotoxicologically less potent than tralopyril by several fold.

In a subchronic inhalation toxicity and neurotoxicity study on tralopyril in rats, animals were exposed to target levels of 0, 20, 40 or 80 mg/m^3 for 6 hours/day, 7 days/week, for 90 days. At concentrations of 40 and 80 mg/m^3, there were decreases in body weight and feed consumption in both sexes, males exhibited decreased total locomotor activity in week 3 and decreased activity throughout the study period, and females exhibited increased alkaline phosphatase levels and increased relative lung weights. Histopathological changes consisted of ulcerations and erosions of the interior nose structures and degeneration of the respiratory and olfactory epithelium at 40 and 80 mg/m^3. At 80 mg/m^3, there were treatment-related mortalities in males.

A related study on chlorfenapyr in which rats were administered a concentration of 0, 5, 20, 40 or 80 mg/m^3 was reviewed by the 2012 JMPR. Although both were 90-day studies and the NOAECs for the end-points were numerically the same (20 mg/m^3) for the two compounds (but note that exposure duration differed: 7 days/week for tralopyril and 5 days/week for chlorfenapyr), tralopyril produced some neurotoxic effects (reduced activity and locomotor activity) as well as systemic effects in both sexes, whereas chlorfenapyr produced effects on lethality, haematological alterations and prolonged prothrombin times at 40 mg/m^3. Thus, it appears that the two chemicals are of similar potency for systemic toxicity in inhalational exposure studies, but produce different toxicological spectra. A major difference is the neurotoxicity produced by tralopyril and not by chlorfenapyr.

Tralopyril did not exhibit evidence of genotoxic activity in in vitro and in vivo assays. The Meeting noted that in the in vivo mouse micronucleus test, gavage doses of 15 mg/kg bw produced deaths, and clinical signs were seen at 12 mg/kg bw.

In a developmental toxicity study in rats on tralopyril, presumed pregnant females were dosed at 0, 5, 10 or 20 mg/kg bw per day on GDs 6–19. The NOAEL for both maternal and embryo/fetal toxicity was 5 mg/kg bw per day. At 10 mg/kg bw per day, dams exhibited an immediate increase in transient salivation after gavage, and there was a decrease in fetal weights. At 20 mg/kg bw per day, there were two treatment-related deaths comorbid with reddish salivation, tremors and convulsions and transient salivation following dosing, decreased feed consumption and decreased body weight gain in surviving dams. In the caesarean section parameters at 20 mg/kg bw per day, uterine and placental weights were decreased, and there was an increase in resorptions. Fetuses at 20 mg/kg bw per day exhibited an increased incidence of skeletal variations, although the changes were considered by the Meeting to be secondary to clear maternal toxicity. The transient salivation at the middle and high doses was determined to be a treatment-related and adverse effect based on the neurotoxicological profile of the compound.

For the developmental toxicity study in rats on c hlorfenapyr, the NOAEL for maternal toxicity was 75 mg/kg bw per day, based on decreased body weight at 225 mg/kg bw per day, and the NOAEL for embryo/fetal toxicity was 225 mg/kg bw per day, the highest dose tested. The NOAEL of 5 mg/kg bw per day for both maternal and embryo/fetal toxicity in the tralopyril study was therefore 15 and 45 times lower than the NOAELs of chlorfenapyr for maternal and embryo/fetal toxicity, respectively. Importantly, the critical end-point observed in pregnant dams exposed to tralopyril (maternal salivation) differed from those with chlorfenapyr (decreased body weight and body weight gain), and there was no evidence of embryo and fetal toxicity in the chlorfenapyr study in the presence of maternal toxicity. Together, the evidence suggests that tralopyril is more potent than chlorfenapyr in developmental toxicity studies and produces more severe maternal toxicity.

In summary, after 13 weeks of oral exposure, tralopyril was several-fold more potent than chlorfenapyr in female and male rats based on a comparison of LOAELs. In developmental toxicity studies in rats, tralopyril exhibited a higher degree of toxicity than chlorfenapyr, with NOAELs for maternal and embryo/fetal toxicity 15 and 45 times lower than those for chlorfenapyr, respectively. Tralopyril produced more severe neurotoxicity in oral and inhalational studies compared with chlorfenapyr on repeated administration. The data therefore indicate that tralopyril is more toxic than chlorfenapyr following repeated oral administration.

Toxicological evaluation

In 2012, the Meeting established an ADI for chlorfenapyr of 0–0.03 mg/kg bw, based on a NOAEL of 2.8 mg/kg bw per day for decreases in body weight gain and vacuolation of the white matter of the brain at 16.6 mg/kg bw per day in an 18-month mouse study and a NOAEL of 2.9 mg/kg bw per day for reduced body weight and body weight gain and increased liver weight associated with hepatocellular enlargement at 15 mg/kg bw per day in a 2-year rat study. This was supported by a NOAEL of 2.6 mg/kg bw per day for reversible vacuolar myelinopathy, vacuolation and/or myelin sheath swelling of the brain and spinal cord in males at 13.6 mg/kg bw per day in a 1-year study of neurotoxicity in rats. A safety factor of 100 was applied. The Meeting established an ARfD for chlorfenapyr of 0.03 mg/kg bw, based on the NOAEL of 3 mg/kg bw for depression of grooming and reactivity and decreased spontaneous motor activity observed at 10 mg/kg bw in a pharmacological study in mice. Based on the available information, it was not possible for the Meeting to determine whether the ADI and ARfD would also cover tralopyril.

There is less than a 10-fold difference in potency between tralopyril and chlorfenapyr for the effects of repeated administration on vacuolation of the peripheral and central nervous systems. However, there is approximately an order of magnitude difference in some acute effects (e.g. LD_{50}s of 27 mg/kg bw for tralopyril compared with 441 mg/kg bw for chlorfenapyr), and direct comparison of the most sensitive end-point in the most sensitive species (mouse) is not possible. The Meeting therefore established a potency factor of 10 for a comparison of exposure of tralopyril with both the ADI and the ARfD:

Estimates of exposure for comparison with the ADI/ARfD = chlorfenapyr + 10 × tralopyril

The Meeting noted that it may be possible to refine this potency factor estimate with additional studies.

Levels relevant to risk assessment of chlorfenapyr

Species	Study	Effect	NOAEL	LOAEL
Studies in metabolites: tralopyril				
Rat	Ninety-day study of toxicity[a]	Neurotoxicity	–	80 ppm, equal to 6.3 mg/kg bw per day[b]
	Developmental toxicity study[c]	Maternal toxicity	5 mg/kg bw per day	10 mg/kg bw per day
		Embryo and fetal toxicity	5 mg/kg bw per day	10 mg/kg bw per day

[a] Dietary administration.
[b] Lowest dose tested.
[c] Gavage administration.

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

Results from metabolism studies to determine systemic exposure to critical chlorfenapyr metabolites

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

Studies in metabolites: tralopyril	
Acute toxicity	
Rat, LD_{50}, oral	27 mg/kg bw
Short-term studies of toxicity	
Target/critical effect	Vacuolation of the central and peripheral nervous systems
Lowest relevant oral NOAEL	< 6.3 mg/kg bw per day, the lowest dose tested (rat)
Lowest relevant inhalation NOAEC	20 mg/m^3 (rat)
Genotoxicity	
	Not genotoxic
Developmental toxicity	
Target/critical effect	Maternal salivation, body weight, fetal weight
Lowest relevant maternal NOAEL	5 mg/kg bw per day
Lowest relevant embryo/fetal NOAEL	5 mg/kg bw per day

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CYANTRANILIPROLE

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Explanation

Cyantraniliprole is the International Organization for Standardization–approved common name for 3-bromo-1-(3-chloro-2-pyridyl)-4′-cyano-2′-methyl-6′-(methylcarbamoyl)pyrazole-5-carboxanilide (International Union of Pure and Applied Chemistry), with Chemical Abstracts Service No. 736994-63-1. It is a new second-generation ryanodine receptor insecticide whose pesticidal mode of action is through unregulated activation of insect ryanodine receptor channels, which leads to internal calcium store depletion and impaired regulation of muscle contraction, causing paralysis and eventual death of the insect. Cyantraniliprole is used to control insect pests in fruit crops, tree nuts, oil seed crops, cotton, grapes, rice, vegetables, ornamentals and turf around the world.

Cyantraniliprole has not been evaluated previously by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) and is being evaluated by the present Meeting at the request of the Codex Committee on Pesticide Residues.

All critical studies were certified as complying with good laboratory practice (GLP).

Evaluation for acceptable daily intake

1. Biochemical aspects

1.1 Absorption, distribution and excretion

The absorption, distribution and excretion of cyantraniliprole in rats following oral administration have been assessed in a quantitative single low-dose and single high-dose material balance study and in a 14-day repeated-dose study. The experiments were performed by dosing rats with [cyano-^{14}C]cyantraniliprole ([CN-^{14}C]cyantraniliprole) (radiochemical purity 99%) and [pyrazole-carbonyl-^{14}C]cyantraniliprole ([PC-^{14}C]cyantraniliprole) (radiochemical purity 98.1–99%), diluted with cyantraniliprole technical (purity 93.4%), either separately (single-dose study) or as a 1 : 1 weight per weight (w/w) mixture (repeated-dose study). The structure of cyantraniliprole used in the metabolism studies and the label positions are given in Fig. 1.

Fig. 1. The chemical structure and the label positions of cyantraniliprole

[CN-^{14}C]cyantraniliprole (cyano label)
* = location of the radiolabel

[PC-^{14}C]cyantraniliprole (pyrazole-carbonyl label)
= location of the radiolabel

(a) Single-dose administration

The absorption, distribution, metabolism and excretion of cyantraniliprole were studied in male and female Sprague-Dawley Crl:CD(SD) rats (four of each sex per group) administered either [CN-^{14}C]cyantraniliprole or [PC-^{14}C]cyantraniliprole. Experiments were performed to study the pharmacokinetic behaviour of radioactive residues in plasma and red blood cells, the disposition and material balance of total ^{14}C residues among tissues and excreta, the percentage and concentration of ^{14}C residues in tissues at selected times after dosing (the time at which the maximum concentration in plasma is reached [T_{max}], $T_{max/2}$ and terminal sacrifice) and the elimination of ^{14}C residues in bile. The profile of metabolites was characterized in urine, faeces and bile (see section 1.2).

All rats received the appropriate levels of radioactivity (MBq/animal) at the targeted dose rates of 10 and 150 mg/kg body weight (bw). The mean radioactivity dose ranged from 0.86 to 1.84 MBq for male rats and from 0.62 to 1.29 MBq for female rats.

The sex-specific kinetics of the two forms of radiolabelled cyantraniliprole were very similar; however, at low and high doses of each label, the peak plasma concentration (C_{max}), half-life ($t_{½}$) and area under the plasma concentration–time curve (AUC) were approximately 2.0- to 2.5-fold higher in female rats than in male rats (Table 1).

Table 1. Kinetic parameters of single-dose treatment of rats with cyantraniliprole at low and high doses

	10 mg/kg bw				150 mg/kg bw			
	[CN-^{14}C]		[PC-^{14}C]		[CN-^{14}C]		[PC-^{14}C]	
	Males	Females	Males	Females	Males	Females	Males	Females
T_{max} (h)	2.0	1.8	2.5	1.6	1.4	2.5	1.0	1.3
C_{max} (µg/g)	6.3	11.5	4.8	10.4	42.2	47.4	42.2	52.2
$t_{½}$ (h)	42.3	129	53.8	117	61.7	64.7	55.3	79.7
AUC (h·µg/g)	195	609	245	638	1 730	3 590	1 830	5 470

AUC: area under the plasma concentration–time curve; bw: body weight; C_{max}: peak plasma concentration; $t_{½}$: half-life; T_{max}: time to reach C_{max}
Source: Gannon (2010a)

Rats with bile duct cannulae were administered both [PC-^{14}C]cyantraniliprole and [CN-^{14}C]cyantraniliprole separately at a dose of either 10 or 150 mg/kg bw to measure the percentage of absorbed oral dose recoverable over 48 hours from bile, urine, carcass and the gastrointestinal tract (excluding contents). There was no significant difference in absorption of the two forms of radiolabelled cyantraniliprole. The mean total recovery (including cage washings) of radioactivity accounted for 89.0–101.6% of the dose. Absorption of the 10 mg/kg bw dose was higher than absorption of the 150 mg/kg bw dose. The percentage recoveries were 75.8–80.4% in male rats and 62.6–74.9% in female rats after the low dose and 38.8–40.0% in male rats and 31.4–32.2% in female rats after the high dose (Table 2) (Gannon, 2010a).

Table 2. Percentage absorption of radioactivity over 48 hours based on biliary elimination and material balance following a 10 or 150 mg/kg bw single oral dose of [CN-^{14}C]cyantraniliprole or [PC-^{14}C]cyantraniliprole

	10 mg/kg bw				150 mg/kg bw			
	[CN-^{14}C]		[PC-^{14}C]		[CN-^{14}C]		[PC-^{14}C]	
	Males	Females	Males	Females	Males	Females	Males	Females
Absorption (%)	75.8	62.6	80.4	74.9	40.0	31.4	38.8	32.2

Source: Gannon (2010a)

The distribution of ^{14}C residues was evaluated as the percentage of the administered dose, concentration of ^{14}C equivalents per gram of tissue and tissue : plasma concentration ratios at T_{max}, $T_{max/2}$ and terminal sacrifice after single oral dose administration. There was no significant difference in tissue distribution between dosing with the [CN-^{14}C]cyantraniliprole or [PC-^{14}C]cyantraniliprole. Most of the dose was initially associated with the gastrointestinal tract contents and subsequently showed uptake and distribution to all tissues, with higher concentrations at the T_{max} (2 hours) in liver, gastrointestinal tract, gastrointestinal tract contents, lungs, pituitary, thyroid, adrenals and urinary bladder. By 168 hours, the concentrations in most organs and tissues were much reduced, with higher concentrations remaining in liver, pituitary, adrenals and thyroid. The percentage recovery and tissue concentration data showed that female rats retained a greater proportion of ^{14}C residues compared with male rats. The reductions in plasma and tissue ^{14}C residue concentrations at high and low doses were generally similar. These observations were consistent with the shorter elimination half-life in male rats compared with female rats. The tissue : plasma concentration ratios were less than or equal to 1 by 168 hours after dose administration. Many of the tissue : plasma concentration ratios were below 1 at the $T_{max/2}$ time point as well.

There was no significant difference in excretion between rats administered [CN-^{14}C]cyantraniliprole or [PC-^{14}C]cyantraniliprole. Rats given a single 10 mg/kg bw dose of either radiolabelled cyantraniliprole compound excreted a greater percentage of the dose in urine compared with rats dosed with 150 mg/kg bw. For both dose levels and labels, most of the dose was excreted by 24–48 hours after administration. The percentage recovery from rats of the 10 mg/kg bw dose level at 168 hours was 22.0–34.6% for urine, 46.8–61.6% for faeces and 1.1–5.4% for tissues. Lower absorption from the gastrointestinal tract occurred in rats given the 150 mg/kg bw dose, as indicated by the lower percentage of the dose in tissues and urine and the greater percentage of the dose excreted in the faeces. For all groups in which material balance was measured, the mean percentage for total recovery by 7 days after dosing ranged from 88.3% to 96.5% (Table 3).

Table 3. Excretion in urine and faeces 168 hours after treatment

	% of total administered radioactivity									
	Single dose								Repeated dose	
	10 mg/kg bw				150 mg/kg bw				10 mg/kg bw	
	[CN-^{14}C]		[PC-^{14}C]		[CN-^{14}C]		[PC-^{14}C]		[CN-^{14}C] + [PC-^{14}C]	
	M	F	M	F	M	F	M	F	M	F
Urine	27.7	22.0	34.6	23.7	14.8	13.2	11.8	12.9	28.8	20.3
Faeces	61.5	61.6	46.8	60.6	77.6	78.6	80.1	77.6	60.8	61.9
Carcass[a]	1.14	4.25	1.67	5.35	0.68	2.45	0.25	2.30	0.8	2.5
Cage washing	5.62	5.35	5.23	3.40	1.66	1.12	2.27	1.08	2.8	4.5
Total[b]	96.5	92.6	88.3	93.0	95.0	95.1	94.5	93.7	93.2	89.1

F: females; M: males

[a] Total of tissues and carcass except erythrocytes and plasma.

[b] The mean percentage for total recovery in individual data.

Source: Gannon (2010a)

(b) Repeated-dose administration

A 1 : 1 w/w mixture of [CN-^{14}C]cyantraniliprole and [PC-^{14}C]cyantraniliprole was administered to groups of three male and three female Sprague-Dawley rats orally by gavage at a dose level of 10 mg/kg bw per day for 14 days. The average daily dose was 10.5 mg/kg bw, corresponding to a radioactivity dose rate of 0.11 MBq/rat per day.

The primary objective of this study was to examine the kinetics of ^{14}C-labelled cyantraniliprole in whole blood, plasma, red blood cells, fat, kidneys, liver and muscle during and following multiple oral dose administration to rats for determination of steady-state kinetics during dose administration and tissue/organ elimination half-lives. The second objective was to establish the metabolite profile in urine and faeces collected for 24 hours after the 1st, 7th and last days of dosing (see section 1.2). The mean body weights during the 14-day dosing period were 297 ± 27 g for males and 203 ± 19 g for females.

Concentration (µg equivalents/g), tissue : plasma ratio and percentage of dose were calculated for tissues collected from male and female rats at various times during and after exposure. In male rats, tissues were collected on day 15 and day 21 (1 day and 7 days after last dose). Tissues were collected from female rats on days 5, 9, 12, 15, 17, 21 and 26. There were more collections from female rats because a previous study had found that systemic availability was higher in females than in males following a single oral gavage dose. Because of the higher frequency of tissue collections from female rats, elimination half-lives could be calculated with greater precision than in males. All tissues were collected from male and female animals on day 15 and day 21, but a more limited set of tissues was collected from female rats on the other collection days.

The data show that the tissue concentrations fall rapidly following the end of dosing. The half-lives ranged from 2.6 days in fat to approximately 6 days in whole blood (Table 4). The lack of accumulation was confirmed by the decreasing concentrations in tissues collected at day 15 and day 21 from both male and female rats. The tissue : plasma ratios were all less than 1 following the end of the dosing period, this being additional evidence for a lack of accumulation in tissues. The cumulative excretion of total radioactivity in urine and faeces was evaluated from day 1 through day 20 as both the percentage of accumulating dose and the percentage of total dose. The accumulating dose in urine, which represents the amount excreted in the urine in a 24-hour period, ranged from 24% to 29% in males and from 13% to 20% in females. The total percentage of dose eliminated in the urine was 29% in male rats and 20% in female rats. The accumulating dose in faeces ranged from 43% to 61% in males and from 42% to 62% in females. The total percentage of dose eliminated in the faeces was 61% in males and 62% in females (Gannon, 2010b).

Table 4. Kinetic parameters of residues in female rats following oral dosing by gavage with ^{14}C-labelled cyantraniliprole at 10 mg/kg bw per day for 14 days

	Plasma	Red blood cells	Total blood	Fat	Liver
T_{max} (day)	15	15	15	15	15
C_{max} (µg/g)	60.1	10.4	30.9	45.0	30.7
$t_{½}$ (day)	5.6	5.4	5.7	2.6	4.0
AUC (day·µg/g)	828	161	463	577	402

AUC: area under the plasma concentration–time curve; C_{max}: peak plasma concentration; $t_{½}$: half-life; T_{max}: time to reach C_{max}
Source: Gannon (2010b)

1.2 Biotransformation

The metabolism of cyantraniliprole was investigated in two studies performed in Sprague-Dawley Crl:CD®(SD)IGS BR rats with a 1 : 1 w/w mixture of [CN-^{14}C]cyantraniliprole (radiochemical purity 99%) and [PC-^{14}C]cyantraniliprole (radiochemical purity 98.1–99%), diluted with cyantraniliprole technical (purity 93.4%). The rats were dosed once by gavage with either 10 or 150 mg/kg bw (four rats of each sex per group) (Gannon, 2010a) or daily for 14 days by gavage at a dose of 10 mg/kg bw per day (three males and three females) (Gannon, 2010b). Metabolites were identified and quantified by radiochromatography and liquid chromatography/mass spectrometry.

The main animal metabolites and degradates of cyantraniliprole are shown in Table 5.

In the single-dose studies, cyantraniliprole was found to be readily hydroxylated to form IN-N7B69 and IN-MYX98, and IN-N7B69 was further metabolized to a glucuronide. Cyantraniliprole underwent ring closure to generate IN-J9Z38, which was in turn hydroxylated to form IN-NBC94, its carboxylic acid and its glucuronide conjugate. IN-MYX98 was also metabolized to the closed-ring metabolite IN-MLA84, which, like IN-NBC94, was further oxidized to a hydroxylated metabolite, a carboxylic acid and the glucuronide of the hydroxyl metabolite. The hydroxylated metabolite IN-MYX98 could be *N*-dealkylated to form IN-HGW87 as well as hydroxylated a second time to form bis-hydroxy cyantraniliprole. Cyantraniliprole was also hydroxylated on the pyridine ring, followed by a ring closure analogous to the conversion of cyantraniliprole to IN-J9Z38. Cyantraniliprole could also be *N*-dealkylated and cleaved at the carbonyl bridge to form IN-DBC80.

Metabolites in urine and faeces during and following multiple dosing were the same as those observed in the single oral dose study (Table 6). In most cases, there was very little difference observed between metabolite distribution on day 1, 7 or 14 in urine. In urine from male rats, IN-N7B69 was present at approximately 5% of the dose on day 1 and day 7, but was not detected on day 14. It was not detected on any day in female rat urine. IN-MYX98 was present at only 0.5% of the

administered dose in male urine on day 1, but was present at significantly higher levels by day 7 (5% of the dose) and day 14 (3% of the dose). In contrast, this same metabolite in urine of female rats was present at 7% on day 1, 11% on day 7, but only 1% on day 14. Cyantraniliprole constituted approximately 5% of the dose in female rat urine on days 1 and 7, but was not detected on day 14. IN-MLA84 made up less than 11% of the dose on days 2 and 7, but was 14% of the dose on day 14. The most notable differences in faecal metabolite profile both occurred in the female rat samples. There was an increase in both IN-MLA84 (day 1, 1% of the dose; day 14, 5% of the dose) and IN-MYX98 (day 1, 10% of the dose; day 14, 16% of the dose) as a percentage of the dose (Gannon, 2010a,b).

Table 5. Main metabolites and/or degradates of cyantraniliprole

Codes	Names
Bis-hydroxy cyantraniliprole	3-Bromo-1-(3-chloro-2-pyridinyl)-*N*-[4-cyano-2-(hydroxymethyl)-6-[[(hydroxy-methyl)amino]carbonyl]phenyl]-1*H*-pyrazole-5-carboxamide
IN-DBC80	3-Bromo-1-(3-chloro-2-pyridinyl)-1*H*-pyrazole-5-carboxylic acid
IN-F6L99	3-Bromo-*N*-methyl-1*H*-pyrazole-5-carboxamide
IN-HGW87	*N*-[2-(Aminocarbonyl)-4-cyano-6-methylphenyl]-3-bromo-1-(3-chloro-2-pyridinyl)-1*H*-pyrazole-5-carboxamide
IN-J9Z38	2-[3-Bromo-1-(3-chloro-2-pyridinyl)-1*H*-pyrazol-5-yl]-3,4-dihydro-3,8-dimethyl-4-oxo-6-quinazolinecarbonitrile
IN-JSE76	4-[[[3-Bromo-1-(3-chloro-2-pyridinyl)-1*H*-pyrazol-5-yl]carbonyl]amino]-3-methyl-5-[(methylamino)carbonyl]benzoic acid
IN-MLA84	2-[3-Bromo-1-(3-chloro-2-pyridinyl)-1*H*-pyrazol-5-yl]-1,4-dihydro-8-methyl-4-oxo-6-quinazolinecarbonitrile
IN-MLA84 carboxylic acid	2-[3-Bromo-1-(3-chloro-2-pyridinyl)-1*H*-pyrazol-5-yl]-6-cyano-1,4-dihydro-4-oxo-8-quinazolinecarboxylic acid
Hydroxy-IN-MLA84	2-[3-Bromo-1-(3-chloro-2-pyridinyl)-1*H*-pyrazol-5-yl]-3,4-dihydro-8-(hydroxymethyl)-4-oxo-6-quinazolinecarbonitrile
Hydroxy-IN-MLA84 glucoside	2-[3-Bromo-1-(3-chloro-2-pyridinyl)-1*H*-pyrazol-5-yl]-8-[(β-D-glucopyranosyloxy)-methyl]-1,4-dihydro-4-oxo-6-quinazolinecarbonitrile
Hydroxy-IN-MLA84 glucuronide	[2-[3-Bromo-1-(3-chloro-2-pyridinyl)-1*H*-pyrazol-5-yl]-6-cyano-1,4-dihydro-4-oxo-8-quinazolinyl]methyl β-D-glucopyranosiduronate
IN-MYX98	3-Bromo-1-(3-chloro-2-pyridinyl)-*N*-[4-cyano-2[[(hydroxymethyl)amino]carbonyl]-6-methylphenyl]-1*H*-pyrazole-5-carboxamide
IN-N5M09	6-Chloro-4-methyl-11-oxo-11*H*-pyrido[2,1-*b*]quinazoline-2-carbonitrile
IN-N7B69	3-Bromo-1-(3-chloro-2-pyridinyl)-*N*-[4-cyano-2-(hydroxymethyl)-6-[(methylamino)-carbonyl]phenyl]-1*H*-pyrazole-5-carboxamide
IN-N7B69 glucuronide	3-Bromo-1-(3-chloropyridine-2-yl)-*N*-[4-cyano-2-(hydroxymethyl)-6-(methylcarbamoyl)phenyl]-1*H*-pyrazole-6-methyl β-D-*O*-hexopyranosiduronate
IN-NBC94	2-[3-Bromo-1-(3-chloro-2-pyridinyl)-1*H*-pyrazole-5-yl]-3,4-dihydro-8-(hydroxymethyl)-3-methyl-4-oxo-6-quinazolinecarbonitrile
IN-NBC94 glucuronide	2-[3-Bromo-1-(3-chloro-2-pyridinyl)-1*H*-pyrazole-5-yl]-6-cyano-3,4-dihydro-3-methyl-4-oxo-8-quinazolynyl]methyl 8-D-glucopyranosiduronate
IN-PLT97	2-[3-Bromo-1-(3-chloro-2-pyridinyl)-1*H*-pyrazol-5-yl]-1,4-dihydro-8-methyl-4-oxo-6-quinazolinecarboxylic acid

Source: Gannon (2010a,b)

Table 6. Metabolites in bile, urine or faeces in single or repeated exposure to cyantraniliprole in rats[a]

Dose (mg/kg bw)	Sex	Sample	% of total administered radioactivity	
			Parent	Metabolites (> 1.0)
Single exposure				
[CN-^{14}C]				
10	M	Urine	0.33	IN-MYX98 (4.52), IN-N7B69 (4.43), bis-hydroxy cyantraniliprole (1.40)
		Faeces	5.06	IN-MYX98 (10.5), bis-hydroxy cyantraniliprole (8.12), IN-N7B69 (4.91), IN-NBC94 (2.41), IN-HGW87 (2.14), IN-MLA84 (1.19)
		Bile	—	IN-NBC94 glucuronide (4.78), IN-N7B69 glucuronide (4.00), hydroxy-IN-MLA84 glucuronide (2.15)
	F	Urine	5.42	IN-MYX98 (11.5)
		Faeces	16.8	IN-MYX98 (14.4), IN-HGW87 (4.10), IN-MLA84 (3.36), IN-NBC94 (3.03), IN-J9Z38 (2.79), IN-N7B69 (2.40), bis-hydroxy cyantraniliprole (2.04)
		Bile	—	IN-NBC94 glucuronide (4.83), IN-N7B69 glucuronide (2.93)
150	M	Urine	1.37	IN-N7B69 (4.53), IN-MYX98 (4.34)
		Faeces	55.8	IN-MYX98 (5.46), bis-hydroxy cyantraniliprole (2.45), IN-N7B69 (1.96), IN-HGW87 (1.14)
		Bile	—	IN-NBC94 glucuronide (3.58), hydroxy-IN-MLA84 glucuronide (1.57), IN-N7B69 glucuronide (1.27)
	F	Urine	1.83	IN-MYX98 (4.88)
		Faeces	55.0	IN-MYX98 (6.73), IN-HGW87 (3.05), IN-MLA84 (2.17), bis-hydroxy cyantraniliprole (1.04)
		Bile	—	IN-NBC94 glucuronide (2.18), IN-N7B69 glucuronide (1.67)
[PC-^{14}C]				
10	M	Urine	1.09	IN-N7B69 (13.6), IN-MYX98 (4.07), bis-hydroxy cyantraniliprole (3.04), IN-DBC80 (2.10)
		Faeces	5.38	IN-MYX98 (9.25), bis-hydroxy cyantraniliprole (5.59), IN-DBC80 (5.30), IN-N7B69 (3.58), IN-NBC94 (2.57), IN-HGW87 (1.46)
		Bile	—	IN-NBC94 (3.41), IN-N7B69 glucuronide (2.78), IN-NBC94 glucuronide (2.62)
	F	Urine	3.58	IN-MYX98 (8.55), IN-N7B69 (1.74)
		Faeces	15.0	IN-MYX98 (17.2), IN-HGW87 (5.52), IN-NBC94 (2.94), IN-MLA84 (2.93), IN-J9Z38 (2.83), IN-DBC80 (2.56), IN-N7B69 (1.96), bis-hydroxy cyantraniliprole (1.93)
		Bile	—	IN-NBC94 glucuronide (3.73), IN-N7B69 glucuronide (3.60), hydroxy-IN-MLA84 glucuronide (2.22), IN-MLA84 (1.55)
150	M	Urine	0.77	IN-N7B69 (3.97), IN-MYX98 (2.10), bis-hydroxy cyantraniliprole (1.08)
		Faeces	65.6	IN-MYX98 (3.59), bis-hydroxy cyantraniliprole (1.64), IN-HGW87 (1.28)
		Bile	—	IN-NBC94 glucuronide (2.25), hydroxy-IN-MLA84 glucuronide (1.15), IN-N7B69 glucuronide (1.07)
	F	Urine	1.35	IN-MYX98 (3.95), IN-MLA84 (1.28), IN-N7B69 (1.21)
		Faeces	59.4	IN-MYX98 (6.37), IN-HGW87 (2.26), IN-MLA84 (2.18), IN-NBC94 (1.08)
		Bile	—	IN-NBC94 glucuronide (2.08), IN-N7B69 glucuronide (1.93), IN-NBC94 (1.21)

Table 6 (continued)

Dose (mg/kg bw)	Sex	Sample	% of total administered radioactivity	
			Parent	Metabolites (> 1.0)
Repeated exposure				
[CN-^{14}C] + [PC-^{14}C]				
10	M	Urine	—	IN-NBC94 (7.95), IN-DBC80 (6.36), IN-MYX98 (3.29), IN-MLA84 (1.91), IN-NBC94 glucuronide (1.48)
		Faeces	9.84	IN-MYX98 (10.7), bis-hydroxy cyantraniliprole (4.55), IN-N7B69 (4.04), IN-NBC94 (3.13), IN-DBC80 (2.27), IN-MLA84 (1.57), IN-HGW87 (1.10)
	F	Urine	—	IN-MLA84 (14.3), IN-DBC80 (1.52), IN-NBC94 (1.30), IN-MYX98 (1.19)
		Faeces	13.5	IN-MYX98 (16.4), IN-MLA84 (5.12), IN-N7B69 (3.65), IN-NBC94 (3.41), IN-HGW87 (2.14), IN-J9Z38 (1.80)

F: female; M: male

[a] Measured 48 and 72 hours after dosing in males and females, respectively.

Source: Gannon (2010a,b)

Analyses for cyantraniliprole and four of its metabolites (IN-J9Z38, IN-MYX98, IN-MLA84 and IN-N7B69) were conducted on plasma samples collected at around test day 60 during the course of 90-day oral toxicity studies in rats (Gannon, 2011b) and mice (Gannon, 2011a) and during 90-day and 1-year (at week 39) oral toxicity studies in dogs (Gannon, 2009; Mawn, 2010). The plasma concentrations of cyantraniliprole and its metabolites in mice, rats and dogs are shown in Table 7.

In mice, IN-MLA84 was by far the most prevalent analyte, and its concentrations were similar in males and females (approximately 400 000 ng/mL) in the highest dietary dose group of 7000 parts per million (ppm) (Table 7). The mean plasma concentrations of cyantraniliprole at this exposure were approximately 4000 ng/mL in male mice and 9000 ng/mL in female mice. All other analytes were present at concentrations less than 800 ng/mL at all dose concentrations. The plasma concentration dose–response curves for IN-MLA84 in particular showed little increase with dose above 300 ppm (Gannon, 2011a).

In rats, the concentration of each analyte was higher in females than in males at all dietary levels, except for IN-J9Z38 at 20 000 ppm, at which concentrations were similar in both males and females (Table 7). In all cases, the most abundant analyte present was IN-MLA84. In the 20 000 ppm dietary group of rats, the mean plasma concentration of IN-MLA84 was 145 890 ng/mL in males and 259 500 ng/mL in females. The parent compound, cyantraniliprole, was the next most abundant analyte, at the very much lower concentrations of 4634 ng/mL in males and 5624 ng/mL in females of the same dietary group. Although these plasma concentrations are quoted for the 20 000 ppm group, there was little increase for any of the analytes compared with the 400 ppm group (Gannon, 2011b).

In contrast to the rats and mice, cyantraniliprole was by far the most abundant analyte in the 90-day dog study; the concentrations were approximately 50 000 ng/mL in both males and females of the 10 000 ppm dietary group. The similarity of cyantraniliprole concentrations in males and females was maintained in all but the lowest (30 ppm) dietary group, where the plasma concentration was approximately 1.5 times higher in females than in males. Concentrations of metabolites IN-J9Z38, IN-MYX98 and IN-N7B69 were not significantly different from each other in females of the high-dose group (10 000 ppm). The concentrations of IN-J9Z38 were similar in males and females. The plasma concentrations of IN-N7B69 and IN-MYX98 were higher in males than in females. The concentrations of the analytes did not increase linearly with dietary concentration in dogs above about 100 ppm (Gannon, 2009). At week 39 of the 1-year dog study, there were no appreciable sex differences in cyantraniliprole concentrations in plasma (Table 7) (Gannon, 2009, 2011a,b; Mawn, 2010).

Table 7. Plasma concentrations of cyantraniliprole and metabolites in mice, rats and dogs

	Plasma concentrations (ng/mL)									
	Males					Females				
Mice in 90-day oral toxicity study	0 ppm	50 ppm	300 ppm	1 000 ppm	7 000 ppm	0 ppm	50 ppm	300 ppm	1 000 ppm	7 000 ppm
Cyantraniliprole	< LOQ	85 ± 33	815 ± 270	1 451 ± 321	3 942 ± 1 045	< LOQ	140 ± 108	1 002 ± 230	2 634 ± 1 073	8 980 ± 9 857
IN-J9Z38	< LOQ	< LOQ	64 ± 18	118 ± 17	278 ± 45	< LOQ	< LOQ	63 ± 24	182 ± 77	312 ± 73
IN-MYX98	< LOQ	26 ± 4	179 ± 29	308 ± 75	839 ± 197	< LOQ	36 ± 16	132 ± 25	334 ± 113	769 ± 147
IN-MLA84	241 ± 76	111 500 ± 11 385	394 100 ± 27 898	402 800 ± 33 320	411 000 ± 34 943	350 ± 87	153 200 ± 16 243	321 200 ± 33 626	502 600 ± 30 146	384 600 ± 108 475
IN-N7B69	< LOQ	< LOQ	68 ± 14	121 ± 29	262 ± 51	< LOQ	< LOQ	59 ± 10	146 ± 39	331 ± 26
Rats in 90-day oral toxicity study	0 ppm	100 ppm	400 ppm	3 000 ppm	20 000 ppm	0 ppm	100 ppm	400 ppm	3 000 ppm	20 000 ppm
Cyantraniliprole	14 ± 4	357 ± 64	1 729 ± 754	3 402 ± 552	4 634 ± 761	< LOQ	1 592 ± 399	4 245 ± 1 232	6 010 ± 1 717	5 624 ± 1 679
IN-J9Z38	< LOQ	173 ± 77	598 ± 195	1 298 ± 590	1 464 ± 304	< LOQ	710 ± 212	1 822 ± 409	1 482 ± 408	1 311 ± 336
IN-MYX98	< LOQ	29 ± 15	110 ± 26	207 ± 70	455 ± 102	< LOQ	108 ± 16	328 ± 83	573 ± 159	716 ± 216
IN-MLA84	< LOQ	16 303 ± 5 758	67 500 ± 13 216	91 605 ± 21 906	145 890 ± 30 076	32 ± 26	29 150 ± 6 457	175 300 ± 40 795	256 800 ± 44 694	259 500 ± 54 056
IN-N7B69	< LOQ	16 ± 4	21 ± 9	35 ± 20	50 ± 25	< LOQ	< LOQ	70 ± 17	137 ± 33	164 ± 35

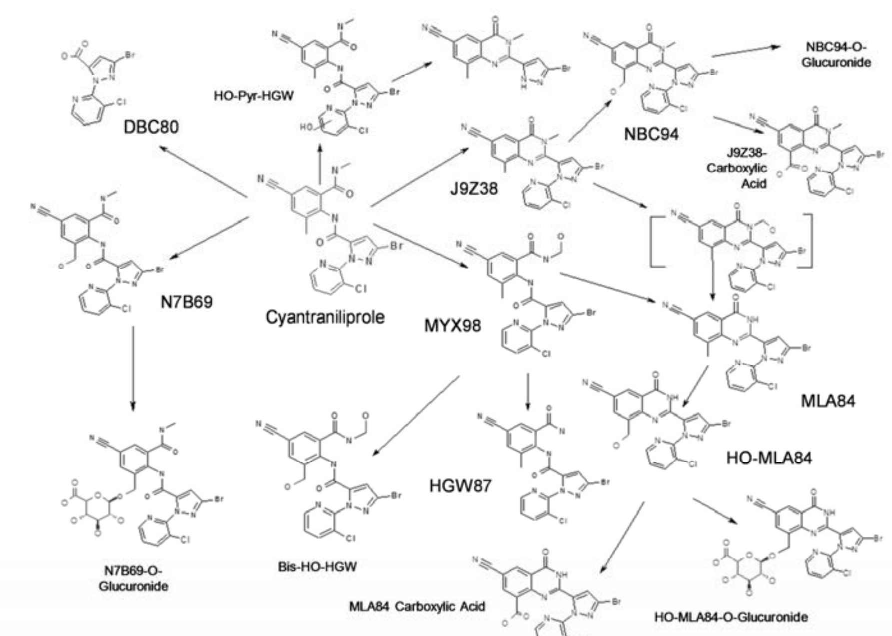
Table 7 (continued)

	Plasma concentrations (ng/mL)							
	Males				Females			
Dogs in 90-day oral toxicity study	30 ppm	100 ppm	1 000 ppm	10 000 ppm	30 ppm	100 ppm	1 000 ppm	10 000 ppm
Cyantraniliprole	1 743 ± 1 110	16 806 ± 6 046	30 963 ± 6 988	51 900 ± 6 597	2 424 ± 1 087	20 600 ± 10 772	28 383 ± 18 056	51 263 ± 26 885
IN-J9Z38	181 ± 61	562 ± 185	1 486 ± 97	2 668 ± 1 205	96 ± 28	661 ± 491	1 176 ± 565	2 043 ± 488
IN-MYX98	256 ± 93	718 ± 159	8 713 ± 2 250	18 717 ± 7 877	77 ± 23	1 021 ± 1 123	1 766 ± 1 070	4 048 ± 895
IN-MLA84	32 ± 7	83 ± 82	217 ± 56	359 ± 183	< LOQ	105 ± 93	158 ± 87	567 ± 255
IN-N7B69	252 ± 154	798 ± 427	11 676 ± 8 501	8 558 ± 8 568	81 ± 21	946 ± 1 402	2 543 ± 1 992	3 801 ± 1 978
Dogs at week 39 in 1-year oral toxicity study	0	5 000	5 000 + recovery		0	5 000	5 000 + recovery	
Cyantraniliprole	< LOQ	62 200	19.7		< LOQ	565	10.8	

LOQ: limit of quantification (5.00 ng/mL)
Source: Gannon (2009, 2011a,b); Mawn (2010)

Proposed pathways for the metabolism of cyantraniliprole in rats are shown in Fig. 2.

Fig. 2. Proposed metabolic pathway of cyantraniliprole in rats



2. Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

The acute toxicities of cyantraniliprole are summarized in Table 8.

Table 8. Summary of acute toxicity of cyantraniliprole

Route	Species and sex	LD_{50} (mg/kg bw)		Abnormalities	Reference
		Males	Females		
Gavage (purity 97.0%; batch no. 9182-1)	SD rat Female (3/group)	—	> 5 000	No treatment-related changes were observed	Carpenter (2009a)
Oral (purity 94.5%; batch no. HGW86-230)	ICR mice Female (5/group)	—	> 5 000	No deaths or abnormal clinical signs	Carpenter (2008a)
Dermal (purity 94.5%; batch no. HGW86-230)	SD rat Male/female (5/sex/group)	> 5 000	> 5 000	No deaths or abnormal clinical signs	Carpenter (2008b)
Inhalation (purity 94.5%; batch no. HGW86-230)	SD rat Male/female (5/sex/group)	> 5.2 mg/L[a]	> 5.2 mg/L[a]	No deaths or abnormal clinical signs	Weinberg (2009)

LD_{50}: median lethal dose

[a] Median lethal concentration (LC_{50}).

(b) Dermal irritation

Cyantraniliprole (purity 97.0%; batch no. HGW86-412) was applied as a single 0.5 g dermal dose to the shaved intact skin of three young adult male New Zealand White rabbits. No dermal irritation was observed (Carpenter, 2009b).

(c) Ocular irritation

A single dose of 62 mg (equivalent to 0.1 mL) of cyantraniliprole (purity 97.0%; batch no. 9182-1) was administered into the lower conjunctival sac of the right eye of three young adult male New Zealand White rabbits. Cyantraniliprole was not irritating to rabbit eyes (Carpenter, 2009c).

(d) Dermal sensitization

The potential of cyantraniliprole (purity 97.0%; batch no. 9182-1) to produce a sensitization response in mice using the local lymph node assay (LLNA) was evaluated. Cyantraniliprole did not produce a sensitization response in mice (Hoban, 2009a).

The dermal sensitization potential of cyantraniliprole (purity 95.6%; lot no. D100487-104; batch no. HGW86-648) was evaluated by the Magnusson-Kligman maximization method in female Hartley albino guinea-pigs. Cyantraniliprole did not produce a dermal sensitization response in guinea-pigs (Nomura, 2011).

Under the conditions of these studies, cyantraniliprole was not a skin sensitizer.

2.2 Short-term studies of toxicity

(a) Oral administration

Mice

In a 28-day feeding study, cyantraniliprole (purity 92.7%; batch no. HWG86-085) was administered to male and female Crl:CD1®(ICR) mice (10 animals of each sex per group) at a concentration of 0, 300, 1000, 3000 or 7000 ppm (equal to 0, 53, 175, 528 and 1261 mg/kg bw per day for males and 0, 63, 212, 664 and 1476 mg/kg bw per day for females, respectively). Parameters evaluated in all mice included body weight, body weight gain, feed consumption, feed efficiency, clinical signs, gross pathology, organ weights, haematology, plasma total protein and microscopic pathology. Cytochrome P450 and β-oxidation in the liver were evaluated. This study was not conducted in accordance with GLP.

No treatment-related deaths or treatment-related changes in clinical observations, body weights, feed intakes, haematology, plasma total protein, organ weights or histopathology were observed. Increased liver weight in males at 3000 ppm and above and in females at 300 ppm and above were considered to be adaptive effects related to induction of drug metabolism enzymes in the liver and not biologically adverse. Cyantraniliprole did not induce hepatic β-oxidation in male or female mice. In male mice, the hepatic total cytochrome P450 (CYP) content was significantly increased at dietary concentrations of 3000 and 7000 ppm. In female mice, total cytochrome P450 content was significantly increased at 300 ppm and higher.

The no-observed-adverse-effect level (NOAEL) for 28-day oral toxicity in mice was 7000 ppm (equal to 1261 mg/kg bw per day), the highest dose tested, based on no adverse effects detected in this study (Carpenter, 2009d).

Cyantraniliprole (purity 93.4%; batch no. HGW86-141) was administered to male and female Crl:CD1®(ICR) mice (10 mice of each sex per group) at a dietary concentration of 0, 50, 300, 1000 or 7000 ppm (equal to 0, 7.2, 47.1, 150 and 1092 mg/kg bw per day for males and 0, 9.7, 58.1, 204 and 1344 mg/kg bw per day for females, respectively) for at least 90 days (97 days for males; 98 days for females). Parameters evaluated included body weight, body weight gain, feed consumption, feed efficiency, clinical signs, haematology, clinical chemistry, ophthalmology, organ weights, and gross

and microscopic pathology. Satellite groups of the same strain of mice (five of each sex per group) were treated similarly for 63 days (non-fasted) to measure plasma concentrations of cyantraniliprole and selected metabolites. The results are shown in Table 7.

No treatment-related deaths occurred, and there were no clinical signs or ophthalmological observations attributable to treatment. No test substance–related effects on body weight or any nutritional parameters were observed. There were no treatment-related changes in clinical pathology parameters (haematology and plasma total protein). A test substance–related increase in liver weights was observed in males at 7000 ppm and in females at 1000 ppm. The increased liver weights correlated with centrilobular hypertrophy of hepatocytes at the same concentrations (Table 9). Both the liver weight increases and the hepatocellular hypertrophy were considered indicative of non-adverse hepatic enzyme induction. Minimal focal necrosis of hepatocytes was increased in females at 7000 ppm. In addition, microscopic examination revealed an increase in mild to slight microvesiculation in fascicular zone cells of adrenal cortex in males fed at 50 ppm and above. The increase in microvesiculation in the adrenal in males was considered to be a non-adverse change within normal physiological limits, because the changes were slight, there was no clear dose dependency and there was no indication of cytotoxicity in organelles by electron microscopic examination. In addition, a lack of increase was detected in a long-term study in mice (Craig, 2011b). Basal urinary corticosterone was comparable between the control and treated groups.

Table 9. Summary of liver effects in a 90-day oral toxicity study in mice

	Males					Females				
	0 ppm	50 ppm	300 ppm	1 000 ppm	7 000 ppm	0 ppm	50 ppm	300 ppm	1 000 ppm	7 000 ppm
No. of rats examined	10	10	10	9	10	10	10	9	10	10
Relative liver weight (% of body weight × 100)	4.800	4.770	5.108	5.209	6.071*	4.912	4.739	4.937	5.449	6.074*
Liver										
Centrilobular hypertrophy of hepatocytes	0	0	0	0	2	0	0	0	1	9
Hepatocellular necrosis, focal	0	0	0	1	1	0	0	1	1	4
Adrenal										
Increased microvesiculation in fascicular zone	0	3	5	4	7	0	0	0	0	0
- Minimal	0	2	2	2	6	0	0	0	0	0
- Slight	0	1	3	2	1	0	0	0	0	0

Source: MacKenzie (2007)

The NOAEL for the 90-day oral toxicity study in mice was 1000 ppm (equal to 204 mg/kg bw per day), based on minimal necrosis in the liver at 7000 ppm (equal to 1344 mg/kg bw per day) in females (MacKenzie, 2007; Gannon, 2011a).

Rats

In a 14-day gavage study, cyantraniliprole (purity 100%; batch no. HGW86-014) was administered to male and female Crl:CD®(SD) IGS BR rats at a dose of 0 (5 males, 5 females), 25 (23 males, 5 females), 300 (23 males, 5 females) or 1000 mg/kg bw per day (8 males, 5 females). For each concentration, the first five males and five females were designated for evaluation of subacute

toxicity and in vivo micronucleus studies (main study group). At the 25, 300 and 1000 mg/kg bw dose levels, the remaining males were designated for pharmacokinetic evaluation. A separate group of five males and five females each received a single 2000 mg/kg bw dose for evaluation of genetic toxicology. Parameters evaluated in the main study group included body weight, clinical signs, clinical chemistry, haematology, urine analysis, macroscopic and microscopic pathology, organ weights, hepatic biochemistry (cytochrome P450, β-oxidation), thyroid hormone levels and genetic toxicology.

No treatment-related changes were observed in the parameters examined except for increases in relative liver and adrenal weights in females at 1000 mg/kg bw per day. The weight increases were not accompanied by any microscopic changes. No significant effects on the thyroid hormones thyroid stimulating hormone (TSH), triiodothyronine (T_3) or thyroxine (T_4) were detected at any dose level. Total cytochrome P450 content in the liver of male or female rats was minimally elevated at 1000 mg/kg bw per day. Cytochrome P450 isozymes of CYP2B1 and CYP1A1 were induced in the liver of both sexes and of males, respectively. Cyantraniliprole did not induce β-oxidation (Nabb, 2010).

In a 28-day feeding study, cyantraniliprole (purity 92.7%; batch no. HGW86D008A) was administered to male and female Crl:CD®(SD) rats (five animals of each sex per group) at a concentration of 0, 600, 2000, 6000 or 20 000 ppm (equal to 0, 53, 175, 528 and 1776 mg/kg bw per day for males and 0, 62, 188, 595 and 1953 mg/kg bw per day for females, respectively). Parameters evaluated included body weight, body weight gain, feed consumption, feed efficiency, clinical signs, ophthalmology, gross pathology, histopathology, organ weights, haematology, clinical chemistry and hepatic biochemical measurements (cytochrome P450, β-oxidation, uridine diphosphate–glucuronyltransferase [UDPGT]).

No deaths and no clinical or ophthalmological observations were attributable to the treatment. No treatment-related effects on body weight or feed intake were observed. There were no adverse effects on blood chemistry or on gross pathology. In haematological examination, increased degrees of change in erythrocyte shape (e.g. echinocytes and acanthocytes) were observed in males and females at 6000 and 20 000 ppm, without any changes in other red cell parameters. Echinocyte is a common in vitro artefactual finding of red blood cells, and the red cells of Sprague-Dawley rats are especially susceptible to this artefact as a result of in vitro crenation (Reagan, Irizarry & DeNicola, 2008). Therefore, the morphological changes of erythrocytes were not considered adverse.

Liver weights were statistically significantly increased in both sexes fed 6000 ppm and higher. Microscopically, centrilobular hepatocellular hypertrophy was observed in male and female rats at 2000 ppm or higher (Table 10). Thyroid weight increases were detected in both sexes at 6000 ppm and above, and follicular cell hypertrophy in the thyroid was observed in males and females at 2000 ppm and higher. The increase of thyroid follicular cell hypertrophy is considered to be indicative of altered homeostasis in the thyroid.

Hepatic UDPGT activity was slightly induced in both sexes, whereas the effect on induction of cytochrome P450 content was slight (Table 11). Cyantraniliprole did not induce hepatic β-oxidation, a measure of peroxisome proliferation, in male or female rats.

The NOAEL for the 28-day oral toxicity study in rats was 600 ppm (equal to 53 mg/kg bw per day), based on liver hypertrophy and thyroid follicular cell hypertrophy observed in both sexes at 2000 ppm (equal to 175 mg/kg bw per day) (Carpenter, 2009e).

In a 90-day feeding study, cyantraniliprole (purity 93.4%; batch no. HGW86-141) was administered to male and female Crl:CD®(SD) rats (10 of each sex per group) at a dietary concentration of 0, 100, 400, 3000 or 20 000 ppm (equal to 0, 5.7, 22, 168 and 1147 mg/kg bw per day for males and 0, 6.9, 27, 202 and 1346 mg/kg bw per day for females, respectively). Parameters evaluated included body weight, body weight gain, feed consumption, feed efficiency, clinical signs,

haematology, clinical chemistry, urine analysis, ophthalmology, organ weights, and gross and microscopic

Table 10. Select data on organ weights and histopathology in a 28-day oral toxicity study in rats

	0 ppm	600 ppm	2 000 ppm	6 000 ppm	20 000 ppm
Liver weight					
Males					
Absolute liver weight (g)	10.7	11.8	12.1	13.7a	13.7a
Relative[a] liver weight (%)	3.3	3.5	3.9	4.2b	4.4b
Females					
Absolute liver weight (g)	7.7	7.8	9.1	9.5	10.6a
Relative[a] liver weight (%)	3.7	4.0	4.4	4.5b	5.2b
Thyroid weight					
Males					
Absolute thyroid weight (g)	0.017	0.021	0.021	0.024a	0.020
Relative[a] thyroid weight (%)	0.005	0.006	0.007	0.007a	0.006
Females					
Absolute thyroid weight (g)	0.013	0.016	0.016	0.018a	0.022a
Relative[a] thyroid weight (%)	0.006	0.008	0.008	0.008	0.011a
Histopathology					
Males					
Liver: Hepatocellular hypertrophy, centrilobular	0/5	1/5	3/5	5/5	5/5
Thyroid: Follicular cell hypertrophy	0/5	1/5	3/5	3/5	4/5
Females					
Liver: Hepatocellular hypertrophy, centrilobular	0/5	1/5	4/5	3/5	5/5
Thyroid: Follicular cell hypertrophy	05	0/5	1/5	1/5	4/5

a, pair-wise test (Dunnett/Tamhane-Dunnett) significant at $P < 0.05$; b, non-parametric comparison with control (Dunn's) significant at $P < 0.05$

[a] Relative to body weight.

Source: Carpenter (2009e)

Table 11. Summary of hepatic β-oxidation, cytochrome P450 content and UDPGT activity of the liver in a 28-day oral toxicity study in rats

	0 ppm	600 ppm	2 000 ppm	6 000 ppm	20 000 ppm
Males					
β-oxidation activity (nmol/mg protein)	11.3 ± 1.1	9.2 ± 1.8	10.9 ± 2.4	9.4 ± 3.5	9.0 ± 1.1
Cytochrome P450 content (nmol/mg protein)	1.03 ± 0.32	1.05 ± 0.58	1.05 ± 0.58	1.05 ± 0.16	1.30 ± 0.35
UDPGT activity (nmol/mg protein)	9.6 ± 2.3	13.3 ± 5.5	16.0 ± 2.2*	23.5 ± 3.4*	22.3 ± 3.6*
Females					
β-oxidation activity (nmol/mg protein)	15.2 ± 3.1	13.0 ± 3.1	15.4 ± 4.0	17.2 ± 2.2	15.0 ± 2.5
Cytochrome P450 content (nmol/mg protein)	0.50 ± 0.08	0.68 ± 0.24	0.67 ± 0.05[a]	0.76 ± 0.08*	0.76 ± 0.11*
UDPGT activity (nmol/mg protein)	12.3 ± 3.0	16.8 ± 4.8	16.3 ± 3.8	17.5 ± 2.9	17.0 ± 2.9

UDPGT: uridine diphosphate–glucuronosyltransferase; *: $P < 0.05$ (Dunn's test)

[a] $n = 4$.

Source: Carpenter (2009e)

pathology. Five satellite groups of the same strain of rats (five of each sex per group) were administered the same dietary doses for 29 days (males) or 30 days (females). Serum thyroid hormone concentrations and hepatic biochemical parameters (cytochrome P450 and UDPGT) were evaluated using the satellite groups and main group at termination. Blood (from non-fasted animals) was collected on test days 63 (males) and 64 (females) for analysis of the concentration of the test substance and metabolites in plasma. The results are shown in Table 7.

No treatment-related deaths occurred, and there were no clinical signs or ophthalmological observations attributable to treatment. No test substance–related deaths and no clinical or ophthalmological observations were attributed to exposure to the test substance. No treatment-related effects on body weight or feed intake were observed. In haematology, the degree of shape change in erythrocytes (echinocytes and acanthocytes) was increased in both sexes at 3000 and 20 000 ppm, but there was a lack of abnormalities in other haematological parameters. The morphological changes in red blood cells were not considered adverse because they were not associated with changes in other haematological parameters, including red cell mass (see the interpretation of these changes in the 28-day oral toxicity study in rats described above). In blood biochemistry, an increase in cholesterol level (135% of control) and a decrease in triglyceride level (61% of control) were observed in females at 20 000 ppm at termination. Decreases in bilirubin levels in both sexes of all treated groups appeared to be the consequence of an adaptive change involving bilirubin metabolism in the liver. Slight decreases in albumin level in females and in urea level in males at 20 000 ppm at 30 days were not considered to be treatment related due to the absence of these changes at 90 days. In the liver, UDPGT activity was significantly increased in both sexes at 29/30 days, in females at 90 days at 400, 3000 and 20 000 ppm and in males at terminal kill at 20 000 ppm (Table 12). Total cytochrome P450 was slightly increased after 29 days only in males at 20 000 ppm and after 90 days in females at 20 000 ppm. A significant increase in total cytochrome P450 in males of the 3000 ppm group after 90 days was not part of a dose-related trend. With regard to thyroid-related hormones, there were consistent reductions in T_3 or T_4 that were frequently statistically significant in both sexes at 400, 3000 and 20 000 ppm at both observation times, whereas TSH concentration was significantly higher only in males of the 20 000 ppm group after 90 days.

Liver weights were increased in both sexes at 400 ppm and higher, and thyroid and adrenal weights were increased in females at 20 000 ppm (Table 13). The increases in liver and thyroid weights were accompanied by minimal to slight centrilobular hypertrophy of hepatocytes in males at 3000 ppm and higher and in females at 400 ppm and higher and minimal thyroid follicular cell hypertrophy in males at 20 000 ppm and in females at 400 ppm and higher. The liver hypertrophy without indication of hepatotoxicity was considered to be adaptive. The changes in thyroid weight, histopathology and related hormones indicated that homeostasis of the pituitary and thyroid negative feedback system was affected by the treatment in rats. Minimal to slight microvesiculation of the fascicular zone in the adrenal cortex was increased in males at 20 000 ppm at 90 days. This change was not detected in a long-term study in rats (Craig, 2011a). In a mechanistic study involving measurement of serum corticosterone levels and ultrastructural analysis of the adrenals, no functional or structural changes in the adrenal cortex were observed that were indicative of an adverse effect (see section 2.6). Therefore, the minimal to slight adrenal change was considered to be treatment related but not adverse and within normal physiological limits.

The NOAEL for the 90-day oral toxicity study in rats was 100 ppm (equal to 5.7 mg/kg bw per day), based on liver hypertrophy, decreases in thyroid hormones in both sexes and histopathological changes in the thyroid in females at 400 ppm (equal to 22 mg/kg bw per day) (Carpenter, 2007; Gannon, 2011b).

Dogs

In a 28-day feeding toxicity study, cyantraniliprole (purity 92.7%; batch no. HGW86-085) was administered to male and female Beagle dogs (two of each sex per group) at a dietary concentration of 0, 1000, 10 000 or 40 000 ppm (equal to 0, 35, 311 and 1043 mg/kg bw per day for males and 0, 35, 335 and 1240 mg/kg bw per day for females, respectively). Parameters evaluated

included body weight, body weight gain, feed consumption, feed efficiency, clinical signs, neurobehavioural

Table 12. Thyroid metabolism–related biochemical parameters in a 90-day oral toxicity study in rats

	0 ppm	100 ppm	400 ppm	3 000 ppm	20 000 ppm
Interim kill (29/30 days)					
Males					
T_4 (µg/dL)	3.7 ± 0.9	3.1 ± 0.7	2.3 ± 0.5*	2.1 ± 0.3*	2.1 ± 0.7*
T_3 (ng/dL)	70.4 ± 15.0	68.9 ± 16.4	60.6 ± 15.0	61.0 ± 23.8	55.7 ± 18.4
TSH (ng/mL)	6.8 ± 1.5	6.5 ± 1.4	6.7 ± 2.2	8.2 ± 2.9	8.3 ± 5.1
Total P450 (nmol/mg protein)	0.68 ± 0.16	0.68 ± 0.12	0.80 ± 0.20	0.81 ± 0.08	0.93 ± 0.15*
UDPGT (nmol/mg per minute)	31.2 ± 3.3	40.7 ± 7.2	45.4 ± 12.7*	45.7 ± 3.9*	55.4 ± 8.1*
Females					
T_4 (µg/dL)	3.0 ± 0.6	2.6 ± 0.8	2.1 ± 0.7*	1.4 ± 0.5*	1.3 ± 0.5*
T_3 (ng/dL)	81.3 ± 11.0	79.2 ± 11.8	62.3 ± 6.2*	68.1 ± 15.9	63.3 ± 17.4*
TSH (ng/mL)	5.9 ± 1.6	5.7 ± 0.8	5.9 ± 1.4	6.0 ± 1.2	5.9 ± 1.4
Total P450 (nmol/mg protein)	0.48 ± 0.06	0.47 ± 0.04	0.46 ± 0.08	0.50 ± 0.06	0.56 ± 0.06
UDPGT (nmol/mg per minute)	31.6 ± 2.6	40.1 ± 4.5	61.2 ± 20.0*	59.9 ± 7.7*	66.4 ± 12.2*
Terminal kill (90 days)					
Males					
T_4 (µg/dL)	4.1 ± 0.7	3.7 ± 0.5	3.1 ± 0.8*	2.8 ± 0.5*	2.9 ± 0.6**
T_3 (ng/dL)	67.4 ± 5.3	64.5 ± 9.8	55.7 ± 11.6*	53.6 ± 9.4*	52.4 ± 0.5**
TSH (ng/mL)	7.1 ± 1.0	8.2 ± 2.3	7.9 ± 2.1	10.1 ± 4.1	11.0 ± 2.0*
Total P450 (nmol/mg protein)	0.86 ± 0.09	0.95 ± 0.27	0.79 ± 0.15	1.15 ± 0.17*	1.10 ± 0.08
UDPGT (nmol/mg per minute)	51.7 ± 14.5	48.0 ± 4.5	44.9 ± 6.6	66.7 ± 8.8	75.5 ± 8.4*
Females					
T_4 (µg/dL)	2.4 ± 0.6	2.0 ± 0.7	1.4 ± 0.6	0.8 ± 0.7*	0.5 ± 0.5*
T_3 (ng/dL)	92.1 ± 7.9	91.1 ± 7.9	86.0 ± 16.3	63.3 ± 13.3*	65.0 ± 17.1*
TSH (ng/mL)	6.5 ± 0.7	6.9 ± 1.1	6.8 ± 1.0	6.7 ± 1.1	7.4 ± 1.8
Total P450 (nmol/mg protein)	0.55 ± 0.19	0.60 ± 0.10	0.67 ± 0.11	0.70 ± 0.14	0.87 ± 0.13*
UDPGT (nmol/mg per minute)	32.0 ± 5.8	38.4 ± 9.1	51.2 ± 8.0*	51.8 ± 10.7*	67.7 ± 13.1*

T_3: triiodothyronine; T_4: thyroxine; TSH: thyroid stimulating hormone; UDPGT: uridine diphosphate–glucuronosyltransferase; * $P < 0.05$

Source: Carpenter (2007)

observations, ophthalmoscopic observations, thyroid hormones, gross pathology, organ weights, histopathology, and hepatic cytochrome P450 and microsomal enzymes. This study was conducted as a non-GLP study.

Table 13. Summary of organ weights and histopathology in a 90-day oral toxicity study in rats

	0 ppm	100 ppm	400 ppm	3 000 ppm	20 000 ppm
Interim kill (29/30 days)					
Organ weights					
Males					
- Absolute liver weight (g)	13.1	12.5	13.5	14.7	15.6a
- Relative[a] liver weight (%)	3.2	3.0	3.2	3.5a	3.8a
Females					
- Absolute liver weight (g)	7.40	7.7	8.3	9.2a	9.4a
- Relative[a] liver weight (%)	3.1	3.2	3.6	4.0b	4.0b
- Absolute thyroid weight (g)	0.011	0.017	0.015	0.017	0.021
- Relative[a] thyroid weight (%)	0.005	0.007	0.006	0.008	0.009b
Histopathology					
Liver: Hypertrophy of hepatocytes, centrilobular					
- Males	0/5	0/5	0/5	0/5	0/5
- Females	0/5	0/5	0/5	3/5	5/5
Terminal kill (90 days)					
Organ weights					
Males					
- Absolute liver weight (g)	16.0	16.1	17.3	16.5	18.0a
- Relative[a] liver weight (%)	2.7	2.8	3.0a	2.9a	3.3a
- Absolute kidney weight (g)	4.3	4.2	4.5	3.9b	4.4
- Relative[a] kidney weight (%)	0.74	0.72	0.77	0.69	0.80
- Absolute thyroid weight (g)	0.032	0.028	0.033	0.034	0.034
- Relative[a] thyroid weight (%)	0.005	0.005	0.006	0.006	0.006
Females					
- Absolute liver weight (g)	8.4	8.4	9.2	10.3a	11.2a
- Relative[a] liver weight (%)	2.9	3.0	3.2	3.6a	4.1a
- Absolute kidney weight (g)	2.21	2.09	2.33	2.24	2.37
- Relative[a] kidney weight (%)	0.76	0.76	0.82	0.79	0.86a

	0 ppm	100 ppm	400 ppm	3 000 ppm	20 000 ppm
- Absolute thyroid weight (g)	0.024	0.024	0.028	0.026	0.028
- Relative[a] thyroid weight (%)	0.009	0.009	0.010	0.009	0.010
Histopathology					
Males					
- Liver: Hypertrophy of hepatocytes, centrilobular	0/10	0/10	0/10	4/10	7/10
- Thyroid: Follicular cell hypertrophy	0/10	0/10	0/10	0/10	5/10
- Adrenal: Microvesiculation in fascicular zone of cortex	0/10	0/10	0/10	1/10	6/10
Females					
- Liver: Hypertrophy of hepatocytes, centrilobular	0/10	0/10	2/10	6/10	9/10
- Thyroid: Follicular cell hypertrophy	1/10	0/10	3/10	4/10	6/10
- Adrenal: Microvesiculation in fascicular zone of cortex	0/10	0/10	0/10	2/10	2/10

a: $P < 0.05$ (Dunnett/Tamhane-Dunnett criterion); b: $P < 0.01$
[a] Relative to body weight.
Source: Carpenter (2007)

No treatment-related effects on survival, clinical or neurobehavioural findings or physical or ophthalmoscopic examinations were observed. A dose-related decreased body weight gain in both sexes at 1000 and 10 000 ppm and/or body weight losses in both sexes at 40 000 ppm, corresponding to decreases in feed consumption and feed efficiency, were observed. Effects on clinical chemistry, including increased alkaline phosphatase (AP) activity and decreased albumin levels in both sexes at 1000 ppm and higher, decreased cholesterol levels in males at 1000 ppm and higher and in females at 10 000 ppm and higher, increased gamma-glutamyltransferase (GGT) in females and increased aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT) or sorbitol dehydrogenase (SDH) in both sexes at 40 000 ppm, were detected. Liver weights were increased in males at 10 000 ppm and above and in all female dose groups, but the increases were not associated with microscopic pathology except for minimal hepatocyte apoptosis observed in one female at 40 000 ppm. Induction of hepatic total cytochrome P450 and CYP2B1, CYP3A2 and CYP4A1/2/3 isozymes was observed in all male and female dose groups, but without any monotonic responses. No adverse or primary test article–related effects on thyroid hormones (TSH, total T_3, total T_4) were observed in any dose group. One male dog in the 40 000 ppm group was diagnosed with canine juvenile polyarteritis syndrome (idiopathic arteritis in dogs) because of the characteristic clinical signs and morphological features reported (Hartman, 1987; Hayes, Roberts & Halliwell, 1989; Snyder et al., 1995; Kerns, Roth & Hosokawa, 2001).

A NOAEL for the 28-day oral toxicity study in dogs was not determined, based on changes in body weight, nutritional parameters and clinical chemistry indicating hepatotoxicity in both sexes at 1000 ppm (equal to 35 mg/kg bw per day), the lowest dose tested (Luckett, 2007a).

Cyantraniliprole (purity 93.4%; batch no. HGW86-141) was administered to male and female Beagle dogs (four of each sex per group) at a dietary concentration of 0, 30, 100, 1000 or 10 000 ppm (equal to 0, 0.98, 3.08, 31.9 and 281 mg/kg bw per day for males and 0, 0.97, 3.48, 34.3 and 294 mg/kg bw per day for females, respectively) for 90 days. Parameters evaluated included mortality and morbidity, body weight, body weight gain, feed consumption, feed efficiency, compound consumption, clinical signs, neurobehavioural signs, haematology, urine analysis, clinical chemistry, ophthalmology, organ weights, and gross and microscopic pathology. Blood was collected on test day 57 (unfasted) and analysed for concentrations of parent compound and selected metabolites. The results are shown in Table 7.

One male in the 10 000 ppm diet group was dead on day 52, possibly caused by canine juvenile polyarteritis syndrome. Treatment-related reductions of body weight gain (and/or loss of body weight), feed consumption and feed efficiency were observed in both sexes at 10 000 ppm. In blood biochemistry, decreases in total protein and albumin levels in both sexes at 1000 ppm and above, a decrease in calcium levels in males at 1000 ppm and above and in females at 10 000 ppm, decreases in cholesterol and glucose levels in both sexes at 10 000 ppm and an increase in ALAT in females at 10 000 ppm were observed (Table 14). Statistically significant increases in AP were found in both sexes at 1000 ppm and above. Age-matched reduction of AP was not observed at 30 and 100 ppm in both sexes. The changes at the lower doses were considered to be treatment related, but not adverse, as there was no indication of hepatotoxicity, including histopathology, at these doses. No treatment-related effects on haematology or urine analysis were detected.

Liver weights were increased in both sexes at 1000 ppm and above. Microscopically, minimal to mild bile duct hyperplasia in all males and three females and minimal or mild focal hepatocellular necrosis in one male and three females detected at 10 000 ppm were considered to be treatment-related adverse effects. Three dogs (two males, including the dead one on day 52, and one female) at 10 000 ppm were affected by canine juvenile polyarteritis syndrome, which showed systemic distribution (e.g. heart, liver, other organs). One male affected was dead as a result of cardiac dysfunction caused by a secondary effect of this syndrome. This syndrome has been reported as idiopathic (Hartman, 1987; Hayes, Roberts & Halliwell, 1989; Snyder et al., 1995; Kerns, Roth & Hosokawa, 2001), but a possibility that the treatment exacerbated the syndrome could not be excluded, due to the occurrence of the condition only at the highest doses in both sexes and detection in both the 1-year (Luckett, 2010) and 28-day (Luckett, 2007a) oral toxicity studies of cyantraniliprole in dogs. Hepatocellular necrosis and leukocyte infiltration in sinusoids were also found in both sexes at 10 000 ppm.

The NOAEL for the 90-day oral toxicity study in dogs was 100 ppm (equal to 3.08 mg/kg bw per day), based on increased total protein, albumin concentrations and AP in males at 1000 ppm (equal to 31.9 mg/kg bw per day) (Luckett, 2007b; Gannon, 2009).

In a 1-year feeding study, cyantraniliprole (purity 94.5%; batch no. HGW86-230) was administered to groups of four Beagle dogs of each sex at a dietary concentration of 0, 40, 200, 1000 or 5000 ppm (equal to 0, 0.96, 5.67, 27.0 and 144 mg/kg bw per day for males and 0, 1.12, 6.00, 27.1 and 133 mg/kg bw per day for females, respectively) for 364 consecutive days. An additional seven dogs of each sex received cyantraniliprole at a single dietary concentration of 5000 ppm for 12 weeks. At week 12, four dogs of each sex at 5000 ppm continued to receive the treatment, whereas the remaining surviving dogs (recovery animals: two males, three females) were placed on control diet for 40 weeks to evaluate reversibility. Parameters evaluated included body weight, body weight gain, feed consumption, feed efficiency, compound consumption, clinical signs, neurobehavioural

observations, haematology, coagulation, clinical chemistry, urine analysis, ophthalmology, organ weights, and gross and

Table 14. Summary of clinical chemistry in a 90-day oral toxicity study in dogs

	0 ppm	30 ppm	100 ppm	1 000 ppm	10 000 ppm
Males					
Albumin (g/dL)					
Week 4	2.83 ± 0.49	2.95 ± 0.06	2.90 ± 0.09	2.45 ± 0.13	2.25 ± 0.06*
Week 8	2.58 ± 0.34	2.55 ± 0.10	2.50 ± 0.08	2.03 ± 0.13**	1.63 ± 0.12**
Week 12	3.00 ± 0.34	2.98 ± 0.10	2.88 ± 0.05	2.23 ± 0.10**	1.83 ± 0.31**
AP (U/L)					
Week −2	109.0 ± 30.84	91.5 ± 16.42	111.5 ± 14.43	141.3 ± 59.20	90.3 ± 20.89
Week −1	105.5 ± 21.02	103.5 ± 16.74	116.3 ± 18.32	141.5 ± 58.87	97.5 ± 20.57
Week 4	74.3 ± 12.0	83.5 ± 11.7	141.3 ± 41.3	233.0 ± 74.6**	189.3 ± 93.9*
Week 8	76.5 ± 14.4	102.0 ± 35.0	169.8 ± 78.6	276.5 ± 89.2	426.7 ± 362.3*
Week 12	65.5 ± 12.0	93.5 ± 38.9	138.3 ± 60.1	293.3 ± 115.2*	363.7 ± 246.2*
Cholesterol (mg/dL)					
Week 4	170.0 ± 37.6	185.3 ± 15.6	175.8 ± 39.7	142.5 ± 29.4	100.3 ± 14.1*
Week 8	168.3 ± 41.7	184.8 ± 15.7	161.3 ± 39.7	130.5 ± 32.2	108.0 ± 49.4
Week 12	162.8 ± 48.0	173.3 ± 18.4	154.5 ± 36.8	127.5 ± 36.4	125.3 ± 72.2
Total protein (g/dL)					
Week 4	5.48 ± 0.40	5.50 ± 0.25	5.43 ± 0.17	5.00 ± 0.22	4.83 ± 0.25*
Week 8	5.33 ± 0.15	5.25 ± 0.17	5.20 ± 0.25	4.55 ± 0.33**	4.63 ± 0.38*
Week 12	5.78 ± 0.10	5.63 ± 0.28	5.60 ± 0.26	4.83 ± 0.33**	5.07 ± 0.65
Females					
ALAT (U/L)					
Week 4	22.0 ± 4.8	27.3 ± 5.7	28.3 ± 3.4	24.0 ± 7.1	36.0 ± 2.2**
Week 8	21.3 ± 2.6	25.5 ± 3.7	26.3 ± 4.0	28.0 ± 7.5	42.5 ± 23.5
Week 12	22.8 ± 5.0	26.0 ± 6.6	27.3 ± 27.3	31.0 ± 12.8	87.5 ± 30.6**
Albumin (g/dL)					
Week 4	2.80 ± 0.29	2.85 ± 0.27	2.65 ± 0.39	2.38 ± 0.19	2.20 ± 0.08*
Week 8	2.55 ± 0.27	2.60 ± 0.14	2.40 ± 0.18	1.98 ± 0.13**	1.58 ± 0.25**
Week 12	2.95 ± 0.30	2.90 ± 0.12	2.78 ± 0.17	2.30 ± 0.00**	1.80 ± 0.20**
AP (U/L)					
Week −2	121.3 ± 24.35	108.3 ± 14.36	121.3 ± 72.02	128.8 ± 35.66	90.8 ± 14.57
Week −1	121.8 ± 29.28	98.8 ± 21.47	129.5 ± 32.42	118.3 ± 23.30	127.3 ± 15.46
Week 4	91.5 ± 12.6	113.0 ± 33.2	160.8 ± 39.7	266.3 ± 57.1*	257.3 ± 143.3*
Week 8	90.5 ± 19.5	120.316.9 ±	179.0 ± 40.4	307.0 ± 104.5	483.5 ± 321.6**
Week 12	77.3 ± 13.4	98.8 ± 21.6	142.0 ± 22.7	259.3 ± 98.7**	357.8 ± 85.9**
Cholesterol (mg/dL)					
Week 4	154.8 ± 30.6	150.3 ± 12.4	179.8 ± 27.0	156.0 ± 23.9	97.8 ± 43.6*
Week 8	155.5 ± 23.5	147.8 ± 18.9	173.3 ± 27.0	134.3 ± 15.4	114.3 ± 72.8
Week 12	161.0 ± 28.2	146.3 ± 20.6	155.3 ± 14.0	143.8 ± 17.8	90.8 ± 46.4**
Total protein (g/dL)					
Week 4	5.08 ± 0.30	5.33 ± 0.13	5.38 ± 0.25	5.13 ± 0.30	4.63 ± 0.05*
Week 8	4.98 ± 0.24	5.08 ± 0.05	5.33 ± 0.42	4.60 ± 0.25	4.45 ± 0.38
Week 12	5.33 ± 0.26	5.43 ± 0.13	5.58 ± 0.38	4.93 ± 0.32	4.50 ± 0.12**

ALAT: alanine aminotransferase; AP: alkaline phosphatase; U: units; *: $P < 0.05$; **: $P < 0.01$

[a] $n = 3$ for 10 000 ppm males at weeks 8 and 12.

Source: Luckett (2007b)

microscopic pathology. Concentrations of test article and selected environmental metabolites in plasma were evaluated at week 39. The results are shown in Table 7.

One male and one female at 5000 ppm were euthanized in extremis on days 80 and 176, respectively. Their causes of death were possibly associated with juvenile canine polyarteritis, which especially affected the coronary artery or artery adjacent to the aorta in the male and the artery adjacent to the brachiocephalic trunk in the female, respectively. Mean body weight gains in both sexes at 5000 ppm and in females at 1000 ppm were decreased (Table 15).

Table 15. Mean body weight gain in the 1-year oral toxicity study in dogs

	Mean body weight gain (kg)					
	Main study					Recovery[a]
	0 ppm	40 ppm	200 ppm	1 000 ppm	5 000 ppm	5 000 ppm
Males						
Weeks −1 to 12	1.2 ± 0.5	1.6 ± 0.3	1.4 ± 0.5	1.1 ± 0.3	0.9 ± 0.4	1.2 ± 0.1
Weeks −1 to 52	1.7 ± 0.4	2.1 ± 1.4	2.1 ± 0.7	1.6 ± 0.9	1.1 ± 1.1	1.7 ± 0.6
Females						
Weeks −1 to 12	0.7 ± 0.5	1.3 ± 0.8	0.6 ± 0.4	0.7 ± 0.3	0.5 ± 0.4	1.2 ± 0.5
Weeks −1 to 52	1.5 ± 0.4	2.1 ± 1.4	1.3 ± 0.3	0.9 ± 0.5	0.1 ± 0.7	2.0 ± 1.2

[a] Two males and three females at 5000 ppm were placed on recovery following 12 weeks (84 days) of administration.
Source: Luckett (2010)

Adverse test article–related increases in ALAT at 1000 ppm and higher and in GGT at 5000 ppm and decreases in total protein and albumin levels at 1000 ppm and higher were observed in males and females (Table 16). Consistently decreased levels of cholesterol observed in both sexes at 1000 ppm and above were also considered treatment related. The decrease in females at 200 ppm at termination was not considered toxicologically significant due to the consistently lower values before the treatment in this group (Table 16).

Clear increases in AP observed in both sexes at 1000 ppm and above were considered to be adverse due to other changes suggesting liver toxicity at those doses, described below. AP was slightly increased in both sexes at 200 ppm. In addition, increased liver weights were observed in males, suggesting that the marginal increase in AP was adverse. The age-matched decrease in AP was not observed in males at 40 ppm, but no histopathological or functional change in the liver was found at 40 ppm. In addition, individual values of AP in the male control group before treatment were lower compared with other treated groups and the control group in the 90-day dog study (Luckett, 2007b). Thus, the increase in males at 40 ppm was not considered to be adverse.

Liver (with gallbladder) weights (absolute and/or relative to body weight and to brain weight) at 200 ppm and above in both sexes and thyroid weight (absolute and relative) at 5000 ppm in males were increased. Microscopically, the increased liver weights were associated with degenerative changes in hepatocytes, with enlargement, rarefaction and margination of cytoplasm, vacuolation, cytoplasmic membrane inclusion and/or single-cell necrosis in the centrilobular area and chronic inflammatory change in the portal area at 1000 ppm and above in both sexes (Table 17). In addition, minimal cholestasis was detected in males at 1000 ppm and above and in females at 5000 ppm. Mucosal hyperplasia in the gallbladder was observed at 5000 ppm in both sexes. These effects were reversible, as there was no clinical pathology or microscopic findings at termination in the 5000 ppm recovery group. Arteritis and/or perivasculitis in individual organs (e.g. coronary artery, heart, liver, kidney or brain) were observed in control and treated groups in both sexes. Systemic arteritis was observed in two males and one female at 5000 ppm. These arterial changes were similar to those observed at 10 000 ppm in an earlier 90-day study with cyantraniliprole (Luckett, 2007b). The

morphological characteristics of arteritis and/or perivasculitis were identical to those of canine juvenile polyarteritis (Hartman, 1987; Hayes, Roberts & Halliwell, 1989; Snyder et al., 1995). Whereas the arteritis has been reported to be idiopathic (Hartman, 1987; Hayes, Roberts & Halliwell, 1989; Snyder et al., 1995), a possibility of acceleration by the treatment could not be excluded due to the consistency of the observations with the previous 90-day (Luckett, 2007b) and 28-day (Luckett, 2007a) dog studies at the high dose in both sexes.

Table 16. Selected clinical chemistry parameters in the 1-year oral toxicity study in dogs

	Week	Main study					Recovery[a]
		0 ppm	40 ppm	200 ppm	1 000 ppm	5 000 ppm	5 000 ppm
Males							
AP (U/L)	−3	41.0 ± 9.38	61.5 ± 15.36	57.8 ± 11.58	52.0 ± 9.38	58.2 ± 20.86	54.5 ± 27.58
	−2	37.8 ± 9.60	65.5 ± 14.62	48.5 ± 10.28	46.8 ± 9.84	53.6 ± 19.60	58.0 ± 33.94
	13	26.8 ± 6.8	70.0 ± 9.9**	109.8 ± 10.2**	174.3 ± 51.0**	318.8 ± 159.7**	354.5 ± 24.8
	26	23.5 ± 8.7	79.0 ± 15.1**	125.0 ± 25.2**	207.8 ± 76.1	402.8 ± 198.8	37.0 ± 17.0
	52	17.3 ± 4.6	71.5 ± 17.2**	117.5 ± 9.6**	209.5 ± 105.7	401.0 ± 171.9	28.5 ± 13.4
GGT (U/L)	13	3.0 ± 0.0	3.3 ± 0.5	4.0 ± 0.8	3.8 ± 1.0	5.0 ± 2.2	4.0 ± 1.4
	26	2.5 ± 0.6	3.0 ± 0.8	3.8 ± 1.0	4.0 ± 1.4	4.5 ± 1.3	2.5 ± 0.7
	52	4.5 ± 0.6	4.3 ± 1.0	4.5 ± 1.9	5.8 ± 1.5	8.0 ± 2.2	5.0 ± 0.0
ALAT (U/L)	13	32.3 ± 12.7	31.0 ± 2.9	42.0 ± 18.0	43.0 ± 8.9	67.0 ± 32.7	46.5 ± 9.2
	26	31.5 ± 11.6	33.5 ± 7.5	53.3 ± 13.5	50.8 ± 16.9	94.0 ± 40.2	31.0 ± 0.0
	52	33.0 ± 11.3	36.0 ± 5.7	59.5 ± 27.5	95.8 ± 50.5	112.5 ± 39.2	32.0 ± 0.0
Total protein (g/dL)	13	6.3 ± 0.4	6.0 ± 0.3	5.8 ± 0.2	5.4 ± 0.2*	5.6 ± 0.4	5.4 ± 0.0
	26	6.1 ± 0.5	5.9 ± 0.1	5.5 ± 0.2	5.2 ± 0.1	5.4 ± 0.6	6.0 ± 0.4
	52	6.3 ± 0.5	5.9 ± 0.1	5.6 ± 0.1	5.3 ± 0.2	5.7 ± 0.4	6.1 ± 0.4
Albumin (g/dL)	13	3.2 ± 0.2	2.8 ± 0.2	2.6 ± 0.2*	2.4 ± 0.2**	2.3 ± 0.2**	2.2 ± 0.1
	26	3.2 ± 0.2	2.8 ± 0.2	2.6 ± 0.2*	2.4 ± 0.2**	2.2 ± 0.3**	3.2 ± 0.4
	52	3.2 ± 0.1	2.9 ± 0.1*	2.6 ± 0.2*	2.4 ± 0.1**	2.2 ± 0.4**	3.1 ± 0.4
Cholesterol (mg/dL)	13	155.3 ± 23.8	187.8 ± 27.0	162.8 ± 42.0	102.8 ± 15.5	90. 5 ± 21.63*	128.0 ± 18.4
	26	144.5 ± 17.3	182.5 ± 34.5	148.5 ± 39.7	99.5 ± 13.6*	97.5 ± 32.4	158.5 ± 38.9
	52	145.5 ± 23.2	185.0 ± 24.4	134.3 ± 21.5	95.8 ± 24.1	108.0 ± 30.8	165.0 ± 48.1
Females							
AP (U/L)	−3	74.5 ± 22.83	46.8 ± 1.71	55.8 ± 15.00	60.8 ± 3.95	68.3 ± 14.66	56.3 ± 5.03
	−2	71.3 ± 15.24	41.8 ± 4.19	58.3 ± 17.33	57.8 ± 2.22	70.5 ± 16.13	52.0 ± 8.89
	13	43.8 ± 13.5	54.3 ± 14.9	142.0 ± 76.0	229.8 ± 115.2*	369.0 ± 134.2**	261.7 ± 79.3*
	26	53.5 ± 42.4	65.3 ± 27.4	151.8 ± 69.8	237.3 ± 77.5	445.0 ± 325.6**	43.3 ± 14.0
	52	38.8 ± 14.7	58.0 ± 20.8	151.0 ± 55.4	280.0 ± 77.3*	591.3 ± 254.7**	39.0 ± 4.6
GGT (U/L)	13	3.5 ± 0.6	4.3 ± 2.1	3.8 ± 0.5	4.5 ± 1.3	6.0 ± 0.0*	4.7 ± 1.5
	26	3.3 ± 0.5	3.0 ± 1.2	3.0 ± 0.0	5.0 ± 2.2	7.0 ± 1.0**	2.3 ± 0.6
	52	5.3 ± 1.0	5.0 ± 1.2	5.5 ± 0.6	7.3 ± 2.6	10.3 ± 1.2**	4.3 ± 1.5

Table 16 (continued)

	Week	Main study					Recovery[a]
		0 ppm	40 ppm	200 ppm	1 000 ppm	5 000 ppm	5 000 ppm
ALAT (U/L)	13	26.8 ± 3.0	25.3 ± 3.2	28.8 ± 7.9	41.5 ± 18.1	66.8 ± 29.9*	44.0 ± 18.3
	26	21.0 ± 3.8	26.3 ± 3.6	26.0 ± 6.6	58.8 ± 41.3	117.0 ± 58.4**	21.3 ± 3.1
	52	22.3 ± 3.4	25.5 ± 3.1	26.5 ± 7.1	83.5 ± 74.5	109.0 ± 50.1*	20.7 ± 1.2
Total protein (g/dL)	13	6.0 ± 0.2	6.0 ± 0.4	5.8 ± 0.4	5.2 ± 0.2**	5.2 ± 0.2**	5.1 ± 0.2
	26	5.8 ± 0.3	5.90.4 ±	5.6 ± 0.4	5.0 ± 0.3**	4.9 ± 0.1**	5.8 ± 0.1
	52	6.0 ± 0.2	6.2 ± 0.3	5.6 ± 0.4	5.2 ± 0.2**	4.9 ± 0.2**	6.1 ± 0.3
Albumin (g/dL)	13	3.1 ± 0.2	3.0 ± 0.1	2.9 ± 0.2	2.4 ± 0.1**	2.4 ± 0.2**	2.2 ± 0.3**
	26	3.1 ± 0.3	3.1 ± 0.3	2.9 ± 0.3	2.4 ± 0.2**	2.2 ± 0.0**	2.9 ± 0.3
	52	3.2 ± 0.2	3.1 ± 0.2	2.9 ± 0.2	2.4 ± 0.2**	2.1 ± 0.1**	2.9 ± 0.2
Cholesterol (mg/dL)	−3	173.3 ± 43.1	148.8 ± 14.5	130.8 ± 15.7	156.0 ± 31.9	142.3 ± 8.18	136.3 ± 21.7
	−2	172.0 ± 34.0	144.0 ± 11.34	151.5 ± 23.1	156.0 ± 31.89	161.0 ± 11.46	140.7 ± 24.4
	13	169.8 ± 43.3	158.0 ± 16.0	152.8 ± 12.7	131.5 ± 31.7	115.5 ± 39.8	99.3 ± 2.1*
	26	200.5 ± 31.4	151.8 ± 15.4	149.5 ± 17.9	118.5 ± 32.7**	132.0 ± 12.5	215.0 ± 64.2
	52	249.8 ± 109.2	163.0 ± 13.1	138.5 ± 10.9*	122.3 ± 38.1*	121.7 ± 30.2*	265.7 ± 60.1

ALAT: alanine aminotransferase; AP: alkaline phosphatase; GGT: gamma-glutamyltransferase; U: units; *: $P < 0.05$; **: $P < 0.01$

[a] Recovery group (individuals dosed through week 12); statistics were not run on males of the recovery group because there were two males only.

Source: Luckett (2010)

Table 17. Summary of liver histopathology in a 1-year oral toxicity study in dogs

	Males						Females					
	0 ppm	40 ppm	200 ppm	1 000 ppm	5 000 ppm	Recovery	0 ppm	40 ppm	200 ppm	1 000 ppm	5 000 ppm	Recovery
No. of dogs examined	4	4	4	4	5[a]	2	4	4	4	4	5[a]	2
Canine juvenile polyarteritis												
Systemic	0	0	0	0	2[a]	0	0	0	0	0	1[a]	0
Single organ	1	0	0	3	1	0	1	1	1	0	0	0
Liver												
Cholestasis	0	0	0	1	3	0	0	0	0	0	1	0
Degeneration, hepatocellular	0	0	0	2	4	0	0	0	0	4	3	0
Inflammation, chronic active	0	0	0	0	4[a]	0	0	0	0	2	3	0
Gallbladder												
Hyperplasia, mucosal	0	0	0	0	3	0	0	0	0	0	1	0

[a] Including one male (No. 136) and one female (No. 140) euthanized due to canine juvenile polyarteritis at days 80 and 176, respectively.

Source: Luckett (2010)

The NOAEL in the 1-year oral toxicity study in dogs was 40 ppm (equal to 0.96 mg/kg bw per day), based on marginal increases in AP levels without histopathological change in the liver in both sexes, increased liver weights in males and decreased cholesterol in females at 200 ppm (equal to 5.67 mg/kg bw per day) (Luckett, 2010).

(b) Dermal application

Rats

In a 28-day dermal study, cyantraniliprole (purity 94.5%; batch no. HGW86-230) was applied to the shaved, intact dorsal skin of male and female Hsd:Sprague-Dawley® rats (10 of each sex per group). The test substance was moistened with distilled water and applied for 29 daily (consecutive) applications. The rats were exposed to the test substance for 6 hours/day. Exposure doses were 0, 100, 300 and 1000 mg/kg bw per day. Parameters evaluated included body weight, body weight gain, feed consumption, feed efficiency, clinical signs, dermal irritation, clinical pathology, ophthalmology, organ weights, and gross and microscopic pathology.

There were no effects on mortality, body weights, nutritional parameters, ophthalmology, organ weights, or clinical or anatomic pathology parameters. Slight or mild erythema was observed at the dose site of male and female rats in all treated groups; the occurrence was less frequent in females than in males. One male at 100 mg/kg bw per day exhibited oedema, and one female in the control group exhibited slight erythema but no oedema at the dose site. Although the dermal irritation was considered to be test substance related, it was not considered adverse.

The NOAEL for dermal toxicity in rats was 1000 mg/kg bw per day, the highest dose tested (Lowe, 2009).

2.3 Long-term studies of toxicity and carcinogenicity

Mice

In an 18-month carcinogenicity feeding study, cyantraniliprole (purity 97%; batch no. HGW86-412) was administered to male and female Crl:CD1®(ICR) mice (60 of each sex per group) at a dietary concentration of 0, 20, 150, 1000 or 7000 ppm (equal to 0, 2.0, 15.5, 104 and 769 mg/kg bw per day for males and 0, 2.4, 18.6, 131 and 904 mg/kg bw per day for females, respectively). Parameters evaluated included body weight, body weight gain, feed consumption, feed efficiency, clinical signs, clinical pathology, ophthalmology, organ weights, gross pathology and histopathology.

No treatment-related effects were observed on survival, nutritional parameters, clinical or ophthalmological signs of toxicity or clinical pathology parameters. Body weight gain was depressed in males at 7000 ppm. Thyroid weight (relative to body weight) was increased in males at 7000 ppm, although corresponding morphological findings were not observed. Increases in liver weight in both sexes at 1000 ppm and above were correlated with an increased incidence of hepatocellular hypertrophy at the same concentrations. Hepatocellular hypertrophy in the absence of hepatotoxicity was not considered to be adverse. There were no increases in tumour incidence or in any treatment-related non-neoplastic microscopic pathology.

The NOAEL for toxicity in mice was 1000 ppm (equal to 104 mg/kg bw per day), based on a decrease in body weight gain and increased thyroid weight in males at 7000 ppm (equal to 769 mg/kg bw per day). The NOAEL for carcinogenicity in mice was 7000 ppm (equal to 769 mg/kg bw per day), the highest dose tested (Craig, 2011b).

Rats

In a 2-year chronic toxicity and carcinogenicity feeding study, cyantraniliprole (purity 97%; lot no. 9182-7; batch no. HGW86-412) was administered to male and female Crl:CD®(SD) rats (70 of each sex per group) at a dietary concentration of 0, 20, 200, 2000 or 20 000 ppm (equal to 0, 0.8, 8.3, 84.8 and 907 mg/kg bw per day for males and 0, 1.1, 10.5, 107 and 1161 mg/kg bw per day for females, respectively). Ten rats per group were examined after approximately 1 year on study, and all surviving rats were euthanized after approximately 2 years on study. Parameters evaluated included

body weight, body weight gain, feed consumption, feed efficiency, clinical signs, clinical pathology, ophthalmology, organ weights, and gross and microscopic pathology.

There were no treatment-related effects on survival or on clinical or ophthalmological observations, and there was no increase in the incidence of observed masses. At 20 000 ppm, there were small reductions in body weight, body weight gain and feed efficiency in both sexes, primarily over the first 1–1.5 years. In addition, there was a slight decrease in body weight gain (90% compared with the control value) in females at 2000 ppm during the first 3 months. Three male rats at 20 000 ppm had increases in liver enzyme levels at 12 months. In blood biochemistry at interim kill, increases in GGT, ASAT, ALAT and SDH compared with control values were detected in males at 20 000 ppm. Liver weights were increased at 2000 ppm (12 months) and 20 000 ppm (12 and 24 months) in males and at 2000 ppm and above (12 months only) in females. Test substance–related histopathological changes were observed in liver of males after 12 months and in liver and kidney of females (Table 18). Hepatocellular hypertrophy in the centrilobular area was observed in both sexes at 2000 ppm and above at 12 and 24 months. Increases in focus of cellular alterations (eosinophilic, basophilic or clear) were noted in males at 2000 ppm (clear only) and 20 000 ppm. Incidence of focal vacuolation of hepatocytes was increased in males at 2000 ppm and higher. In females, the incidence of chronic progressive nephropathy was slightly increased at 20 000 ppm; however, the relationship of this finding with the treatment was not clear due to the common occurrence of this disease in aged rats. Erosion/ulcer or epithelial hyperplasia in the non-glandular stomach was slightly increased in females at 20 000 ppm; however, the relationship of these findings with treatment was not determined. No increased incidences of tumours were detected in any treated groups.

Table 18. Summary of non-neoplastic histopathology in a combined oral toxicity and carcinogenicity study in rats

	0 ppm	20 ppm	200 ppm	2 000 ppm	20 000 ppm
Males					
12 months					
Liver: Hepatocyte hypertrophy, centrilobular	0/10	0/10	0/10	5/10*	8/10*
24 months					
Liver					
- Hepatocyte hypertrophy, centrilobular	0/60	0/60	0/60	0/60	6/60#*
- Focus of cellular alteration, clear	0/60	0/60	1/60	4/60#	5/60#
- Focus of cellular alteration, eosinophilic	20/60	15/60	12/60	29/60	32/60#*
- Focus of cellular alteration, basophilic	10/60	9/60	9/60	12/60	20/60#
- Vacuolation of hepatocyte, focal	4/60	4/60	7/60	13/60#*	13/60#*
Females					
12 months					
Liver					
- Hepatocyte hypertrophy, centrilobular	0/10	0/10	0/10	4/10	6/10*
- Hepatocyte hypertrophy, panlobular	0/10	0/10	0/10	0/10	1/10
24 months					
Liver					
- Hepatocyte hypertrophy, centrilobular	0/60	0/60	0/60	9/60#*	22/60#*
Kidney					
- Nephropathy, chronic progressive	34/60	37/60	32/60	44/60	45/60#
Stomach, non-glandular					
- Erosion/ulcer	0/60	1/60	0/60	0/60	5/60
- Epithelial hyperplasia	1/60	2/60	2/60	3/60	6/60

#: $P < 0.05$ (Cochran-Armitage trend test); *: $P < 0.05$ (Fisher's exact test)
Source: Craig (2011a)

The NOAEL in the 2-year oral toxicity study in rats was 200 ppm (equal to 8.3 mg/kg bw per day), based on increased incidences of foci of cellular alteration in the liver in males and hepatocellular vacuolation in both sexes and slight depression of body weights in females at 2000 ppm (equal to 84.8 mg/kg bw per day). The NOAEL for carcinogenicity in rats was 20 000 ppm (equal to 907 mg/kg bw per day), the highest dose tested (Craig, 2011a).

2.4 Genotoxicity

Cyantraniliprole was tested for genetic toxicity and mutagenic potential in a battery of in vitro and in vivo studies (Table 19). These were in vitro tests for gene mutation induction in *Salmonella typhimurium* tester strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* tester strain WP2 *uvr*A and mammalian cells (Chinese hamster ovary fibroblasts), for clastogenicity in cultures of human lymphocytes and for micronucleus induction in bone marrow cells of mice treated in vivo orally by gavage on a single occasion and sampled 24 and 48 hours later. No activity was observed in any of these assays that was considered indicative of adverse effects on genetic material. A statistically significant increase in micronuclei observed at 2000 mg/kg bw in male mice at the 48-hour sampling time (3/2000 cells compared with 0/2000 cells in the controls) was considered spurious by the authors, because it was within the laboratory's historical control range.

Table 19. Summary of genotoxicity studies with cyantraniliprole

Type of study	Batch no.	Test system	Concentration range tested	Result	Reference
In vitro bacterial mutagenicity (Ames)	HGW86-412	*Salmonella typhimurium* and *Escherichia coli*	1.5–5 000 µg/plate (±S9)	Negative	Wagner & VanDyke (2009a)
In vitro chromosomal aberration (clastogenicity)	HGW86-412	Human lymphocytes	125–3 500 µg/mL (4 h, ±S9) 15.7–1 500 µg/mL (20 h, −S9)	Negative	Gudi & Rao (2009)
In vitro mammalian cell mutagenicity (CHO/HPRT)	HGW86-412	CHO cells	10–1 000 µg/mL (±S9)	Negative	Stankowski (2011)
In vivo micronucleus	HGW86-412	Mouse bone marrow	Male and female: 500–2 000 mg/kg bw	Negative	Donner (2011)
In vivo micronucleus	HGW86-014	Rat peripheral blood	Male and female: 2 000 mg/kg bw	Negative	Nabb (2010)

CHO: Chinese hamster ovary; HPRT: hypoxanthine–guanine phosphoribosyltransferase; S9: 9000 × *g* supernatant fraction of rat liver homogenate

2.5 Reproductive and developmental toxicity

(a) Multigeneration reproduction study

Rats

In a two-generation reproduction study, cyantraniliprole (purity 94.5%; batch no. HGW86-230) was administered to male and female Crl:CD®(SD) rats (150 of each sex per group for both the P and F_1 generations) at a dietary concentration of 0, 20, 200, 2000 or 20 000 ppm. The P rats were bred within their treatment groups to produce F_1 litters after 70 days on test. The F_1 rats were bred within their respective treatment groups to produce F_2 litters at least 86 days after weaning.

Parameters evaluated included body weight, body weight gain, feed consumption, feed efficiency, clinical signs, reproductive indices, litter and pup parameters, estrous cycle, sperm parameters, organ weights, and gross and microscopic pathology.

The achieved doses for each parental generation are shown in Table 20.

Table 20. The achieved doses for each parental generation in a reproductive toxicity study in rats

Generation	Feeding period (days)	Achieved doses (mg/kg bw per day)			
		20 ppm	200 ppm	2 000 ppm	20 000 ppm
P males	Premating 1–70	1.1	11.0	110	1 125
P females	Premating 1–70	1.4	13.9	136	1 344
	Gestation 0–21	1.4	13.3	135	1 353
	Lactation 1–15	2.7	27.0	283	2 782
F_1 males	Premating 1–70	1.4	14.6	151	1 583
F_1 females	Premating 1–70	1.9	20.1	203	2 125
	Gestation 0–21	1.4	14.7	149	1 518
	Lactation 1–15	2.7	27.4	277	2 769

Source: Barnett (2011)

In the P and F_1 parental generations, no treatment-related clinical signs were detected. Slight reductions in body weight gain and nutritional parameters in parental animals at 20 000 ppm in both sexes were detected throughout the study, including premating, gestation and lactation periods. There was no adverse treatment-related reproductive toxicity at any level tested in this study up to and including 20 000 ppm. Increases in liver weight accompanied by hepatocellular hypertrophy were observed at 2000 and 20 000 ppm in both sexes of the P and F_1 generations (Table 21). Thyroid weights were marginally increased at 2000 and 20 000 ppm in males and at 200, 2000 and 20 000 ppm in females in the P and F_1 generations. Follicular cell hypertrophy was observed in the thyroid in the P and F_1 generations of both sexes at 2000 and 20 000 ppm. The liver hypertrophy with the change in thyroid metabolism at 2000 ppm and above was considered adverse. The minimal increases in thyroid weight with no accompanying morphological changes or liver effects in P and F_1 females at 200 ppm were not toxicologically significant (Table 22).

Thymus weights were decreased in P females at 2000 and 20 000 ppm and in F_1 females at 20 000 ppm (Table 21). Microscopically, atrophy of thymus was observed in P females at these doses (1/29, 2/29, 2/30, 6/29 and 10/29 at 0, 20, 200, 2000 and 20 000 ppm, respectively), but not in F_1 females. Vacuoles in the fascicular zone cells of the adrenal cortex accompanied by slightly increased adrenal weights were similar to changes observed in the 90-day oral toxicity studies in mice and rats (see section 2.2). The vacuolation was not considered to be adverse because there was no functional or ultrastructural damage to the adrenal cortex (see section 2.6). Therefore, the minimal to slight adrenal change was considered to be treatment related but not adverse and within normal physiological limits.

Data on reproductive toxicity, including estrous cyclicity and sperm analyses, mating behaviour, conception and fertility, parturition, gestation length, lactation, weaning and the development of offspring, except onset of puberty, were similar across all groups tested. In F_1 female rats, the average date on which vaginal opening occurred was delayed (2.2 days) at 20 000 ppm compared with the control rats. The mean value of 35.1 days at 20 000 ppm was within the testing laboratory's historical control range (mean 32.7 days; range 30.1–35.3 days). Further, the delay was not statistically significant when adjusted for body weight. The delay in time to vaginal opening was considered to be due to overall lower body weight in the F_1 generation females at weaning and persistence of the lower body weight after weaning. Therefore, the delay of puberty in F_1 females at

20 000 ppm was considered to be a secondary effect of reduced body weight at this dose. In addition, an increase in the number of F_1 pups observed with mild dehydration was noted at 20 000 ppm.

Table 21. Summary of organ weights in the P and F_1 parental generations in a reproductive toxicity study in rats

	0 ppm	20 ppm	200 ppm	2 000 ppm	20 000 ppm
Liver weight					
P males					
Absolute weight (g)	23.5	23.9	23.4	25.2	26.1*
Relative weight[a] (%)	3.6	3.6	3.6	4.0*	4.2*
P females					
Absolute weight (g)	15.8	15.5	15.6	18.8*	21.3*
Relative weight[a] (%)	4.5	4.5	4.6	5.5*	6.2*
F_1 males					
Absolute weight (g)	23.8	25.2	24.4	25. 5	25.3
Relative weight[a] (%)	3.9	3.9	3.9	4.2*	4.6*
F_1 females					
Absolute weight (g)	16.0	15.9	16.6	19.1**	21.2**
Relative weight[a] (%)	4.5	4.4	4.7	5.5**	6.2**
Thyroid/parathyroid weight					
P males					
Absolute weight (g)	0.05	0.05	0.04	0.05	0.06*
Relative weight[a] (% × 1000)	7.424	6.980	6.653*	7.588	8.892*
P females					
Absolute weight (g)	0.04	0.04	0.05*	0.05*	0.05*
Relative weight[a] (% × 1000)	10.97	10.85	13.61*	15.01*	13.80*
F_1 males					
Absolute weight (g)	0.04	0.05	0.05	0.05	0.05*
Relative weight[a] (% × 1000)	7.088	7.139	7.509	7.563	8.827*
F_1 females					
Absolute weight (g)	0.03	0.03	0.04*	0.04*	0.04*
Relative weight[a] (% × 1000)	9.3	9.0	10.4*	10.5*	11.5*
Adrenal (left adrenal)					
F_1 females					
Absolute weight (g)	0.04	0.04	0.05*	0.05*	0.05*
Relative weight[a] (% × 1000)	11.3	11.9	13.1*	14.4*	14.4*
Thymus					
P females					
Absolute weight (g)	0.18	0.17	0.18	0.14*	0.13*
Relative weight[a] (%)	0.05	0.05	0.05	0.04	0.04
F_1 females					
Absolute weight (g)	0.26	0.27	0.28	0.22	0.17*
Relative weight[a] (%)	0.07	0.08	0.08	0.06	0.06

*: $P < 0.05$; **: $P < 0.01$

[a] Relative to body weight.

Source: Barnett (2011)

Table 22. Selected histopathological changes in P and F_1 generations in a reproductive toxicity study in rats

	Males					Females				
	0 ppm	20 ppm	200 ppm	2 000 ppm	20 000 ppm	0 ppm	20 ppm	200 ppm	2 000 ppm	20 000 ppm
P parent										
Liver: Centrilobular hypertrophy of hepatocyte	0/25	0/30	0/29	28/30	29/29	1/29	0/29	0/30	27/29	30/30
Thyroid: Follicular cell hypertrophy	4/30	2/30	3/29	19/30	28/30	3/29	5/29	5/29	16/29	25/30
F_1 parent										
Liver: Centrilobular hypertrophy of hepatocyte	0/30	0/30	0/29	29/30	30/30	1/30	0/29	0/29	23/30	28/30
Thyroid: Follicular cell hypertrophy	1/30	2/30	7/29	18/30	27/30	1/30	1/29	5/29	16/30	24/30

Source: Barnett (2011)

In pups, lower body weights observed at 20 000 ppm in the F_1 generation on postnatal days (PNDs) 15 and 22 (14% lower at PND 22) and in the F_2 generation during lactation (15% lower at PND 22) were considered to be treatment related (Table 23). In the 200 and 2000 ppm groups, lower body weights detected in F_2 generation pups during the lactation period were minimal (6% and 7% lower at PND 22, respectively) (Table 23). Therefore, the lower body weights at 200 and 2000 ppm were not considered to be adverse. Weights of thymus, spleen or adrenal were decreased at 2000 or 20 000 ppm, but the decreases were not accompanied by histopathological changes (Table 24). The decreases at those doses were considered to correspond to the lower body weights at those doses and not to a direct effect on the thymus and spleen. Therefore, the lower weights of thymus and spleen were not considered to be adverse. Lower brain weights at 200 ppm and above in the F_2 generation were very minimal (within 6%), and therefore the reduced weights were not considered to be toxicologically significant.

Table 23. Body weight changes in F_1 and F_2 pups in the reproductive toxicity study in rats

	0 ppm	20 ppm	200 ppm	2 000 ppm	20 000 ppm
F_1 generation					
At birth	7.0 ± 0.6	7.1 ± 1.2	6.8 ± 0.6	6.7 ± 0.7	7.0 ± 0.8
Day 5 post-culling	10.7 ± 1.6	12.0 ± 2.7	10.8 ± 1.1	10.3 ± 1.3	10.9 ± 1.3
Day 8	16.7 ± 2.9	18.5 ± 3.8	16.8 ± 1.6	16.7 ± 2.6	16.6 ± 2.2
Day 15	34.7 ± 5.4	35.7 ± 5.0	32.8 ± 4.6	33.7 ± 4.7	31.0 ± 6.2*
Day 22	56.7 ± 7.9	57.5 ± 6.4	53.4 ± 6.5	53.6 ± 6.6	49.0 ± 7.8**
F_2 generation					
At birth	6.8 ± 0	6.5 ± 0.6	6.3 ± 0.7*	6.3 ± 0.7*	6.1 ± 0.5*
Day 5 post-culling	11.0 ± 1.6	10.7 ± 1.4	10.3 ± 1.4	9.9 ± 1.4*	9.6 ± 1.0*
Day 8	18.2 ± 2.0	17.8 ± 2.3	16.7 ± 1.7*	16.7 ± 2.0*	15.5 ± 1.6*
Day 15	37.5 ± 2.9	37.1 ± 3.4	35.0 ± 2.4*	34.7 ± 3.0*	32.4 ± 2.5*
Day 22	59.7 ± 4.9	57.6 ± 5.2	55.9 ± 4.3*	55.4 ± 4.5*	50.7 ± 4.4*

*: $P < 0.05$
Source: Barnett (2011)

Table 24. Summary of organ weights of F_1 and F_2 pups in the reproductive toxicity study in rats

	F_1 and F_2 pup organ weights (g/litter)				
	0 ppm	20 ppm	200 ppm	2 000 ppm	20 000 ppm
Thymus					
F_1 males	0.27 ± 0.06	0.28 ± 0.05	0.25 ± 0.07	0.24 ± 0.05	0.21 ± 0.06*
F_1 females	0.27 ± 0.05	0.28 ± 0.04	0.24 ± 0.04	0.24 ± 0.06	0.21 ± 0.06*
F_2 males	0.26 ± 0.05	0.25 ± 0.05	0.25 ± 0.04	0.22 ± 0.05*	0.21 ± 0.03*
F_2 females	0.27 ± 0.05	0.26 ± 0.05	0.26 ± 0.04	0.23 ± 0.05*	0.20 ± 0.03*
Spleen					
F_1 males	0.29 ± 0.07	0.30 ± 0.05	0.27 ± 0.06	0.25 ± 0.06	0.26 ± 0.18
F_1 females	0.30 ± 0.07	0.30 ± 0.07	0.27 ± 0.05	0.26 ± 0.06	0.22 ± 0.07*
F_2 males	0.30 ± 0.07	0.27 ± 0.06*	0.28 ± 0.06	0.26 ± 0.05*	0.21 ± 0.04*
F_2 females	0.30 ± 0.06	0.26 ± 0.06*	0.28 ± 0.04	0.26 ± 0.05*	0.22 ± 0.04*
Brain					
F_1 males	1.60 ± 0.10	1.63 ± 0.09	1.59 ± 0.11	1.61 ± 0.11	1.55 ± 0.14
F_1 females	1.54 ± 0.11	1.60 ± 0.09	1.54 ± 0.09	1.55 ± 0.08	1.47 ± 0.14*
F_2 males	1.64 ± 0.08	1.62 ± 0.07	1.60 ± 0.09*	1.59 ± 0.07*	1.54 ± 0.06*
F_2 females	1.60 ± 0.07	1.58 ± 0.07	1.56 ± 0.06*	1.55 ± 0.07*	1.51 ± 0.07*
Adrenal					
F_2 males	0.019 ± 0.005	0.019 ± 0.004	0.018 ± 0.003	0.019 ± 0.004	0.017 ± 0.003
F_2 females	0.020 ± 0.005	0.018 ± 0.003	0.018 ± 0.002	0.017 ± 0.004	0.016 ± 0.004*

*: $P < 0.05$
Source: Barnett (2011)

In the multigeneration reproduction study in rats, the NOAEL for parental toxicity was 200 ppm (equal to 11.0 mg/kg bw per day), based on hepatocellular hypertrophy and thyroid follicular cell hypertrophy in both sexes at 2000 ppm (equal to 110 mg/kg bw per day) in the P generation. The NOAEL for reproductive toxicity was 20 000 ppm (equal to 1344 mg/kg bw per day), based on no effects on reproductive indicators at the highest dose tested. The NOAEL for offspring toxicity was 2000 ppm (equal to 280 mg/kg bw per day, the mean dose for P and F_1 parental females during lactation), based on lower body weights of F_1 and F_2 generation pups at 20 000 ppm (equal to 2776 mg/kg bw per day, the mean dose for P and F_1 parental females during lactation) (Barnett, 2011).

(b) Developmental toxicity

Rats

In a developmental toxicity study, cyantraniliprole (purity 94.5%; batch no. HGW86-230) was administered by oral gavage to time-mated Crl:CD®(SD) female rats (22 per dose group) on gestation days 6–20. Gavage doses in 0.5% aqueous methyl cellulose were 0, 20, 100, 300 and 1000 mg/kg bw per day. The dosing volume was 5 mL/kg bw. Parameters evaluated in dams were body weight, body weight gain (absolute and adjusted for the products of conception), feed consumption, survival, clinical signs, reproductive outcomes and gross pathology. Parameters evaluated in fetuses were body weight, incidences of dead fetuses and/or fetal resorptions, and incidences of external, visceral, head and skeletal malformations and variations.

No test substance–related effects on maternal clinical observations, body weight, body weight gain, feed consumption or gross postmortem observations were observed at any dose level. Unscheduled mortality did not occur. The mean numbers of corpora lutea, implantation sites, resorptions and live fetuses, fetal weight and sex ratio were comparable across all groups. There were no test substance–related fetal external, visceral or skeletal malformations or variations.

The NOAELs for both maternal and embryo/fetal toxicity in rats were 1000 mg/kg bw per day, the highest dose tested (Munley, 2009a).

Rabbits

In a developmental toxicity study, cyantraniliprole (purity 94.5%; batch no. HGW86-230) was administered by oral gavage to time-mated Hra:(NZW)SPF female rabbits (22 per group) on gestation days 7–28. Gavage doses in 0.5% aqueous methyl cellulose were 0, 25, 100, 250 and 500 mg/kg bw per day. The dosing volume was 5 mL/kg bw. Parameters evaluated in dams were body weight, body weight gain (absolute and adjusted for the products of conception), feed consumption, survival, clinical signs, reproductive outcomes and gross pathology. Parameters evaluated in fetuses were body weight, incidences of dead fetuses and/or fetal resorptions, and incidences of external, visceral and skeletal malformations and variations.

Evidence of maternal toxicity was observed at 100 mg/kg bw per day and above and included increased clinical signs of toxicity, including diarrhoea, and lower body weights and feed consumption. Maternal toxicity was sufficiently severe to result in the early euthanasia of two does at 100 mg/kg bw per day. At 250 and 500 mg/kg bw per day, abortions in late gestation and/or deliveries on the day of scheduled termination occurred in three does at 500 mg/kg bw per day and four does at 250 mg/kg bw per day. The abortions/deliveries were considered to be secondary effects of the adverse maternal toxicity that was observed at these doses. Treatment-related effects on offspring were limited to test substance–related reductions in mean fetal weight at 250 and 500 mg/kg bw per day. The lower fetal weight was considered to be related to the severe decrease in maternal body weight gain at 250 mg/kg bw per day and above.

The NOAEL for maternal toxicity in rabbits was 25 mg/kg bw per day, based on mortality, increased clinical signs of toxicity, including diarrhoea, and lower body weights and feed consumption at 100 mg/kg bw per day. The NOAEL for embryo and fetal toxicity in rabbits was 100 mg/kg bw per day, based on reductions in fetal weight at 250 mg/kg bw per day (Munley, 2009b).

2.6 Special studies

(a) Neurotoxiciy

Acute oral neurotoxicity study in rats

In an acute neurotoxicity study, cyantraniliprole (purity 93.4%; batch no. HGW86-141) was administered to male and female Crl:CD®(SD)IGS BR rats (12 of each sex per dose) by single-dose oral gavage in polyethylene glycol (PEG 400). Doses were 0, 250, 1000 and 2000 mg/kg bw. The dosing volume was 4 mL/kg bw. A neurobehavioural test battery, consisting of motor activity and functional observational battery assessments, was conducted on all study rats prior to dosing, approximately 2 hours after dosing (day 1) and on days 8 and 15. Other parameters evaluated included body weight, body weight gain and clinical signs. On test day 17, six rats of each sex per group were perfused in situ with fixative. A microscopic neuropathological evaluation of the peripheral and central nervous systems and selected muscle tissues from control and high-dose rats was conducted.

There were no treatment-related changes in body weight, body weight gain, feed consumption, feed efficiency, mortality, clinical observations, forelimb or hindlimb grip strength, hindlimb foot splay, body temperature, rearing, duration or number of movements, or any of the other behavioural parameters evaluated in the functional observational battery in either males or females administered any dose of the test substance. In addition, there were no gross or microscopic changes related to the treatment in the nervous system tissues.

The NOAEL for acute neurotoxicity in rats was 2000 mg/kg bw, the highest dose tested (Malley, 2006).

Ninety-day neurotoxicity study in rats

In a 90-day neurotoxicity feeding study, cyantraniliprole (purity 94.5%; batch no. HGW86-230) was administered to male and female Crl:CD®(SD) rats (12 of each sex per group) in the diet at a concentration of 0, 200, 2000 or 20 000 ppm (equal to 0, 11.4, 115 and 1195 mg/kg bw per day for males and 0, 14.0, 137 and 1404 mg/kg bw per day for females, respectively). Parameters evaluated included body weight, body weight gain, feed consumption, feed efficiency, clinical signs and gross pathology. A neurobehavioural test battery consisting of motor activity and functional observational battery assessments was conducted on 12 rats of each sex per group prior to the 1st day of dietary administration and during weeks 4, 8 and 13. On test days 92 and 93, six rats of each sex per group were perfused in situ with fixative. The peripheral and central nervous systems and selected muscle tissues from control and high-concentration rats (six rats of each sex per group) were prepared for histopathological evaluation to detect neurotoxicity.

There were no test substance–related effects on body weight, body weight gain, feed consumption, feed efficiency, clinical signs of toxicity, survival, neurobehavioural parameters, or gross and microscopic morphology of the nervous system in either male or female rats administered up to 20 000 ppm of test substance in the diet.

The NOAEL for neurotoxicity and systemic toxicity in rats was 20 000 ppm (equal to 1195 mg/kg bw per day), the highest dose tested, based on the absence of adverse effects of the test substance at this dose (Mukerji, 2009).

(b) Immunotoxicity

Twenty-eight-day dietary immunotoxicity study in mice

In a 28-day immunotoxicity feeding study, cyantraniliprole (purity 94.5%; batch no. HGW86-0603-1) was administered to male and female Crl:CD1®(ICR) mice (10 of each sex per group) at a concentration of 0, 20, 150, 1000 or 7000 ppm (equal to 0, 3.0, 23, 154 and 1065 mg/kg bw per day for males and 0, 4.1, 32, 224 and 1386 mg/kg bw per day for females, respectively). Body weights, feed consumption measurements and clinical observations were recorded during the in-life period. Prior to sacrifice, the immune system was stimulated by injecting sheep red blood cells (sRBCs) on test day 23 or 24, and blood samples were collected from each mouse on test day 28 or 29. The serum samples were assayed for their concentrations of sRBC-specific immunoglobulin M (IgM) antibodies to provide a quantitative assessment of humoral immune response. Serum from animals similarly challenged with a positive control immunosuppressive agent was analysed concurrently to provide confirmation that the assay performance was acceptable for detection of immunosuppression. At sacrifice, each animal was examined grossly, and selected organs were weighed (brain, spleen and thymus).

There were no adverse effects on body weight or nutritional parameters in male or female mice at any dose level. No clinical signs of systemic toxicity were observed. No adverse effects were observed on gross pathology, absolute and relative brain, spleen and thymus weights, or humoral immune response.

The NOAEL for immunotoxicity in mice was 7000 ppm (equal to 1065 mg/kg bw per day), the highest concentration tested (Hoban, 2011).

Twenty-eight-day dietary immunotoxicity study in rats

In a 28-day immunotoxicity feeding study, cyantraniliprole (purity 94.5%; batch no. HGW86-0603-1) was administered to male and female Crl:CD®(SD) rats (10 of each sex per concentration) at a concentration of 0, 20, 200, 2000 or 20 000 ppm (equal to 0, 1.7, 17, 166 and 1699 mg/kg bw per day for males and 0, 1.8, 18, 172 and 1703 mg/kg bw per day for females, respectively). Body weights, feed consumption measurements and clinical observations were recorded during the in-life period. Prior to sacrifice, the immune system was stimulated by injecting sRBCs on test day 22 for males or 23 for females, and blood samples were collected from each rat on test day 28 for males or 29 for females. The serum samples were assayed for their concentration of sRBC-specific IgM

antibodies to provide a quantitative assessment of humoral immune response. Serum from animals similarly challenged with a positive control immunosuppressive agent were analysed concurrently to provide confirmation that the assay performance was acceptable for detection of immunosuppression. At sacrifice, each animal was examined grossly, and selected organs were weighed (brain, spleen and thymus).

There were no adverse effects on body weight or nutritional parameters in male or female rats at any dose level. No clinical signs of systemic toxicity were observed. No adverse effects were observed on gross pathology, absolute and relative brain, spleen and thymus weights, or humoral immune response.

The NOAEL for immunotoxicity in rats was 20 000 ppm (equal to 1699 mg/kg bw per day), the highest concentration tested (Hoban, 2009b).

(c) Mechanistic study of thyroid effects

In vitro study of thyroid peroxidase inhibition

Thyroid peroxidase catalyses the first two steps in thyroid hormone synthesis, oxidation of iodide to iodine and the iodination of tyrosine residues on thyroglobulin. Severe inhibition of thyroid peroxidase affects the homeostasis of the hypothalamic–pituitary–thyroid axis. The objective of this study was to evaluate the ability of cyantraniliprole (purity 94.5%; batch no. HGW86-230) to inhibit thyroid peroxidase activity in vitro using thyroid preparations from the Yucatan pig (microswine). Cyantraniliprole concentrations ranged from 2 to 400 µmol/L, the maximum concentration being the limit of solubility in the assay system. Propylthiouracil (PTU), a substance known to inhibit thyroid peroxidase, was used as a positive control to verify test system performance. The concentration that caused a 50% reduction in enzyme activity (IC_{50}) for PTU was 7.3 µmol/L. Cyantraniliprole did not cause inhibition of thyroid peroxidase at any concentration tested when using guaiacol as the substrate; therefore, it was not possible to determine an IC_{50} value for cyantraniliprole. Under the conditions of this in vitro study, cyantraniliprole at concentrations up to 400 µmol/L did not inhibit thyroid peroxidase activity (Snajdr, 2010).

In vivo study of thyroid changes

A study was performed to evaluate potential mechanisms of thyroid gland changes following exposure of rats to cyantraniliprole. Two groups of young adult female Crl:CD®(SD) rats (15 per group) were fed control diet or diet containing 20 000 ppm (equal to 1903 mg/kg bw per day) cyantraniliprole (purity 94.5%; batch no. HGW86-230) for 29 days. Body weights, feed consumption and feed efficiency were evaluated weekly, and clinical observations were evaluated daily. Thyroid-related end-points (hormone measurements, anatomic pathology, organ weights and hepatic biochemistry) were evaluated. Females were used, as this was the sex more sensitive to effects on these parameters in previous studies.

No effects on mean feed consumption, mortality or clinical observations were observed. Female rats fed 20 000 ppm cyantraniliprole in the diet had lower body weight, body weight gain and feed efficiency compared with controls. Increased liver and thyroid weights and minimal thyroid follicular cell hypertrophy were induced by 29 days of treatment. These effects were associated with increased hepatic UDPGT activity and alterations in thyroid hormone homeostasis, including reduced serum T_4 concentration and increased TSH levels. These effects support increased clearance of T_4 due to increased induction of UDPGT in the liver, leading to lower T_4 levels, activated negative feedback on the hypothalamus and pituitary and subsequent increased TSH stimulation of the thyroid gland, as the predominant mechanism of the observed thyroid follicular cell hypertrophy. A reduction in hepatic microsomal 5′-deiodinase activity was also observed, but there were no effects on T_3 or reverse T_3 (rT_3) levels (MacKenzie, 2010b).

Based on the mechanistic studies described above, the effect of cyantraniliprole on thyroid follicles is considered to be secondary due to excess excretion of thyroid hormones from blood by

treatment-related induction of drug metabolism enzymes in the liver (Hill et al., 1989; Capen, 1997), but the biological significance of the reduction in hepatic microsomal metabolism enzymes was unclear due to the absence of any difference in T_3 or rT_3 levels.

It is well established that rats are more sensitive than dogs or primates (including humans) to thyroid hormone perturbations; the difference in sensitivity is likely due to the difference in half-life of the thyroid hormones, which is considerably shorter in the rat. This difference is due to the presence of thyroxine binding globulin in humans, which is not present in rats. Differences in plasma half-life are considered to be one of the primary reasons the rat, unlike humans, is prone to developing thyroid tumours as a result of TSH stimulation (Capen, 1997; Tucker, 1998). Therefore, this type of effect on the thyroid gland in rats is not considered relevant to human risk assessment.

(d) Mechanistic study of adrenal changes

Mice

A study was performed in mice to evaluate the impact of cyantraniliprole exposure on adrenal gland function and ultrastructure. Two groups of young adult male Crl:CD1® mice (10 per group) were fed control diet or diet containing 7000 ppm cyantraniliprole (purity 91.5%; batch no. HGW86-141) (equal to 1120 mg/kg bw per day) for 93 days. Body weights and feed consumption were evaluated weekly, and clinical observations were evaluated daily. At week 12, urine corticosterone, urine volume and creatinine were measured using an overnight collection of urine. After 93 days of exposure, mice were euthanized, and adrenal glands were weighed and collected. The right adrenal gland was evaluated microscopically from all mice. Left adrenal glands from four mice per group were used for electron microscopic examination.

No treatment-related clinical signs were detected. Body weight gain and feed intakes were decreased in the treated group. No treatment-related effects on histopathology, including ultrastructure in the adrenal cortex cells, urine analysis or hormone assays of corticosterone, were observed (MacKenzie, 2010a).

Rats

Three groups of young adult male Crl:CD®(SD) rats (10 per group) were fed control diet (groups 1 [adrenocorticotrophic hormone (ACTH)–stimulated] and 3 [non-ACTH-simulated]) or diet containing 20 000 ppm of cyantraniliprole (purity 94.5%; batch no. HGW86-230) (group 2), equal to 1230 mg/kg bw per day, for 93 days. The ACTH stimulation test is a well established clinical procedure in human and veterinary medicine that evaluates the responsiveness of the adrenal cortex to ACTH. For this test, exogenous ACTH is administered to simulate conditions of physiological stress, and the adrenal cortical response is evaluated by measuring serum glucocorticoid concentrations. In addition, body weights, feed consumption and feed efficiency were evaluated weekly, and clinical observations were evaluated daily. Adrenal end-points (urine corticosterone, adrenal response to ACTH, organ weights and pathology, including electron microscopic examination) were evaluated in males only, because males were previously observed to be more sensitive than females to these respective effects.

No adverse effects were observed on mean body weight gain or feed efficiency in male rats or on mean feed consumption, mortality or clinical observations. In radioimmunoassay, basal urinary corticosterone and ACTH-induced serum corticosterone levels were comparable to levels in the control group (Table 25). Daily dietary exposure at 20 000 ppm for 93 days resulted in increased microvesiculation of the adrenal cortex, with no evidence of cytotoxicity or degeneration. In electron microscopic examination, a minimal to mild increase in lipid vacuoles was observed in the cytoplasm of fascicular zone cells, but no effects on cellular organelles or evidence of cytotoxicity or degeneration was detected. The results indicate that the increased microvesiculation in fascicular zone cells in the adrenal cortex was not associated with changes in adrenal cortical function (MacKenzie, 2010b).

Table 25. Summary of effects on the adrenal in male rats

	0 ppm	20 000 ppm	0 ppm
No. of rats examined	10[a]	10	10
Before treatment with ACTH (day 86)			
Treatment with ACTH (12.5 µg/rat, intravenous)	No	No	No
Urine volume	6.7	9.4	NE
Urine creatinine (ng/mL × 10^4)	181	150	NE
Urine corticosterone (ng/mL)	84.2	87.3	NE
After treatment with ACTH (day 93)			
Treatment with ACTH (12.5 µg/rat, intravenous)	Yes	Yes	No
Serum corticosterone (ng/mL)	410	473	87[b]
Histopathology: Increased microvesiculation in fascicular zone cell, minimal	0	4	0

ACTH: adrenocorticotrophic hormone; NE, not examined

[a] Number of rats for measurement of serum corticosterone level was 9.

[b] No increase in serum corticosterone level after ACTH treatment in the control group.

Source: MacKenzie (2010b)

Based on the results from the above two mechanistic studies, mild increased microvesiculation of fascicular zone cells in the adrenal cortex and increased small lipid droplets in the cytoplasm in rats and mice in short-term studies and the reproductive toxicity study in rats were considered to be treatment related but not adverse and within normal physiological limits (MacKenzie, 2010a,b).

3. Studies on metabolites and/or degradates

Acute toxicity and genotoxicity studies on the metabolites and/or degradates of cyantraniliprole from high-temperature food processing (IN-JSE76 and IN-PLT97) and the degradates of cyantraniliprole in soil (IN-N5M09 and IN-F6L99) were conducted. A 28-day toxicity study on IN-JSE76 was conducted in rats.

3.1 Acute toxicity

Acute toxicity studies of cyantraniliprole metabolites and/or degradates are summarized in Table 26.

Table 26. Acute toxicity of cyantraniliprole metabolites and/or degradates

Metabolite/degradate (purity; batch no.)	Species	LD_{50} (mg/kg bw)	Clinical signs	Reference
IN-JSE76 (purity 93.8%; batch no. IN-JSE76-005)	Female SD rats (6/group)	> 5 000	No deaths or clinical signs	Oley (2009)
IN-PLT97 (purity 98.1%; batch no. E115107-77B)	Three female mice	> 5 000	No deaths or clinical signs	Carpenter (2010a)
IN-F6L99 (purity 98.6%; batch no. 004)	Three female mice treated with 2 000 mg/kg bw; one mouse treated with 175 or 550 mg/kg bw	> 2 000	No deaths or clinical signs	Finlay (2006)
IN-N5M09 (purity 99.9%; batch no. D100855-058)	Three female mice treated with 5 000 mg/kg bw	> 5 000	No deaths or clinical signs	Carpenter (2010b)

LD_{50}: median lethal concentration

3.2 Short-term studies of toxicity

In a 28-day feeding study, IN-JSE76 (purity 97.8%; batch no. IN-JSE76-005) was administered to male and female Crl:CD®(SD) rats (10 of each sex per group) at a dietary concentration of 0, 100, 400, 3000 or 20 000 ppm (equal to 0, 7, 29, 212 and 1445 mg/kg bw per day for males and 0, 8, 31, 232 and 1471 mg/kg bw per day for females, respectively). Parameters evaluated included body weight, body weight gain, feed consumption, feed efficiency, clinical signs, serum thyroid hormone levels, hepatic microsomal and peroxisomal enzymes (cytochrome P450, β-oxidation and UDPGT), gross pathology, organ weights, haematology, clinical chemistry, urine analysis and histopathology. Blood (from non-fasted animals) was collected on test days 23 (males) and 24 (females) for analysis of the concentration of IN-JSE76 and selected other metabolites in plasma.

No deaths occurred, and no clinical or ophthalmological observations were attributed to exposure to the test substance. No test substance–related effects on body weight or any nutritional parameters were observed. There were no adverse effects on any clinical pathology parameters (haematology, clinical chemistry, coagulation, urine analysis), organ weights or pathology findings related to treatment. In male rats, serum T_4 levels were statistically significantly decreased at 400, 3000 and 20 000 ppm. Although not significant, there were also corresponding increases in TSH levels at 3000 and 20 000 ppm. These changes were considered to be treatment related, but not adverse, due to the lack of corresponding organ weight and microscopic findings.

The plasma concentrations of IN-JSE76 were approximately linear with respect to dose in both male and female rats over the range of doses tested. There was no sex difference in the plasma concentration of IN-JSE76. The only targeted metabolite that had quantifiable levels in plasma was IN-K5A78, which was reported for the highest-dose group in females and in the two highest-dose groups in males.

The NOAEL for short-term toxicity of IN-JSE76 in rats was 20 000 ppm (equal to 1445 mg/kg bw per day), the highest dose tested (Anand, 2010; Mawn, 2011).

3.3 Genotoxicity

Genotoxicity studies on cyantraniliprole metabolites and/or degradates are summarized in Table 27.

4. Observations in humans

No information on medical surveillance or poisoning incidents was available.

Comments

Biochemical aspects

Cyantraniliprole is readily absorbed in rats, and the absorption is higher at 10 mg/kg bw than at 150 mg/kg bw. The majority of the absorption occurs during the first 48 hours (80% of the absorbed radioactivity), and the peak plasma concentration (C_{max}) is reached approximately 2 hours after dosing, regardless of the sex or dose level. The C_{max} and AUC values demonstrate a 2- to 3-fold greater exposure in female rats than in male rats. Following oral dosing, the majority of the dose is extensively distributed throughout the body. The half-life is shorter in male rats than in female rats (42–54 hours in males and 117–129 hours in females). The absorbed cyantraniliprole is readily and extensively metabolized, mainly by hydroxylation of methylphenyl and *N*-methyl carbon. Further metabolism of the hydroxylated metabolites includes *N*-methylation, nitrogen-to-carbon cyclization with loss of a water molecule, oxidation of alcohols to carboxylic acids, amide bridge cleavage, amine hydrolysis and *O*-glucuronidation. The bile is found to be very rich in metabolites, and most of the metabolites are found in both urine and faeces. IN-MLA84 (2-[3-bromo-1-(3-chloro-2-pyridinyl)-1*H*-pyrazol-5-yl]-1,4-dihydro-8-methyl-4-oxo-6-quinazoline carbonitrile) is the most abundant

Table 27. Summary of genotoxicity studies of cyantraniliprole metabolites and/or degradates

Metabolite (purity; batch no.)	Type of study	Test system	Concentration range tested	Result	Reference
IN-JSE76 (purity 93.8%; batch no. JSE76-005)	In vitro bacterial mutagenicity (Ames)	*Salmonella typhimurium* and *Escherichia coli*	50–5 000 µg/plate (±S9)	Negative	Wagner & VanDyke (2009b)
IN-JSE76 (purity 93.8%; batch no. JSE76-005)	In vitro chromosomal aberration (clastogenicity)	Human lymphocytes	313–2 500 µg/mL (4 h, −S9) 156–2 500 µg/mL (4 h, +S9) 156–2 000 µg/mL (20 h, −S9)	Negative	Gudi & Rao (2010)
IN-JSE76 (purity 93.8%; batch no. JSE76-005)	In vitro mammalian cell mutagenicity (CHO/HPRT)	CHO cells	100–1 500 µg/mL (±S9)	Negative	Clarke (2009)
IN-PLT97 (purity 98.1%; batch no. PLT97-003)	In vitro bacterial mutagenicity (Ames)	*S. typhimurium* and *E. coli*	50–5 000 µg/plate (±S9)	Negative	Wagner & Jois (2010)
IN-PLT97 (purity 98.1%; batch no. PLT97-003)	In vitro chromosomal aberration (clastogenicity)	Human lymphocytes	25–1 550 µg/mL (4 h, ±S9) 25–1 550 µg/mL (20 h, −S9)	Negative	Madraymootoo & Jois (2011)
IN-PLT97 (purity 98.1%; batch no. PLT97-003)	In vitro mammalian cell mutagenicity (CHO/HPRT)	CHO cells	10–150 µg/mL (±S9)	Negative	Clarke (2010)
IN-F6L99 (purity 98.6%; batch no. F6L99-004)	In vitro bacterial mutagenicity (Ames)	*S. typhimurium* and *E. coli*	33.3–5 000 µg/plate (±S9)	Negative	Wagner & Jois (2010)
IN-N5M09 (purity 99.9%; batch no. N5M09-003)	In vitro bacterial mutagenicity (Ames)	*S. typhimurium* and *E. coli*	1.5–5 000 µg/plate (±S9)	Negative	Wagner & VanDyke (2009c)

CHO: Chinese hamster ovary; HPRT: hypoxanthine–guanine phosphoribosyltransferase; S9: 9000 × *g* supernatant fraction of rat liver homogenate

analyte in the blood of rats and mice of both sexes, whereas the highest concentrations in dogs are of the parent compound, cyantraniliprole.

Toxicological data

In rats, the oral LD_{50} was greater than 5000 mg/kg bw, the dermal LD_{50} was greater than 5000 mg/kg bw and the inhalation LC_{50} was greater than 5.2 mg/L. Cyantraniliprole was not a skin irritant in rabbits, an eye irritant in rabbits or a skin sensitizer.

Liver was the interspecies target of cyantraniliprole in short- and long-term studies, although dogs appeared to be more sensitive than rats. In rodents, the thyroid was also a target organ, with adverse effects on thyroid hormone metabolism.

Short-term toxicity of cyantraniliprole was examined in mice, rats and dogs. The NOAEL in a 28-day oral toxicity study in mice was 7000 ppm (equal to 1261 mg/kg bw per day), the highest dose tested. The NOAEL in a 90-day oral toxicity study in which mice were administered cyantraniliprole in the diet at a concentration of 0, 50, 300, 1000 or 7000 ppm (equal to 0, 7.2, 47.1, 150 and 1092 mg/kg bw per day for males and 0, 9.7, 58.1, 204 and 1344 mg/kg bw per day for females,

respectively) was 1000 ppm (equal to 204 mg/kg bw per day), based on minimal necrosis in the liver at 7000 ppm (equal to 1344 mg/kg bw per day) in females.

In a 28-day oral toxicity study in which rats were administered cyantraniliprole in the diet at a concentration of 0, 600, 2000, 6000 or 20 000 ppm (equal to 0, 53, 175, 528 and 1776 mg/kg bw per day for males and 0, 62, 188, 595 and 1953 mg/kg bw per day for females, respectively), the NOAEL was 600 ppm (equal to 53 mg/kg bw per day), based on liver hypertrophy and thyroid follicular cell hypertrophy observed in both sexes at 2000 ppm (equal to 175 mg/kg bw per day). In a 90-day oral toxicity study in which rats were administered a dietary concentration of 0, 100, 400, 3000 or 20 000 ppm (equal to 0, 5.7, 22, 168 and 1147 mg/kg bw per day for males and 0, 6.9, 27, 202 and 1346 mg/kg bw per day for females, respectively), the NOAEL was 100 ppm (equal to 5.7 mg/kg bw per day), based on liver hypertrophy, decreases in thyroid hormones in both sexes and histopathological changes in the thyroid in females at 400 ppm (equal to 22 mg/kg bw per day).

Three feeding studies (28 days, 90 days and 1 year) were conducted with cyantraniliprole in dogs. A NOAEL for the 28-day oral toxicity study in dogs was not determined, based on changes in body weight, nutritional parameters and clinical chemistry indicating hepatotoxicity in both sexes at 1000 ppm (equal to 35 mg/kg bw per day), the lowest dose tested. The NOAEL for the 90-day oral toxicity study in which dogs were administered cyantraniliprole at 0, 30, 100, 1000 or 10 000 ppm (equal to 0, 0.98, 3.08, 31.9 and 281 mg/kg bw per day for males and 0, 0.97, 3.48, 34.3 and 294 mg/kg bw per day for females, respectively) was 100 ppm (equal to 3.08 mg/kg bw per day), based on increased total protein, albumin and AP levels in males at 1000 ppm (equal to 31.9 mg/kg bw per day). In a 1-year dog study utilizing concentrations of 0, 40, 200, 1000 and 5000 ppm (equal to 0, 0.96, 5.67, 27.0 and 144 mg/kg bw per day for males and 0, 1.12, 6.00, 27.1 and 133 mg/kg bw per day for females, respectively), the increased levels of AP at 40 ppm were not considered adverse in view of the absence of histopathological or functional changes at this and the next higher dose (200 ppm). Therefore, the NOAEL for the 1-year oral toxicity study in dogs was 40 ppm (equal to 0.96 mg/kg bw per day), based on marginal increases in AP levels without histopathological change in the liver in both sexes, increased liver weights in males and decreased cholesterol in females at 200 ppm (equal to 5.67 mg/kg bw per day). The Meeting concluded that the overall NOAEL for oral toxicity in dogs was 100 ppm (equal to 3.08 mg/kg bw per day), and the overall lowest-observed-adverse-effect level (LOAEL) was 200 ppm (equal to 5.67 mg/kg bw per day).

Long-term toxicity studies were conducted in mice and rats. In an 18-month carcinogenicity study in which mice were administered a dietary concentration of 0, 20, 150, 1000 or 7000 ppm (equal to 0, 2.0, 15.5, 104 and 769 mg/kg bw per day for males and 0, 2.4, 18.6, 131 and 904 mg/kg bw per day for females, respectively), the NOAEL for toxicity was 1000 ppm (equal to 104 mg/kg bw per day), based on a decrease in body weight gain and increased thyroid weight in males at 7000 ppm (equal to 769 mg/kg bw per day). No increase in neoplastic incidence was observed. The NOAEL for carcinogenicity in mice was 7000 ppm (equal to 769 mg/kg bw per day), the highest dose tested.

In a 2-year toxicity and carcinogenicity feeding study in which rats were administered cyantraniliprole in the diet at 0, 20, 200, 2000 or 20 000 ppm (equal to 0, 0.8, 8.3, 84.8 and 907 mg/kg bw per day for males and 0, 1.1, 10.5, 107 and 1161 mg/kg bw per day for females, respectively), the NOAEL for toxicity was 200 ppm (equal to 8.3 mg/kg bw per day), based on increased incidences of foci of cellular alteration in the liver in males and hepatocellular vacuolation in both sexes and slight depression of body weights in females at 2000 ppm (equal to 84.8 mg/kg bw per day). No increase in neoplastic incidence was observed, and the NOAEL for carcinogenicity in rats was 20 000 ppm (equal to 907 mg/kg bw per day), the highest dose tested.

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

Cyantraniliprole was tested for genotoxicity in vitro and in vivo in an adequate range of assays. In these assays, there was no evidence of genotoxic potential.

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

In a multigeneration reproductive toxicity study in which rats were given cyantraniliprole at a concentration of 0, 20, 200, 2000 or 20 000 ppm (in P generation: equal, respectively, to 0, 1.1, 11.0, 110 and 1125 mg/kg bw per day for males, 0, 1.4, 13.9, 136 and 1344 mg/kg bw per day for premating females, 0, 1.4, 13.3, 135 and 1353 mg/kg bw per day for females during gestation, and 0, 2.7, 27.0, 283 and 2782 mg/kg bw per day for females during lactation; in F_1 generation: equal, respectively, to 0, 1.4, 14.6, 151 and 1583 mg/kg bw per day for males, 0, 1.9, 20.1, 203 and 2125 mg/kg bw per day for premating females, 0, 1.4, 14.7, 149 and 1518 mg/kg bw per day for females during gestation and 0, 2.7, 27.4, 277 and 2769 mg/kg bw per day for females during lactation), the NOAEL for parental toxicity was 200 ppm (equal to 11.0 mg/kg bw per day), based on hepatocellular hypertrophy and thyroid follicular cell hypertrophy in both sexes at 2000 ppm (equal to 110 mg/kg bw per day) in the P generation. The NOAEL for reproductive toxicity was 20 000 ppm (equal to 1344 mg/kg bw per day), the highest dose tested. The NOAEL for offspring toxicity was 2000 ppm (equal to 280 mg/kg bw per day, mean value for P and F_1 parental females during lactation), based on lower body weights of F_1 and F_2 generation pups at 20 000 ppm (equal to 2776 mg/kg bw per day, mean value for P and F_1 parental females during lactation).

In a developmental toxicity study in rats administered a dose of 0, 20, 100, 300 or 1000 mg/kg bw per day, the NOAELs for both maternal and embryo/fetal toxicity in rats were 1000 mg/kg bw per day, the highest dose tested.

In a developmental toxicity study in rabbits administered a dose of 0, 25, 100, 250 or 500 mg/kg bw per day, the NOAEL for maternal toxicity was 25 mg/kg bw per day, based on mortality, increased clinical signs of toxicity, including diarrhoea, and lower body weights and feed consumption at 100 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 100 mg/kg bw per day, based on reductions in fetal weight at 250 mg/kg bw per day.

The Meeting concluded that cyantraniliprole is not teratogenic in rats or rabbits.

In an acute neurotoxicity study in rats, the NOAEL was 2000 mg/kg bw, the highest dose tested.

In a 90-day study of neurotoxicity in which rats were administered a dose of 0, 200, 2000 or 20 000 ppm (equal to 0, 11.4, 115 and 1195 mg/kg bw per day for males and 0, 14.0, 137 and 1404 mg/kg bw per day for females, respectively), the NOAEL was 20 000 ppm (equal to 1195 mg/kg bw per day), the highest dose tested.

The Meeting concluded that cyantraniliprole is not neurotoxic.

Immunotoxicity studies were conducted in mice and rats. In a 28-day immunotoxicity study in mice, the NOAEL was 7000 ppm (equal to 1065 mg/kg bw per day), the highest dose tested. In a 28-day immunotoxicity study in rats, the NOAEL was 20 000 ppm (equal to 1699 mg/kg bw per day), the highest dose tested.

Toxicological data on metabolites and/or degradates

Acute toxicity and genotoxicity studies of metabolites and/or degradates were conducted. 4-[[[3-Bromo-1-(3-chloro-2-pyridinyl)-1*H*-pyrazol-5-yl]carbonyl]amino]-3-methyl-5-[(methylamino)-carbonyl]benzoic acid (IN-JSE76), 2-[3-bromo-1-(3-chloro-2-pyridinyl)-1*H*-pyrazol-5-yl]-1,4-dihydro-8-methyl-4-oxo-6-quinazolinecarboxylic acid (IN-PLT97), 6-chloro-4-methyl-11-oxo-11*H*-pyrido[2,1-*b*]quinazoline-2-carbonitrile (IN-N5M09) and 3-bromo-*N*-methyl-1*H*-pyrazole-5-carboxamide (IN-F6L99) were degradates in soil. All metabolites and/or degradates exhibited low acute toxicities and no genotoxicity. The NOAEL in a 28-day toxicity study of IN-JSE76 in rats was 20 000 ppm (equal to 1445 mg/kg bw per day), the highest dose tested.

Human data

No information on medical surveillance or poisoning incidents was available.

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

Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) of 0–0.03 mg/kg bw on the basis of the overall NOAEL of 3.08 mg/kg bw per day in dog studies, based on liver effects at 5.67 mg/kg bw per day. A safety factor of 100 was applied.

The metabolites IN-N7B69, IN-MLA84, IN-MYX98 and IN-J9Z38 have been included in the residue definition. As the estimated exposure to IN-N7B69 is below the threshold of toxicological concern for Cramer class III compounds, there is no concern for this metabolite. For the other three metabolites, these have been tested in rodents through their formation from the parent compound and are therefore covered by the ADI for cyantraniliprole.

The Meeting concluded that it was not necessary to establish an acute reference dose (ARfD) for cyantraniliprole 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 cyantraniliprole

Species	Study	Effect	NOAEL	LOAEL
Mouse	Eighteen-month study of toxicity and carcinogenicity[a]	Toxicity	1 000 ppm, equal to 104 mg/kg bw per day	7 000 ppm, equal to 769 mg/kg bw per day
		Carcinogenicity	7 000 ppm, equal to 769 mg/kg bw per day[b]	–
Rat	Ninety-day study of toxicity[a]	Toxicity	100 ppm, equal to 5.7 mg/kg bw per day	400 ppm, equal to 22 mg/kg bw per day
	Two-year study of toxicity and carcinogenicity[a]	Toxicity	200 ppm, equal to 8.3 mg/kg bw per day	2 000 ppm, equal to 84.8 mg/kg bw per day
		Carcinogenicity	20 000 ppm, equal to 907 mg/kg bw per day[b]	–
	Multigeneration reproductive toxicity study[a]	Parental toxicity	200 ppm, equal to 11.0 mg/kg bw per day	2 000 ppm, equal to 110 mg/kg bw per day
		Reproductive toxicity	20 000 ppm, equal to 1 344 mg/kg bw per day[b]	–
		Offspring toxicity	2 000 ppm, equal to 280 mg/kg bw per day	20 000 ppm, equal to 2 776 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]	–
	Acute neurotoxicity	Toxicity	2 000 mg/kg bw per day[b]	–
	Ninety-day study of neurotoxicity[a]	Neurotoxicity	20 000 ppm, equal to 1 195 mg/kg bw per day[b]	–

Species	Study	Effect	NOAEL	LOAEL
Rabbit	Developmental toxicity study[c]	Maternal toxicity	25 mg/kg bw per day	100 mg/kg bw per day
		Embryo and fetal toxicity	100 mg/kg bw per day	250 mg/kg bw per day
Dog	Ninety-day and 1-year studies of toxicity[a,d]	Toxicity	100 ppm, equal to 3.08 mg/kg bw per day	200 ppm, equal to 5.67 mg/kg bw per day

LOAEL: lowest-observed-adverse-effect level; NOAEL: no-observed-adverse-effect level
[a] Dietary administration.
[b] Highest dose tested.
[c] Gavage administration.
[d] Two or more studies combined.

Estimate of acceptable daily intake

0–0.03 mg/kg bw

Estimate of acute reference dose

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 cyantraniliprole

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid (in 2 h) and extensive (> 80%)
Dermal absorption	No data
Distribution	Extensive; all tissues
Potential for accumulation	Low
Rate and extent of excretion	Rapid (mainly in 48 h samples) and extensive; faeces > urine
Metabolism in animals	IN-MLA84 is abundant in mice and rats, less in dogs
Toxicologically significant compounds in animals, plants and the environment	Cyantraniliprole, IN-MLA84, IN-MYX98 and IN-J9Z38
Acute toxicity	
Rat, LD_{50}, oral	> 5 000 mg/kg bw
Rat, LD_{50}, dermal	> 5 000 mg/kg bw
Rat, LC_{50}, inhalation	> 5.2 mg/L
Rat, dermal irritation	Non-irritating
Rabbit, ocular irritation	Non-irritating
Dermal sensitization	Non-sensitizing (LLNA in mice; maximization test in guinea-pigs)
Short-term toxicity	
Target/critical effect	Liver and thyroid / increases in AP and liver weights (dogs)
Lowest relevant oral NOAEL	3.08 mg/kg bw per day (dog)
Lowest relevant dermal NOAEL	1 000 mg/kg bw per day (rat)

Lowest relevant inhalation NOAEC	No data
Long-term toxicity and carcinogenicity	
Target/critical effect	Liver and thyroid / increased incidence of altered foci of hepatocytes in the liver, decreased body weight gain in females
Lowest relevant NOAEL	8.3 mg/kg bw per day (rat)
Carcinogenicity	Not carcinogenic
Genotoxicity	
	Not genotoxic
Reproductive toxicity	
Reproduction target/critical effect	No reproductive toxicity
Lowest relevant parental NOAEL	11.0 mg/kg bw per day
Lowest relevant offspring NOAEL	280 mg/kg bw per day
Lowest relevant reproductive NOAEL	1 344 mg/kg bw per day, the highest dose tested
Developmental toxicity	
Target/critical effect	Mortality, increased clinical signs, decreased body weight gain and lower feed consumption of dams
Lowest relevant maternal NOAEL	25 mg/kg bw per day (rabbit)
Lowest relevant developmental NOAEL	100 mg/kg bw per day (rabbit)
Neurotoxicity	
Acute and subchronic neurotoxicity	Not neurotoxic
Immunotoxicity	
Lowest relevant immunotoxicity NOAEL	1 065 mg/kg bw per day, the highest dose tested (mouse)
Medical data	
	No information available

LC_{50}: median lethal concentration; LD_{50}: median lethal dose; NOAEC: no-observed-adverse-effect concentration; NOAEL: no-observed-adverse-effect level

Summary

	Value	Study	Safety factor
ADI	0–0.03 mg/kg bw	Ninety-day and 1-year toxicity studies (dog)	100
ARfD	Unnecessary	–	–

ADI: acceptable daily intake; ARfD: acute reference dose

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DIQUAT (addendum)

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Explanation

Diquat is the International Organization for Standardization–approved name for 6,7-dihydrodipyrido[1,2-a:2′,1′-c]pyrazinediium dibromide (International Union of Pure and Applied Chemistry), for which the Chemical Abstracts Service (CAS) number is 85-00-7. The CAS number for diquat ion is 2764-72-9. Diquat is a non-selective, quick-acting contact herbicide and desiccant, causing injury only to the parts of the plant to which it is applied. Diquat interacts with the electron transfer components associated with Photosystem I, which causes inhibition of photosynthesis. Diquat is referred to as a desiccant because it causes a leaf or an entire plant to dry out quickly. It is also used as an aquatic algicide.

Diquat was previously evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 1970, 1972, 1977 and 1993. An acceptable daily intake (ADI) of 0–0.008 mg diquat ion/kg body weight (bw) was allocated in 1977. In 1993, JMPR established an ADI of 0–0.002 mg diquat ion/kg bw based on a no-observed-adverse-effect level (NOAEL) of 0.19 mg diquat ion/kg bw per day identified in a 2-year study of toxicity and carcinogenicity in rats, using a safety factor of 100.

Diquat was reviewed at the present Meeting as part of the periodic review programme of the Codex Committee on Pesticide Residues.

Since the last review by JMPR, the following new studies on diquat have been submitted: acute and subchronic neurotoxicity studies, an immunotoxicity study, metabolism studies and a re-evaluation of cataract observations in previous long-term studies. All dose values are expressed as diquat ion. Some of the studies do not comply with good laboratory practice (GLP), as most of the data were generated before the implementation of GLP regulations. Overall, the Meeting considered that the database was adequate for the risk assessment.

Evaluation for acceptable daily intake

1. Biochemical aspects

1.1 Absorption, distribution and excretion

The absorption and excretion of diquat were studied by Daniel & Henson (1960) to seek an explanation for the large difference between the acute oral and subcutaneous median lethal dose (LD_{50}) values in the rat. From limited experiments, they found that after single oral administration of [^{14}C]diquat, all of the radioactivity was excreted within 48 hours, about 94% in the faeces and about 6% in the urine. After subcutaneous administration, most of the dose appeared in the urine, which suggested that diquat was poorly absorbed from the gut. Using a chemical method of analysis, they found the excretion in urine to be similar to that measured by radiochemical analysis, but the percentage of the dose appearing in faeces after oral dosing was much lower, ranging between 10% and 40%. This low result in faeces was attributed to the metabolism of diquat by the intestinal flora: when diquat was incubated with washings from rat intestine for 24 hours at 37 °C, only 50% could be recovered by chemical analysis.

The work of Daniel & Henson (1960) was further investigated in a study using radiolabelled diquat dibromide or diquat dichloride in male Wistar rats. In this study, ^{14}C-labelled diquat dibromide (5 or 10 mg diquat ion/kg bw) or dichloride (22 or 24 mg diquat ion/kg bw) was administered by gavage; the dibromide was also administered subcutaneously at a dose of 5 or 6 mg diquat ion/kg bw. Treated rats were housed individually in metabolism cages from which urine and faeces were collected frozen over solid carbon dioxide for up to 96 hours. Urine and faeces were collected daily for the duration of each experiment.

Following oral or subcutaneous administration of diquat, most of the radioactivity appeared in the excreta within 2 days; in a few cases, a small amount was excreted on the 3rd day. Diquat was poorly absorbed from the gut; the bulk of an oral dose appeared in the faeces (84–97%), with limited urinary excretion (4–11%). After a subcutaneous dose, little or none appeared in the faeces (Table 1). The presence of a small amount of metabolites in urine after oral dosing of diquat has been detected. As no such metabolism has been observed after subcutaneous administration, it seems probable that it derives from the absorption of degradation products formed within the gut (Daniel & Gage, 1964, 1966).

The absorption, distribution and excretion of diquat were studied in Wistar rats following a single oral gavage dose of 1 or 100 mg [^{14}C]diquat ion/kg bw in water. Diquat dibromide was radiolabelled in the [2,2′,6,6′-^{14}C] positions. For serial blood collection, three rats of each sex per dose were used, and blood from the tail vein was collected at 1, 2, 4, 8 and 24 hours post-dosing. For the tissue distribution study, three rats of each sex per dose were terminated at 2, 4, 8, 24, 48 and 96 hours post-dosing. The positions of the ^{14}C label of diquat dibromide are shown in Fig. 1.

Following a single oral dose of [^{14}C]diquat at the 1 mg diquat ion/kg bw dose level, the maximum mean blood concentration (0.012 µg equivalents [eq] diquat ion/g) in blood was observed at 2 hours; by 24 hours, all values had declined to near background values (Table 2). Following a 100 mg/kg bw dose, the maximum mean blood concentration (1.428 µg eq diquat ion/g) was observed at 1 hour. By 4 hours, the mean blood concentration had declined to 0.265 µg eq/g, and by 24 hours, to 0.259 µg eq/g. There was no sex difference in blood concentrations.

Table 1. Group mean excretion of radioactivity after single oral or subcutaneous administration of [14C]diquat to male rats

Compound	Dose group	Dose (mg/kg bw)	Dose route	Sample	Daily % excretion					Total % excretion (urine + faeces)
					Day 1	Day 2	Day 3	Day 4	Total	
Diquat dibromide	A	5	Oral	Urine	0	4	0	–	4	101
				Faeces	7	90	0	–	97	
	B	10	Oral	Urine	5	1	0	–	6	96
				Faeces	80	10	0	–	90	
	C	5	Subcutaneous	Urine	94	4	0	–	98	98
				Faeces	0	0	–	–	0	
	D	6	Subcutaneous	Urine	84	3	1	0	88	90
				Faeces	2	0	–	–	2	
	D	6	Subcutaneous	Urine	89	4	0	–	93	93
				Faeces	0	0	–	–	0	
Diquat dichloride	E	22	Oral	Urine	7	2	0	0	9	93
				Faeces	13	57	14	0	84	
	F	24	Oral	Urine	8	2	1	0	11	101
				Faeces	8	44	38	0	90	

Source: Daniel & Gage (1964)

Fig. 1. Radiolabel positions of diquat dibromide

Table 2. Mean blood concentrations of radioactivity over a time course following a single oral dose of 1 or 100 mg [14C]diquat ion/kg bw

Time after dosing (h)	Mean blood concentrations (µg eq diquat ion/g)[a]	
	Group A: 1 mg/kg bw	Group B: 100 mg/kg bw
1	0.011	1.428
2	0.012	1.042
4	0.008	0.265
8	0.005	0.240
12	0.003	0.300
24	0.002	0.259

[a] Mean of three males and three females.
Source: Johnston et al. (1991)

Following single oral administration of [14C]diquat at a dose level of 1 mg diquat ion/kg bw, the concentration of radioactivity in tissues fell from a maximum at 2–4 hours to near background levels at 24 hours. Excluding the gastrointestinal tract, highest concentrations of radioactivity at the low dose level were found in kidney. In male rats at 2 hours post-dosing, the highest tissue concentration was present in the kidney (0.096 µg eq diquat ion/g), which progressively declined to

0.04 µg eq/g by 4 hours and to 0.006 µg eq/g by 24 hours. In female rats, at 2 hours post-dosing, the highest tissue concentration was present in the kidney (0.064 µg eq diquat ion/g), which progressively declined to 0.04 µg eq/g by 4 hours and to 0.006 µg eq/g by 24 hours.

Following single oral administration of [^{14}C]diquat at a dose level of 100 mg diquat ion/kg bw, the rate of elimination of radioactivity was generally slower than at the low dose level. In male rats at 2 hours post-dosing, the highest tissue concentrations were present in the kidney, lung and liver (5.75, 2.43 and 0.87 µg eq/g, respectively). All other tissue concentrations were below 0.075 µg eq/g. At 4 and 8 hours after dosing, the kidney (3.06 and 1.76 µg eq/g) and lung (2.41 and 0.95 µg eq/g) again showed the highest concentrations. By 8 hours, all other tissue concentrations were lower than 0.47 µg eq/g. In female rats, at 2 hours post-dosing, the highest tissue concentrations were present in the lung, kidney and liver (3.80, 2.27 and 0.83 µg eq/g, respectively). After 4 hours in female rats, concentrations in kidney (2.59 µg eq/g) and liver (1.06 µg eq/g) had risen slightly, but concentrations in lung (1.53 µg eq/g) had declined; by 96 hours, most other tissue concentrations were approaching background values.

There was no apparent sex difference in blood concentrations over a time course following a single oral dose of [^{14}C]diquat at 1 or 100 mg diquat ion/kg bw, and radioactivity concentrations appeared to be broadly in proportion to dose levels. In general terms, there was no apparent sex difference in the tissue distribution of radioactivity, although concentrations in kidney, liver and lung at 48 hours after a 100 mg/kg bw dose were lower in the female group. In both sexes, ^{14}C concentrations in lung were disproportionately high at the high dose level (Johnston et al., 1991).

The absorption, distribution, excretion and metabolism of diquat were studied in five male and five female Wistar rats following a single oral gavage dose of 1 mg [^{14}C]diquat ion/kg bw in water. Diquat dibromide was radiolabelled in the [2,2′,6,6′-^{14}C] positions. Treated animals were housed individually in metabolism cages for collection of urine and faeces. After 7 days, treated animals were killed, and various tissues and blood samples were removed for further analysis.

About 90% of the administered dose was recovered in faeces in 24 hours (Table 3). Approximately 93% of the administered dose was recovered in faeces in 7 days. Approximately 3% of the administered dose was recovered in urine in 7 days. At 168 hours after dosing, the levels of radioactivity in tissues, organs and body fluids were at or close to background values.

Radio–thin-layer chromatographic (TLC) analysis of both pooled urine (0–8 hours) and pooled faecal extract (0–24 hours) samples indicated the presence of a single major radiolabelled component with a retention factor similar to that of [^{14}C]diquat. Faecal extraction efficiencies were 92.8% for males and 82.5% for females. No urinary or faecal radioactivity co-chromatographed with any of the six reference samples used.

There was apparently no sex difference in the pattern of excretion or the radio-TLC profiles of excreta. An oral dose of diquat was poorly absorbed in the rat. Most of the orally administered dose of diquat was excreted in the first 24 hours, primarily in faeces (about 90%) and to some extent in urine (about 3%). Radio-TLC analysis of pooled urine and faecal samples indicated the presence of a single major radiolabelled component, which co-chromatographed with [^{14}C]diquat (Johnston, Mutch & Scott, 1994).

The absorption, distribution, excretion and metabolism of diquat were studied in five male and five female Wistar rats following a single oral gavage dose of 100 mg [^{14}C]diquat ion/kg bw in water. Diquat dibromide was radiolabelled in the [2,2′,6,6′-^{14}C] positions. Treated animals were housed individually in metabolism cages for collection of urine and faeces. After 7 days, treated animals were killed, and various tissues and blood samples were removed for further analysis.

Following a single oral dose of 100 mg diquat ion/kg bw to male and female rats, there was no sex difference in excretion profiles. A mean of 85.5% of the dose was recovered in faeces over

Table 3. Group mean cumulative excretion of radioactivity over 168 hours after single oral administration of [¹⁴C]diquat to male and female rats at a dose level of 1 mg/kg bw

	Collection period (h)	Group mean cumulative excretion (%)	
		Males (*n* = 5)	Females (*n* = 5)
Urine	0–8	1.87	2.71
	0–24	2.61	3.36
	0–48	2.68	3.48
	0–72	5.67	3.52
	0–168	2.72	3.55
Faeces	0–24	91.67	88.50
	0–48	94.65	92.41
	0–72	94.74	92.61
	0–168	94.85	92.67
Cage wash	0–24	0.20	0.23
	0–48	0.24	0.26
	0–72	0.25	0.29
	0–168	0.34	0.38
Stomach + contents	168	< LRD	< LRD
Small intestine + contents	168	< LRD	< LRD
Large intestine + contents	168	< LRD	< LRD
Tissues + residual carcass	168	< LRD	< LRD

LRD: limit of reliable determination
Source: Johnston, Mutch & Scott (1994)

168 hours. Excretion via urine accounted for a mean of 5.4% of the administered dose. The mean total radioactivity recovered was 93.2% of the dose (Table 4).

At 168 hours after dosing, a very small proportion of the dose remained in the carcass, accounting for 0.02% of the administered dose in males and 0.01% in females. Seven days after the oral administration, the levels of radioactivity in tissues, organs and body fluids were close to background values. The highest concentrations were detected in the organs of excretion (gastrointestinal tract). There were residues in kidney, lung, liver and lens, with mean concentrations ranging from 0.038 to 0.251 µg eq/g. At 96 hours post-dosing, significant residues were seen in the eye lens (Table 5). Radio-TLC analysis of both pooled urine (0–48 hours) and pooled faecal extract (0–72 hours) samples indicated the presence of a single major radiolabelled component with a retention factor similar to that of [¹⁴C]diquat (Johnston et al., 1994).

A distribution study was conducted in rats to determine the residue of diquat photodecomposition product. In this study, mature barley plants were sprayed with [¹⁴C]diquat at a rate equivalent to 0.77 kg/ha. The plants were then kept in sunlight to allow the diquat to photodegrade. The plants were harvested after 2 weeks, and the straw was fed to rats as 10% of their total diet for up to 20 days. The known photodegradation and oxidation products of diquat are diquat monopyridone, 1,2,3,4-tetrahydro-1-oxopyrido-(1,2-a)-5-pyrazinium salt (TOPPS) and diquat dipyridone. To analyse the acid-extractable radioactivity on the barley straw, a sample of the powdered straw was extracted with 2 N HCl for 3 hours. The extract was analysed for diquat, diquat monopyridone, TOPPS, diquat dipyridone and picolinic acid by isotope dilution. All of these compounds have been shown to be stable to the acid extraction conditions used. The picolinic acid analysis also included any picolinamide that was hydrolysed during the acid extraction. The photodegradation products and relative proportions in the acid extraction from the barley plants are shown in Table 6.

Table 4. Group mean cumulative excretion of radioactivity over 168 hours after single oral administration of [^{14}C]diquat to male and female rats at a dose level of 100 mg/kg bw

	Collection period (h)	Group mean cumulative excretion (%)	
		Males (*n* = 5)	Females (*n* = 5)
Urine	0–8	1.09	1.11
	0–24	2.94	2.79
	0–48	5.31	4.78
	0–72	5.67	5.02
	0–168	5.70	5.11
Faeces	0–24	43.44	30.16
	0–48	77.02	69.54
	0–72	82.90	82.40
	0–168	84.17	86.83
Cage wash	0–24	2.55	1.39
	0–48	2.70	1.52
	0–72	2.78	1.58
	0–168	2.90	1.71
Stomach + contents	168	< LRD	< LRD
Small intestine + contents	168	< LRD	< LRD
Large intestine + contents	168	< LRD	< LRD
Tissues + residual carcass	168	0.02	0.01
Total recovery	0–168	92.78	93.86

LRD: limit of reliable determination
Source: Johnston et al. (1994)

Table 5. Distribution of radioactivity in tissues/organs over 96 hours following a single oral dose of 100 mg [^{14}C]diquat ion/kg bw to female rats (Group F)

	Distribution (µg eq diquat ion/g)[a]					
	2 h	4 h	8 h	24 h	48 h	96 h
Bone	0.285	0.441	0.157	0.154	0.140	0.085
Brain	0.131	0.123	0.058	0.056	0.490	0.119
Fat (renal)	0.216	0.127	0.115	0.209	0.322	0.094
Heart	0.440	0.347	0.240	0.249	0.154	0.015
Kidneys	2.272	2.590	1.389	2.054	0.739	0.251
Lens	0.225	0.344	0.112	0.046	0.094	0.097
Liver	0.833	1.060	0.339	0.476	0.164	0.059
Lungs	3.802	1.526	1.854	0.355	0.105	0.038
Muscle	0.247	0.231	0.151	0.111	0.156	0.032
Ovaries	0.570	0.400	0.196	0.272	0.069	0.011
Spleen	0.279	0.435	0.220	0.217	0.100	0.082
Residual carcass	0.630	0.634	0.498	2.265	1.745	0.007
Stomach	134.0	198.2	137.9	154.4	7.823	0.227
Stomach contents	9 259	9 977	9 648	5 756	274.5	9.578
Small intestine	78.97	32.17	26.89	84.24	12.03	0.833
Small intestine contents	1 947	672.8	656.3	1 388	192.8	17.48

	Distribution (µg eq diquat ion/g)[a]					
	2 h	4 h	8 h	24 h	48 h	96 h
Large intestine	49.67	112.5	89.40	84.37	38.86	2.544
Large intestine contents	1 992	2 849	2 050	1 721	1 219	79.25
Whole blood	0.235	0.232	0.108	0.244	< LRD	< LRD
Plasma	0.420	0.352	0.163	0.386	0.055	0.017

LRD: limit of reliable determination
[a] Each value represents the mean of three animals.
Source: Johnston et al. (1994)

Table 6. Percentages of [^{14}C]diquat and its photoproducts acid-extracted from [^{14}C]diquat-treated barley straw

	% of total barley straw radioactivity extracted
Total radioactivity extracted	*81*
Diquat	21
Diquat monopyridone	2
TOPPS	12
Diquat dipyridone	1
Picolinic acid	2
Unidentified photoproducts	62

TOPPS: 1,2,3,4-tetrahydro-1-oxopyrido-(1,2-a)-5-pyrazinium salt
Source: Leahey, Burgess & Mills (1974)

Analysis of the tissues of these rats showed that the maximum radioactive residues in the tissues analysed were as follows: muscle, 0.007 µg eq/g; fat, 0.008 µg eq/g; kidney, 0.03 µg eq/g; liver, 0.02 µg eq/g; heart, 0.01 µg eq/g; and lungs, 0.01 µg eq/g. Necropsy of the rats revealed no gross abnormalities in any of the tissues, and no behavioural abnormalities occurred during the experiment (Leahey, Burgess & Mills, 1974).

In a study investigating the extent of absorption of [^{14}C]diquat residues on wheat chaff, a sample of [^{14}C]diquat-treated wheat chaff was produced at Zeneca Agrochemicals, Jealott's Hill Research Station (Heath & Leahey, 1989). The bioavailability of the radioactivity associated with a nominal dose of 100 mg (84 kBq) of this wheat chaff was investigated in the Alpk:APfSD rat. Three male and three female rats were given three consecutive daily doses of wheat chaff, by gavage, as an aqueous suspension. An additional three male and three female bile duct–cannulated rats were given a similar single oral dose. Urine, faeces and, where applicable, bile were collected over 48 hours from the bile duct–cannulated rats and over 120 hours from the non-cannulated rats. At the end of these periods, the animals were killed, and selected tissues and the residual carcass were sampled. Excreta, tissues and carcasses were analysed for radioactivity.

The radioactivity in bile represented less than 0.7% of the dose for either sex. The urine from bile duct–cannulated rats contained 4.2% and 2.9% of the administered dose for males and females, respectively. The urine from non-cannulated rats contained 2.2% and 2.4% of the cumulative dose to males and females, respectively. The remainder of the administered radioactivity was excreted in the faeces. Very low levels of radioactivity were detected in all tissues for both bile duct–cannulated and non-cannulated rats. All mean measurements in the residual carcass were below the limit of detection. The radioactivity associated with the [^{14}C]diquat-treated wheat chaff was poorly absorbed following either single or repeated oral dosing. The bioavailability of oral doses of radiolabelled diquat residues on wheat chaff is therefore very low in the rat (Lappin, Platt & Davies, 1993).

1.2 Biotransformation

A comparative metabolism study was conducted in Wistar rats via single oral gavage or single subcutaneous administration of diquat dibromide. In this study, a single oral dose of 45 mg [^{14}C]diquat ion/kg bw in water (radiolabelled in 1,1′-[U-^{14}C]ethylene-2,2′-bipyridylium ion) was administered to each of five male rats by gavage. A single subcutaneous dose of 10 mg [^{14}C]diquat ion/kg bw in water was administered to each of five male rats. Urine and faeces were collected at 24-hour intervals for 96 hours after dosing. Diluted urine samples and acid extracts of faecal samples were further subjected to metabolic identification. A separate in vitro study was conducted to evaluate the nature of the radioactivity present in the rat caecal content.

Rats given a single oral dose of 45 mg diquat ion/kg bw excreted 6.3% and 89.3% of the administered dose in urine and faeces, respectively, within 4 days, mainly in the first 48 hours. Diquat was the major radioactive component in the urine (5.1% of the dose). Diquat monohydrate represented 0.2% of the dose in the urine. Diquat dipyridone and unknown degradation products represented 0.1% and 0.3% of the dose, respectively. The chromatographic analysis of the faecal extract showed that diquat represented 57.1% and diquat monopyridone 4.3% of the dose. The unidentified radioactivity (4.1% of the dose) included one discrete unidentified metabolite. Diquat dipyridone was not detected.

Following subcutaneous injection of 10 mg diquat ion/kg bw, rats excreted 87.1% of the dose in the urine and 4.6% in the faeces within 4 days. The urine contained mainly diquat (75% of the dose), together with diquat monopyridone (about 3% of the dose) and diquat dipyridone (at least 6% of the dose). Diluted sulfuric acid extracted 4.3% of the radioactivity from faeces, 0.6% of which was diquat. No further analysis of radiolabelled faecal metabolites was done.

Methanol extracted 98% of the radioactivity present in caecal contents following incubation with [^{14}C]diquat. Chromatographic analysis showed that two compounds were present: 88% was identified as parent diquat, and 7.8% as diquat monopyridone. This finding confirmed that the gut microflora possesses the capacity to metabolize diquat at least to diquat monopyridone.

The study author concluded that the extent of diquat metabolism in rats is considerably lower than predicted previously (Daniel & Gage, 1966), those predictions being based on erroneous comparisons of radioactivity measurements and chemical analyses (Mills, 1976).

Urine and faecal samples from the previous study (Johnston et al., 1994) in which male and female Wistar rats were administered a single oral gavage dose of 100 mg [^{14}C]diquat ion/kg bw in water were subjected to metabolic identification. Urine samples used for metabolite identification represented separate 0- to 48-hour pools for male and female rats administered a single oral gavage dose of 100 mg diquat ion/kg bw. Faecal samples used for metabolite identification represented separate 0- to 72-hour pools for male and female rats administered a single oral gavage dose of 100 mg diquat ion/kg bw.

The mean proportion of administered radioactivity recovered in urine following a single oral dose of 100 mg diquat ion/kg bw was 5.4% (5.7% for males and 5.1% for females). Radio–high-performance liquid chromatographic (HPLC) analyses of 0- to 48-hour pooled urine samples exhibited one major peak with a retention time consistent with that of diquat. This peak accounted for 76.2% and 79.8% of the total excreted radioactivity in 48 hours for males and females, respectively. In addition, three approximately equivalent minor peaks were observed by radio-HPLC analysis, with retention times consistent with picolinic acid, diquat dipyridone and diquat monopyridone (Table 7). The mean proportion of administered radioactivity recovered in faeces following a single oral dose of 100 mg diquat ion/kg bw was 85.5% (84.2% for males and 86.8% for females). Radio-HPLC analyses of 0- to 72-hour pooled faecal samples exhibited one major peak with a retention time consistent with that of diquat. The peak accounted for 86.7% and 75.4% of the total excreted radioactivity within 72 hours for male and female rats, respectively. One minor peak was observed in the female faecal sample with a retention time between 9 and 10 minutes, which did not correspond to any of the analysed reference standards. This accounted for 6.4% of total excreted radioactivity in faeces within

72 hours in females. A broad band of radioactivity was also observed between 2 and 6 minutes, which accounted for 6.2% of total excreted radioactivity within 72 hours for male rats and 9.3% for female rats (Williams, Cameron & McGuire, 1991).

Table 7. Metabolite identification of excreta derived following oral administration of diquat in rats following a single dose of 100 mg diquat ion/kg bw

	% of administered dose					
	Males			Females		
	Urine (0–48 h)	Faeces (0–72 h)	Total excreta	Urine (0–48 h)	Faeces (0–72 h)	Total excreta
Total urinary excretion (0–168 h)	5.70	N/A	N/A	5.11	N/A	N/A
Excretion in 0–48 h urine	5.31	N/A	N/A	4.78	N/A	N/A
Total faecal excretion (0–168 h)	N/A	84.17	N/A	N/A	86.83	N/A
Excretion in 0–72 h faeces	N/A	82.90	N/A	N/A	82.40	N/A
Diquat	4.05	71.87	75.92	3.81	62.13	65.94
Diquat dipyridone		ND			ND	
Diquat monopyridone	0.92	ND	0.92	0.75	ND	0.75
Picolinic acid		ND			ND	
TOPPS	ND	ND	ND	ND	ND	ND
Pyridine-2-carboxamide	ND	ND	ND	ND	ND	ND
Total identified	4.97	71.87	76.84	4.56	62.13	66.69
Unidentified at RT 9–10 min	–	–	–	–	5.27	5.27
Unidentified at RT 2–6 min	–	5.14	5.14	–	7.66	7.66
Total unidentified	–	5.14	5.14	–	12.93	12.93
Total accounted for	4.97	77.01	81.98	4.56	75.06	79.62

N/A: not applicable; ND: not detected; RT: retention time
Source: Williams, Cameron & McGuire (1991)

The biotransformation pathway proposed for diquat is presented in Fig. 2.

Fig.2. Proposed biotransformation pathway for diquat

The biotransformation of diquat is postulated to proceed either by progressive oxidation of the pyridine rings to form diquat monopyridone and diquat dipyridone or by the cleavage of one of the pyridine rings to form picolinic acid, possibly via pyridine-2-carboxamide as an intermediary metabolite, although this was not identified. However, the total proportion of the dose metabolized to diquat monopyridone, diquat dipyridone and picolinic acid amounted to less than 1%. Furthermore, it is possible that these various metabolites were produced by the intestinal flora and absorbed as such into the systemic circulation and were then subject, like the parent diquat, to rapid urinary excretion. Similarly, the unidentified minor metabolites in faeces are also likely to be formed by gut flora metabolism, and the available evidence suggests that these were not absorbed into systemic circulation and were therefore not bioavailable to the rat, but were excreted directly in the faeces (Williams, Cameron & McGuire 1991).

1.3 Dermal absorption

(a) In vivo

^{14}C-ring-labelled diquat dibromide was dissolved in deionized water and applied topically at a single dose of 0.05, 0.5 or 5.0 mg onto an unabraded 10 cm^2 application site on the shaved dorsal trunk of each of 12 male Sprague-Dawley® Crl:CD® (SD)BR rats per dose group. Treated animals were kept in metabolism cages for collection of urine, faeces and volatiles. Four animals per dose were sacrificed at 2, 10 and 24 hours post-exposure. Urine, faeces, blood, carcass and skin from application sites were analysed for radioactivity.

The mean recovery of radioactivity ranged from 93.5% to 99.6% of the administered dose. The absorbed dose included the radioactivity in urine, faeces, cage wash, residual carcass and blood. The radioactivity remaining at the application site after skin washing was considered the absorbable dose. The mean systemic absorption following dermal application of 0.05 mg diquat dibromide per rat was 1.3%, 2% and 2.5% at 2, 10 and 24 hours, respectively. At these times, 2.6%, 3.1% and 3.3% of the dose were recovered from the skin application site after skin washing. The mean systemic absorption following dermal application of 0.5 mg diquat dibromide per rat was 1.2%, 3.6% and 2.1% at 2, 10 and 24 hours, respectively. At these times, 1.2%, 1.3% and 1.0% of the dose were recovered from the application site of the skin. The mean systemic absorption following dermal application of 5.0 mg diquat dibromide per rat was 1.8%, 3.4% and 3.4% at 2, 10 and 24 hours, respectively. At these times, 0.6%, 0.8% and 0.7% of the dose were recovered from the application site of the skin.

The dermal absorption of diquat dibromide through the skin of male rats is low. Approximately 6% of the administered dose was absorbed through rat skin in 24 hours when skin-bound residue after skin washing was included (Brorby & Griffis, 1987).

(b) In vitro

In vitro dermal absorption of ^{14}C-ring-labelled diquat dibromide from a soluble concentrate formulation through human epidermis was studied. The doses were applied as the concentrate formulation (200 g diquat ion/L) and as 1 : 100 volume per volume (v/v) (2 g/L) and 1 : 200 v/v (1 g/L) spray strength dilutions of the formulation in water. The doses were applied to the epidermal membranes at a rate of 10 μL/cm^2 and left unoccluded for an exposure period of 24 hours. The absorption rate of diquat from the concentrate formulation through human epidermis was 0.082 μg/cm^2 per hour during the 24-hour exposure period. The absorption rate of diquat from the 1 : 100 v/v dilution through human epidermis was 0.001 μg/cm^2 per hour during the 24-hour exposure period. The absorption rate of diquat from the 1 : 200 v/v dilution through human epidermis was 0.001 μg/cm^2 per hour during the 24-hour exposure period (Johnson, 2009).

2. Toxicological studies

2.1 Acute toxicity

The results of acute toxicity studies with diquat (including skin and eye irritation and dermal sensitization studies; see below) are summarized in Table 8.

Table 8. Acute toxicity of diquat

Species	Strain	Sex	Route	Purity; vehicle	LD_{50} (mg ion/kg bw) or LC_{50} (mg ion/L)	Reference
Rat	Wistar derived (Alpk:APfSD)	M + F	Oral	21.2% w/w diquat ion; deionized water	LD_{50} M = 214 (180–271) F = 222 (203–241)	McCall & Robinson (1990a)
Rat	Wistar derived (Alpk:APfSD)	M + F	Dermal	21.2% w/w diquat ion; undiluted	LD_{50} > 424	McCall & Robinson (1990b)
Rat	Sprague-Dawley	M + F	Inhalation (whole body)	19.5% w/w diquat ion; distilled water	LC_{50} (4 h) M = 0.121 F = 0.132 Combined = 0.125	Bruce, Griffis & Wong (1985)
Rabbit	New Zealand White	F	Skin irritation	20.7% diquat ion; undiluted	Moderate to severely irritating	Robinson (1998a)
Rabbit	New Zealand White	F	Eye irritation	20.7% diquat ion	Mildly irritating	Robinson (1998b)
Guinea-pig	Dunkin Hartley	F	Skin sensitization (Magnusson and Kligman method)	267 g/L; deionized water	Sensitizer	Rattray & Robinson (1990)

F: female; LC_{50}: median lethal concentration; LD_{50}: median lethal dose; M: male; w/w: weight per weight

(a) Lethal doses

In an acute oral toxicity study, groups of five male and five female fasted young adult Alpk:APfSD (Wistar-derived) rats were given a single oral dose of diquat dibromide (purity 21.2% weight per weight [w/w] diquat ion) in deionized water at a dose of 100, 150, 200, 225 or 250 mg diquat ion/kg bw and observed up to 15 days.

Following a dose of 100 mg/kg bw, there were no mortalities and no signs of toxicity. At 150 mg/kg bw, one male rat died, and at 200 mg/kg bw, there were two mortalities (one male and one female). Surviving animals at these two dose levels showed signs of slight to moderate toxicity, which persisted until day 7. Signs of extreme toxicity were seen in all animals at 225 and 250 mg/kg bw. There were four mortalities (two males and two females) at 225 mg/kg bw by day 8, but surviving animals recovered by day 12. All animals dosed at 250 mg/kg bw were either found dead or killed in extremis by day 5. At 150 and 250 mg/kg bw, clinical signs of toxicity seen included decreased activity, hypothermia, piloerection, reduced splay reflex, distended abdomen, sides pinched in, ungroomed, upward curvature of the spine, increased breathing depth, decreased breathing depth, increased breathing rate, breathing irregular, stains around mouth and/or nose, dehydrated, urinary incontinence, diarrhoea, chromodacryorrhoea and splayed gait.

The acute oral LD_{50} for males was 214 mg diquat ion/kg bw (95% confidence interval [CI] 180–271). The acute oral LD_{50} for females was 222 mg diquat ion/kg bw (95% CI 203–241) (McCall & Robinson, 1990a).

In an acute dermal toxicity study, five male and five female young adult Alpk:APfSD rats were each given a single 24-hour dermal application of diquat dibromide (purity 21.2% w/w diquat ion) at 2000 mg/kg bw. The test substance was applied undiluted to the shorn backs of the rats and held in place under an occlusive dressing. The animals were observed daily for 15 days.

There were no mortalities, and no significant signs of toxicity were seen. Signs of moderate skin irritation were seen. No treatment-related effects were observed on body weight or upon macroscopic examination.

The acute dermal LD_{50} of diquat dibromide to males and females was greater than 2000 mg/kg bw (equivalent to > 424 mg diquat ion/kg bw) (McCall & Robinson, 1990b).

In an acute inhalation toxicity study, groups of young adult Sprague-Dawley CD rats (five of each sex) were exposed by inhalation (whole body) for 4 hours to aerosols containing 19.5% w/w diquat ion in distilled water at target concentrations of 0 (controls), 0.15, 1.0 and 4.0 mg/L. Approximately 97–99% of each aerosol was smaller than 10 µm (mass median aerodynamic diameter 2.2–2.8 µm). The average total measured concentrations were 0, 0.16, 1.1 and 3.9 mg/L. Specified tissues were submitted for histological examination.

All animals exposed to 3.9 mg/L (211 µg diquat ion/L) died 2–10 days after exposure. Three males and three females exposed to 1.1 mg/L (118 µg diquat ion/L) died 3–5 days after exposure. There were no deaths in animals exposed to 0.16 mg/L (86.1 µg diquat ion/L).

Signs of toxicity included squinted eyes and salivation (seen during exposure at all concentrations); laboured breathing, increased respiration rate, abnormal respiratory sounds; nasal, oral and/or ocular discharge; slight hindquarter ataxia/weakness; sores/alopecia on the throat; and weight loss. At necropsy, treatment-related lung changes were present in animals exposed to 1.1 and 3.9 mg/L. Histopathological examination revealed exposure-related, subchronic inflammation, congestion, oedema and alveolar/septal thickening in the lungs of all rats exposed to 1.1 and 3.9 mg/L. Three males and two females in the 0.16 mg/L group had subchronic inflammation and alveolar/septal thickening of the lungs.

It was concluded that the acute inhalation median lethal concentration (LC_{50}) of diquat ion was 121 µg/L for males, 132 µg/L for females and 125 µg/L for both sexes combined (Bruce, Griffis & Wong, 1985).

(b) Dermal and ocular irritation

In a primary dermal irritation study, three young adult female New Zealand White albino rabbits were dermally exposed to 0.5 mL of diquat dibromide technical concentrate (20.7% w/w diquat ion) on an area (approximately 2.5 cm × 2.5 cm) of the shorn flank for 4 hours. The animals were assessed for up to 23 days for any signs of skin irritation. Irritation was scored by the method of Draize.

Very slight or well defined erythema was seen in two animals for up to 7 days and in one animal for up to 17 days. Very slight or slight oedema was seen in all animals for up to 2 or 7 days. There were no additional signs of irritation in one animal. Additional signs seen in the remaining two animals included desquamation, skin thickening and development of new skin with associated sparse hair growth. All signs of irritation had completely regressed by day 23 in both animals.

Diquat dibromide technical concentrate is a moderate to severe irritant following a single 4-hour application to rabbit skin (Robinson, 1998a).

In a primary eye irritation study, 0.1 mL of diquat dibromide technical concentrate (20.7% w/w diquat ion) was instilled into the conjunctival sac of the left eye of each of three young adult female New Zealand White rabbits. The eyes were examined for up to 8 days to assess the grade of ocular reaction. Irritation was scored by the method of Draize.

No corneal or iridial effects were observed. Conjunctival effects included slight to moderate redness, slight chemosis and slight to moderate discharge. All effects had completely regressed by day 8.

Diquat dibromide technical concentrate is a mild irritant (class 4 on a 1–8 scale) to the rabbit eye (Robinson, 1998b).

(c) Dermal sensitization

Groups of 20 test and 20 control young adult female Alpk:Dunkin Hartley guinea-pigs were used to assess the sensitization potential of diquat using a method based on the maximization test of Magnusson and Kligman. For the main study, the concentrations used were 0.1% weight per volume (w/v) in deionized water for the induction intradermal injections, 100% for the topical induction applications, and 10% w/v in deionized water and 100% for the challenge applications. A positive control study was conducted using essentially the same methodology and using formaldehyde as the test substance.

Following challenge with the undiluted test sample, scattered mild redness was seen in 5/16 test and 0/17 control animals. The net response was calculated to be 31%. Following challenge with a 10% w/v preparation of the test sample in deionized water, scattered mild redness was seen in 1/16 test and 0/17 control animals. The net response was calculated to be 6%. No erythematous reactions were seen in any animal, test or control, following challenge with deionized water. The net response was zero. The positive control study provided a response as expected. Challenge of previously induced guinea-pigs with undiluted diquat elicited a moderate sensitization response, and challenge with a 10% w/v preparation elicited a weak sensitization response.

Diquat is considered to be a skin sensitizer in the guinea-pig (Rattray & Robinson, 1990).

2.2 Short-term studies of toxicity

(a) Oral administration

Mice

No data are available.

Rats

In a 90-day toxicity study, diquat technical, as the dibromide (26.9% diquat ion), was administered to 12 Alpk:APfSD rats of each sex per dose in the diet at a dose level of 0, 20, 100 or 500 ppm diquat ion (equal to 0, 1.7, 8.5 and 39.5 mg diquat ion/kg bw per day for males and 0, 1.9, 9.2 and 41.5 mg diquat ion/kg bw per day for females, respectively). The clinical condition of the animals, including body weight gain and feed consumption, was monitored during the study, and the eyes were examined ophthalmoscopically. Urine and blood were examined during the study. At necropsy, blood was taken for clinical chemistry and haematology, and selected organs were weighed and examined histologically.

There were no mortalities. All rats survived the experimental period until scheduled termination. Males and females fed 500 ppm diquat ion showed a statistically significant decrease in growth throughout the study, resulting in a mean body weight at termination that was 26% below that of the control group for males and 15% lower for females. Feed consumption was reduced (approximately 25% below control values) throughout the study for males and females receiving 500 ppm diquat ion. During the later stages of the study, the majority of male and female rats fed 500 ppm diquat ion showed clinical changes in the eyes, which were opaque and pale. Cataract formation was also observed at this dose at 8 weeks, and at necropsy, corneal opacity was observed in 7/12 males and 4/12 females. At histological examination, cataract was seen in 12/12 males and 11/12 females. Additionally, a low incidence of focal inflammation of the tongue and epithelium of the palate was observed.

Reduced plasma total protein and albumin levels were seen at 500 ppm, possibly caused by low feed intake. In the high-dose males (Table 9), cholesterol, triglycerides and alanine transaminase (ALT) were statistically significantly reduced compared with control values at termination. In the high-dose females, ALT and aspartate transaminase (AST) were statistically significantly reduced compared with control values at termination. A marked reduction in urinary protein was seen in males

in weeks 3 and 13 (2 and 12 weeks of treatment, respectively). At week 3, urinary pH was statistically significantly increased and specific gravity was decreased in both males and females dosed with 500 ppm diquat ion. All organ weights (absolute, but not relative) were reduced, almost certainly a reflection of poor feed intake. Treatment-related effects were not seen at 100 ppm.

Table 9. Intergroup comparison of selected blood clinical chemistry parameters (week 13)

Parameter	Males[a]				Females[a]			
	0 ppm	20 ppm	100 ppm	500 ppm	0 ppm	20 ppm	100 ppm	500 ppm
Albumin (g/100 mL)	4.62	4.70	4.57	4.47*	4.65	4.61	4.57	4.52
Total protein (g/100 mL)	6.32	6.35	6.27	5.98**	6.19	6.16	6.16	6.04
ALT (mU/mL)	57.8	54.1	55.1	45.8**	46.9 (11)	43.8	45.8	24.7**
AST (mU/mL)	59.6	61.1	56.0	58.5	64.3 (11)	64.0	57.6	50.9**
Cholesterol (mg/100 mL)	60.9	62.1	60.4	49.6*	61.6	64.0	60.0	60.1
Triglycerides (mg/100 mL)	143	143	134	77**	86 (11)	75	73	71

ALT: alanine transaminase; AST: aspartate transaminase; U: units; *: $P < 0.05$; **: $P < 0.01$ (Student's *t*-test, two-sided; compared with control group mean)

[a] Number of animals, when less than 12, shown in parentheses.

Source: Hodge (1989a)

The NOAEL was 100 ppm (equal to 8.5 mg diquat ion/kg bw per day), based on decreased body weight gain and feed consumption, changes in clinical chemistry parameters, increased urine volume, decreased urinary specific gravity, minor changes in haematological values, erosion of the tongue and oral cavity and ocular changes at 500 ppm (equal to 39.5 mg/kg bw per day) (Hodge, 1989a).

The purpose of a second 90-day toxicity study in Sprague-Dawley rats (described below) was to determine the NOAEL for cataract formation in rats. In a previous 90-day study in the Alpk:APfSD strain of rat (Hodge, 1989a), the no-effect level for ocular toxicity of diquat ion was 100 ppm. In a 2-year chronic toxicity/carcinogenicity study conducted in the Sprague-Dawley strain of rat, the no-effect level for ocular toxicity of diquat ion at 90 days, based on ophthalmoscopy, was 15 ppm. At the next higher dose of 75 ppm, 1/50 males and 3/50 females showed evidence of treatment-related cataracts (Colley et al. 1985).

In this second 90-day toxicity study, groups of 12 male and 12 female Sprague-Dawley rats were fed diets containing 0, 30, 60 or 300 ppm diquat ion (equal to 0, 2.4, 4.7 and 23.2 mg diquat ion/kg bw per day for males and 0, 2.7, 5.0 and 25.3 mg diquat ion/kg bw per day for females, respectively) for 90 consecutive days. Clinical observations, body weight and feed consumption were measured. An ophthalmoscopic examination was performed on all rats prior to commencement and prior to termination of treatment. At the end of the study, all animals were killed and examined postmortem, and the eyes were removed and stored.

There were no toxicologically significant clinical observations. At 300 ppm, body weight and feed consumption were generally low compared with control values throughout the study for males and low compared with control values during the 1st week of treatment for females. Posterior lens opacities were present in week 13 for 10/12 males and 9/12 females at 300 ppm. A lens opacity

plaque was also seen for one female at this dose level. There were no ophthalmoscopic abnormalities in animals in the control, 30 and 60 ppm diquat ion groups.

The NOAEL was 60 ppm (equal to 4.7 mg diquat ion/kg bw per day) for ocular lesions and lens opacities evident at 300 ppm (equal to 23.2 mg diquat ion/kg bw per day) (Noakes, 2003).

When the results of the two 90-day toxicity studies in rats were combined, the overall NOAEL was 100 ppm (equal to 8.5 mg diquat ion/kg bw per day), with an overall lowest-observed-adverse-effect level (LOAEL) of 300 ppm (equal to 23.2 mg diquat ion/kg bw per day).

Dogs

A 1-year toxicity study in dogs was carried out using diquat dibromide technical (26.7% w/v diquat ion) added to the feed. Groups of four male and four female Beagles received diquat at a dose of 0, 0.5, 2.5 or 12.5 mg diquat ion/kg bw per day (achieved intakes 0, 0.46, 2.42 and 11.48 mg diquat ion/kg bw per day for males and 0, 0.53, 2.53 and 13.21 mg diquat ion/kg bw per day for females, respectively) for 52 weeks. Clinical condition, body weight and feed consumption were monitored throughout. Ophthalmoscopy, haematology and clinical chemistry were carried out. The animals were killed and necropsied at 52 weeks, and a range of organs was examined and processed for histological examination.

No treatment-related effects on survival, clinical signs, haematology, clinical chemistry, urine analysis or gross pathology (except eye) were observed at any dose level. Decreased body weight gains were observed only during the first 2 weeks of dosing in both sexes at the high dose level (males 46% and females 32% of control; Table 10), although there was no decrease in feed consumption at any time point. There was a statistically significant decrease in white blood cells and neutrophil count in males of all treatment groups at a single time point (week 4), which was ascribed to raised counts in one control. There were decreased platelet counts in top-dose females at 4, 26 and 52 weeks. Raised plasma chloride levels observed in the top-dose animals were attributed to bromide ion interference. Plasma triglyceride levels in the males given 12.5 mg diquat ion/kg bw per day were higher than in the controls throughout the study; at 4 and 26 weeks, these increases were statistically significant. Statistically significant increases in relative and absolute kidney weights were observed in both sexes at 12.5 mg diquat ion/kg bw per day. There were decreases in absolute and relative adrenal weights in all treatment groups in the males, which were statistically significant only in the case of relative weights. Additionally, there was a decrease in the absolute and relative weights of the epididymides in all test groups compared with the controls; this finding was statistically significant only for absolute weights in the 2.5 mg diquat ion/kg bw per day group and for relative weights in the top-dose group. Changes in organ weights did not correspond to any histopathological changes.

Table 10. Intergroup comparison of body weight gain from start of study: selected time points

Week	Body weight gain (kg)							
	Males				Females			
	0 mg diquat ion/kg bw per day	0.5 mg diquat ion/kg bw per day	2.5 mg diquat ion/kg bw per day	12.5 mg diquat ion/kg bw per day	0 mg diquat ion/kg bw per day	0.5 mg diquat ion/kg bw per day	2.5 mg diquat ion/kg bw per day	12.5 mg diquat ion/kg bw per day
1	0.27	0.32	0.30	0.17	0.25	0.15	0.07	0.00*
2	0.70	0.88	0.75	0.32*	0.47	0.38	0.30	0.15**
3	0.92	1.13	1.00	0.67	0.60	0.55	0.50	0.35
12	2.67	3.30	2.75	2.60	1.82	1.65	1.90	1.47

*: $P < 0.05$; **: $P < 0.01$ (Student’s *t*-test, two-sided; compared with control group mean)
Source: Hopkins (1990)

At necropsy, bilateral lens opacity was observed in all high-dose males and 3/4 high-dose females. Unilateral lens opacity was observed in two females that received 2.5 mg diquat ion/kg bw per day at necropsy. The first occurrences of lens opacity were at week 8 and week 40 in one female each in the 2.5 mg diquat ion/kg bw per day dose group. The first occurrences of lens opacity at the high dose level were at week 16 in males and week 24 in females. Inflammatory changes were seen at the top dose in the large intestine, consisting of reduction in mucosal thickness, loss and abnormality of mucosal glands, epithelial hyperplasia in crypts and increased goblet cell activity.

The NOAEL was 0.53 mg diquat ion/kg bw per day, based on lens opacity (cataracts) in females at 2.53 mg diquat ion/kg bw per day (Hopkins, 1990).

(b) Dermal application

No data are available.

(c) Exposure by inhalation

No data are available.

2.3 Long-term studies of toxicity and carcinogenicity

Mice

In a 2-year toxicity and carcinogenicity study, groups of 60 male and 60 female mice (C57BL/10JfCD-1/Alpk) were fed a diet containing 0, 30, 100 or 300 ppm diquat (equal to 0, 3.56, 11.96 and 37.83 mg diquat ion/kg bw per day for males and 0, 4.78, 16.03 and 48.27 mg diquat ion/kg bw per day for females, respectively). The test material was technical-grade diquat dibromide containing 26.7% w/v diquat ion. Haematological parameters were assessed at weeks 53 and 79 and at study termination. Various tissues were taken at necropsy and processed for histological examination. At 12 months, 10 mice of each sex per dose were sacrificed.

There were no treatment-related adverse effects on survival of either sex, although a greater number of treated females (37%, 47% and 40% with increasing dose) were killed in extremis compared with the control females (25%). Treatment-related clinical signs were observed in both sexes and included an increased incidence of eye discharge (males in the 100 ppm dose group and mice of both sexes in the 300 ppm dose group) and an increased incidence of thin appearance (males) and subdued behaviour (females in the 100 ppm group and males and females at 300 ppm). Decreased body weight was observed throughout the study in both sexes (males 3.5–8.7% and females 2.9–9.4% below control) at 300 ppm and in males at 100 ppm (3.8–4.7% below control) from week 95 until termination, compared with the controls. Feed consumption was decreased for both sexes at the high dose level. Changes in certain haematological parameters at 300 ppm were also seen – namely, statistically significantly decreased neutrophil count and increased lymphocyte count in both sexes at weeks 53 and 79. There was also a significant increase in total white blood cells at 2 years in males. At termination, platelet count, white blood cell count and lymphocyte count were all slightly higher than control values in males fed 300 ppm diquat, with marginal increases in females receiving 300 ppm diquat. White blood cell counts in males only showed a statistically significant difference. Relative kidney weights were increased significantly in male mice fed 100 or 300 ppm diquat by approximately 5% and 7%, respectively. There was a slight treatment-related increase in microscopic lesions in the kidney (tubular dilatation in both sexes and tubular hyaline droplet formation in females), liver (extramedullary haematopoiesis in both sexes) and mesenteric lymph nodes (lymphoid proliferation in females) at the high dose level, mainly at the terminal kill.

Diquat was not carcinogenic in this study. The number of tumour-bearing mice was the same in the control and diquat-treated male groups. In the females, there was a reduction in the number of tumour-bearing mice in the mid-dose (100 ppm) and high-dose (300 ppm) groups, relative to the controls. Most of the tumour-bearing mice in each group, including the controls, had single, malignant and metastatic tumours.

The NOAEL was 30 ppm (equal to 3.56 mg diquat ion/kg bw per day), based on reduction in body weight gain, increased relative kidney weights and ocular discharges at 100 ppm (equal to 11.96 mg diquat ion/kg bw per day) and above. There was no evidence of carcinogenicity in mice at doses up to and including 300 ppm (equal to 37.83 mg diquat ion/kg bw per day), the highest dose tested (Hodge, 1992).

Rats

In a combined chronic toxicity and carcinogenicity study, male and female Sprague-Dawley rats (50 of each sex per dose) were fed diets containing 0, 5, 15, 75 or 375 ppm diquat dibromide (purity 19.54% w/w diquat ion; equal to 0, 0.19, 0.58, 2.91 and 14.88 mg diquat ion/kg bw per day for males and 0, 0.24, 0.72, 3.64 and 19.44 mg diquat ion/kg bw per day for females, respectively) for 104 weeks. In addition, 10 rats of each sex per dose were treated similarly and killed at 12 months. Ophthalmoscopy was carried out before dosing and periodically during the study. Haematological and biochemical variables were measured on 10 animals from each main group before dosing and at 26, 52, 78 and 104 weeks and in five satellite animals at week 52.

Treatment had no adverse effect on survival. With the exception of effects on the eye, there were no treatment-related clinical signs of toxicity. Changes in the eyes were observed at the two highest dose levels and consisted of an initial appearance of the eye being clearer and more transparent than usual. This was followed temporally by opacity, which first occurred in week 10 at 75 ppm (one male) and in week 11 at 375 ppm (two females). From week 45, a number of rats with total opacity showed a dark red discolouration of the eyes, which became darker with time. This was due to extensive haemorrhage in the anterior chamber of the eye, revealed by ophthalmoscopic examination. Body weight gains were decreased at the high dose level in both sexes throughout the study (weeks 0–13: males 84% and females 80% of control; overall: males 86% and females 75% of control), and a corresponding depression in average feed consumption was observed at the high dose level for both sexes for the first 26 weeks and for females throughout week 78 of the study. No treatment-related effects on body weight or feed consumption were observed at 5, 15 or 75 ppm. After 26 weeks, there was a reduction in mean corpuscular volume and in haemoglobin in females and males, respectively. Minimal reductions in red blood cell parameters were observed in males at 15 ppm and above at 52 and 78 weeks. Other changes were prolonged activated partial thromboplastin times at 75 and 375 ppm in females at 52 and 104 weeks and at 15 ppm in females at 52 weeks. Blood urea nitrogen was elevated at 52 weeks at 75 and 375 ppm and at 78 weeks in the 375 ppm females. At 52 weeks, the 75 and 375 ppm groups exhibited lower total protein and albumin levels. On ophthalmoscopic examination (Table 11), lenticular opacities were seen in the 75 and 375 ppm groups at 13 weeks, and the findings progressed at subsequent examinations. Ophthalmoscopy at 104 weeks showed severe lens opacities at 375 ppm in all surviving rats, whereas less severe ones were seen at 75 ppm. At 15 ppm, a single instance of cataract was seen in each sex. Histological examination of the eyes postmortem showed advanced cataractogenesis in all animals at 375 ppm and in about 80% of the 75 ppm group. There was a low prevalence of cataract at 15 ppm: cataract-type changes were seen in 0/22 control and 3/22 treated males and 0/22 control and 2/20 treated females.

There were no differences in urine clinical chemistry parameters that were considered to be related to treatment. There were no compound-related effects on organ weights. Males at the two highest dose levels displayed an increased incidence of nephropathy in the kidneys, and the high-dose males displayed increased incidences of arteritis/periarteritis in blood vessels and paracortical cell hyperplasia in the lymph nodes. There was no treatment-related increase in any tumour type in either sex.

Benign phaeochromocytoma of the adrenal gland was seen in males receiving 75 or 375 ppm diquat dibromide, but this was not dose related and achieved statistical significance only at the 75 ppm level. There were two instances in males of statistically significant positive trends with dose: osteosarcoma and thyroid follicular cell adenoma. In both cases, these findings reflected elevated incidences at the 375 ppm level only, which were not statistically significant by Fisher’s exact test, and there was no evidence of any increasing incidence below this dose level. There was a significant

Table 11. Intergroup comparison of ophthalmology: selected lens lesions

Finding	Week	Incidence of lens lesions									
		Males					Females				
		0 ppm	5 ppm	15 ppm	75 ppm	375 ppm	0 ppm	5 ppm	15 ppm	75 ppm	375 ppm
Point posterior subcapsular polar opacities	13	0/50	0/50	0/50	8/50	6/49	0/50	2/50	1/50	5/50	0/50
	26	0/50	0/50	0/50	0/49	0/49	0/50	0/50	0/50	0/50	2/49
	52	0/50	0/49	0/50	1/49	0/48	0/49	0/50	0/47	2/50	0/48
	104	0/22	0/16	0/22	0/21	0/24	0/20	0/22	1/20	0/20	0/27
Posterior subcapsular polar opacities	13	0/50	0/50	1/50	0/50	0/49	0/50	0/50	0/50	0/50	0/50
	26	1/50	2/50	3/50	3/49	0/49	0/50	1/50	0/50	5/50	0/49
	78	0/46	1/44	0/44	0/40	0/40	0/39	0/41	0/42	0/41	0/46
Triangular posterior subcapsular polar opacities	13	0/50	0/50	0/50	1/50	32/49	0/50	0/50	0/50	3/50	40/50
	26	0/50	0/50	0/50	2/49	9/49	0/50	0/50	0/50	5/50	10/49
	52	0/50	0/49	0/50	1/49	3/48	0/49	0/50	0/47	5/50	3/48
	78	0/46	0/44	1/44	3/40	0/40	0/39	0/41	0/42	4/41	1/46
	104	0/22	0/16	1/22	9/21	0/24	0/20	0/22	0/20	1/20	0/27
Total cataract	13	0/50	0/50	0/50	1/50	11/49	0/50	0/50	1/50	0/50	2/50
	26	0/50	0/50	0/50	2/49	38/49	0/50	0/50	0/50	0/50	37/49
	52	0/50	0/49	0/50	2/49	45/48	0/49	0/50	0/47	0/50	43/48
	78	1/46	0/44	0/44	2/40	40/40	0/39	0/41	0/42	2/41	45/46
	104	0/22	0/16	1/22	3/21	24/24	0/20	0/22	1/20	3/20	27/27
Plaque opacities	52	1/50	0/49	3/50	1/49	0/48	0/49	1/50	0/47	0/50	0/48
	78	3/46	2/44	3/44	2/40	0/40	0/39	2/41	0/42	6/41	0/46
	104	5/22	4/16	6/22	3/21	0/24	5/20	3/22	0/20	3/20	0/27

Source: Colley et al. (1985)

increase in the number of females with multiple neoplasia and with malignant neoplasms at 5 and 75 ppm. No other change attributable to the test material was seen.

The NOAEL was 5 ppm (equal to 0.19 mg diquat ion/kg bw per day), based on cataractous changes seen at 15 ppm (equal to 0.58 mg diquat ion/kg bw per day). There was no evidence of carcinogenic potential. The NOAEL for carcinogenicity was 375 ppm (equal to 14.88 mg diquat ion/kg bw per day), the highest dose tested (Colley et al., 1985). This was the study on which the ADI was based at the 1993 JMPR.

Subsequent to the 1993 JMPR, the ophthalmoscopy and ocular histopathology data from the 2-year chronic toxicity and carcinogenicity study in rats by Colley et al. (1985) were re-evaluated by the same laboratory that had conducted the original study (specifically by the principal pathologist); in particular, histopathology data were correlated with ophthalmoscopy findings. The evaluation demonstrated a consistent pattern of diquat-induced lesion (bilateral triangular posterior subcapsular polar opacities that progress to more extensive opacities and total cataract) in animals at 75 and 375 ppm. However, none of the animals at 15 ppm demonstrated the consistent pattern; when considered with the histopathology, there was no treatment-related increase in overall cataractous change. It was concluded that there was no evidence of diquat cataractogenic response at 15 ppm (Harling, Buist & Gopinath, 1997a,b).

Based on the above re-evaluation of the data, the present Meeting identified the NOAEL as 15 ppm (equal to 0.58 mg diquat ion/kg bw per day), as the incidence and severity of cataracts were comparable with those of the controls.

2.4 Genotoxicity

The results of studies of genotoxicity with diquat technical are summarized in Table 12. All the studies, either in vitro or in vivo, were negative except for the mammalian cell cytogenetic assay (human lymphocytes), which gave an equivocal response in the absence of metabolic activation and a positive response in the presence of metabolic activation at cytotoxic doses. Diquat is not considered to possess any mutagenic or genotoxic potential.

Table 12. Results of studies of genotoxicity with diquat

Type of study	Organism/cell line	Dose range tested	Purity (%)[a]	Result	Reference
In vitro					
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA1538; *Escherichia coli* (WP2 uvrA PMK101)	0.05–100 µg/plate (+S9 mix) 0.01–50 µg/plate (−S9 mix) (in water)	25.8	Negative ±S9 mix	Callander (1986)
Mammalian cell gene mutation assay	L5178Y mouse lymphoma cells	3.13–50 µg/mL (+S9 mix) 6.25–100 µg/mL (−S9 mix) (in 1% DMSO)	25.8	Negative ±S9 mix	Cross (1986)
Mammalian cell cytogenetic assay	Human lymphocytes (chromosomal aberrations)	26.7–534.8 µg/mL (+S9 mix) 13.4–534.8 µg/mL (−S9 mix) (in 0.85% saline)	100	Equivocal −S9 mix Positive +S9 mix	Wildgoose et al. (1986)
In vivo					
Mouse micronucleus	Male and female C57BL/6J/Alpk mice	0, 62.5 and 100 mg/kg bw (gavage in deionized water)	25.8	Negative	Sheldon, Richardson & Shaw (1986)
DNA repair (unscheduled DNA synthesis) assay	Rat liver Alpk:AP rats	0, 225, 450 or 900 mg/kg bw (gavage in deionized water)	25.8	Negative	Trueman (1987)
Dominant lethal assay	CD-1 mice	0, 0.1, 1.0 or 10 mg/kg bw for 5 consecutive days (gavage in 0.5% Tween 80 in water)	28.6	Negative	McGregor (1974)

DMSO: dimethyl sulfoxide; DNA: deoxyribonucleic acid; S9: 9000 × *g* supernatant fraction from rat liver homogenate; w/w: weight per weight

[a] Purity expressed as diquat ion (w/w).

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

Rats

A multigeneration reproduction study was conducted using technical-grade diquat dibromide, containing 26.7% w/v diquat ion. Groups of Alpk:APfSD Wistar-derived rats (30 of each sex) were fed diets containing 0, 16, 80 or 400/240 ppm diquat ion (equal to 0, 1.6, 7.9 and 38.7 mg/kg bw per day for males and 0, 1.7, 8.4 and 40.4 mg/kg bw per day for females, respectively, during the premating period). After 12 weeks, the animals were mated and allowed to rear the litters that resulted (F_{1a}). The process was repeated with 30 male and 30 female parents per group selected from the F_{1a} litters, these F_1 parents being mated 11 weeks after selection. The dose received by the top-dose F_1 rats was reduced after 4 weeks to 240 ppm. Ophthalmoscopic examination of the eyes was carried out on the controls and top-dose group at 12 weeks and on all the F_0 animals at termination (24 weeks). A similar examination was carried out on the F_1 rats 4 weeks after selection, at 11 weeks before mating and at termination (21 weeks).

No treatment-related mortality was observed. Parental toxicity was observed mostly in the high-dose group in both generations as increased incidences of clinical signs (red/brown urine, piloerection and ulcers on palate and tongue) and increased incidences of ophthalmoscopic signs (eye opacity, partial or total lenticular cataracts and iritis). At a dose level of 80 ppm, there was a low incidence of mouth lesions in both generations and a slightly increased incidence of partial cataract in F_1 females. At the high dose in both generations, a statistically significant ($P < 0.01$) decreased body weight gain during the premating period (males, 15–40%; and females, 10–37%), gestation (20–23%) and lactation (32–80%) and statistically significant ($P < 0.05$ or 0.01) decreased feed consumption during the premating period (males, 7–40%; and females, 5–33%), gestation (8–14%) and lactation (16–28%) were observed. The nature of the diquat-induced cataracts varied from animal to animal and also varied according to the time period of examination. The less severe cataracts (partial) were characterized by the following features: increased transparency or clearing of the lens (believed to result from a change in optical density or loss of lines of discontinuity rather than opacity); focal areas of opacity (usually posterior) with or without increased demarcation of the posterior suture lines; and vacuoles (usually posterior). The more severe cataracts (total) were characterized either by opacity of the whole or greater part of the lens or by shrinkage of the lens with opacification. The cataracts tended to be bilateral, although the degree of change in each eye was not necessarily the same. In both F_0 and F_1 parents, the severity and incidence of cataracts increased with time, and females were more severely affected than males. Cataracts, first seen at week 12 in F_0 females and at week 13 in males and 2 weeks earlier in each sex in the F_{1a} and F_{1b} generations, were also observed in the F_1 adults. Although cataract formation was mostly confined to the 240 ppm group, a low incidence was seen at 80 ppm in the F_1 female parents (3/30 at premating ophthalmoscopic examination and 4/30 at examination before termination of the study). Diquat had no effect on fertility in either sex. At the terminal histopathological examination, cataract was seen in the highest-dose group only of F_0 and F_1 parents. There was an increase in pathological changes in the renal tract in the F_1 and F_2 pups. Pup weight gain was lower in the top dose in both generations compared with controls. A statistically significant decrease in total litter weight was apparent for most time points for the F_{1a} litters. The slightly lower values for pup weight gain in the F_{1a} litters at 80 ppm were considered not to be related to treatment, as no similar effects were seen in the F_{2a} litters, and total litter weight was not affected.

The NOAEL for parental systemic toxicity was 16 ppm (equal to 1.6 mg diquat ion/kg bw per day), based on a low incidence of mouth lesions in both generations and a slightly increased incidence of cataracts at 80 ppm (equal to 7.9 mg diquat ion/kg bw per day). The NOAEL for reproductive toxicity was 400/240 ppm (equal to 38.7 mg diquat ion/kg bw per day), the highest dose tested. The NOAEL for offspring toxicity was 80 ppm (equal to 7.9 mg diquat ion/kg bw per day), based on decreased pup body weights seen in both generations at 400/240 ppm (equal to 38.7 mg diquat ion/kg bw per day) (Hodge, 1990).

(b) Developmental toxicity

Rats

In a developmental toxicity study, diquat dibromide (purity 26.2% w/v diquat ion) was administered by gavage, in deionized water, to pregnant Alpk:APfSD Wistar-derived rats (23–24 per group) from gestation days 7 through 16. The dose levels used were 0, 4, 12 and 40 mg/kg bw per day, expressed as diquat ion. Animals were sacrificed on gestation day 22, and uteri were examined for live fetuses and intrauterine deaths.

A dose-related, statistically significant decrease in body weight gain compared with control values was seen across all dose groups during the first 3 days of dosing. There was a clear statistically significant decrease in body weight gain (about 22% compared with controls) in the 40 mg diquat ion/kg bw per day dose group throughout the dosing period, with the majority of the animals losing weight initially. The effect seen at 4 and 12 mg diquat ion/kg bw per day was evident only during the first few days of dosing and was very mild at 4 mg diquat ion/kg bw per day. A dose-related, statistically significant reduction in feed consumption compared with controls was seen across all dose groups during the first 3 days of dosing. This effect persisted throughout the dosing period for animals in the 12 and 40 mg diquat ion/kg bw per day groups and continued at the top dose for the first 3 days post-dosing. It did not persist in the 4 mg diquat ion/kg bw per day group. Significant reductions in fetal weight, litter weight and gravid uterine weight as well as fetal defects in ossification were seen at 40 mg diquat ion/kg bw per day. At the high dose, increased incidences of fetuses with haemorrhagic kidney (five in four litters; $P < 0.05$), compared with none in the control group, were also seen (Table 13). Minor evidence of reduced ossification was seen at the lower doses, but these were considered not to be of biological significance.

Table 13. Intergroup comparison of selected fetal defects and variants in a developmental toxicity study in rats

Observation	0 mg/kg bw per day	4 mg/kg bw per day	12 mg/kg bw per day	40 mg/kg bw per day
No. of litters examined	24	24	23	20
No. of fetuses examined	297	291	269	239
Total incidence of major defects	1	2	1	4
No. of fetuses showing major skeletal defects	1	1	1	1
No. of fetuses showing minor skeletal defects only	106	102	97	132**
Incidence of haemorrhagic kidneys (fetus/litter)	0	1/1	0	5*/4*
Incidence of calcaneum not ossified (fetus/litter)	133/22	139/21	157**/22	149**/19
Incidence of odontoid not ossified (fetus/litter)	107/22	114/19	110/17	102/19
Incidence of partially ossified 4th lumbar transverse process (fetus/litter)	232/24	210/24	182/22	128/19
Mean *manus* score	2.20	2.25	2.18	2.52**
Mean *pes* score	2.92	2.93	2.92	2.98

*: $P < 0.05$; **: $P < 0.01$ (Student's *t*-test, two-sided; compared with control group mean)
Source: Wickramaratne (1989)

The NOAEL for maternal toxicity was 12 mg diquat ion/kg bw per day, based on reduced body weights and feed consumption seen at 40 mg diquat ion/kg bw per day. The NOAEL for embryo and fetal toxicity was 12 mg diquat ion/kg bw per day, based on reduced fetal weight, haemorrhagic kidney and increased incidence of soft tissue and minor skeletal anomalies at 40 mg diquat ion/kg bw per day (Wickramaratne, 1989).

Rabbits

In a developmental toxicity study, groups of 20 New Zealand White rabbits were administered diquat dibromide technical (26.2% w/v diquat ion) in deionized water by gavage at a dose of 0, 1, 3 or 10 mg diquat ion/kg bw per day from days 7 to 19 (inclusive) of gestation. On day 30 of gestation, the females were killed and the uteri examined for live fetuses and intrauterine deaths. The fetuses were weighed, examined externally and for visceral abnormalities, eviscerated and stained for skeletal abnormalities.

Nothing remarkable was observed in the low-dose group (1 mg diquat ion/kg bw per day). The mid-dose group (3 mg diquat ion/kg bw per day) was associated with decreased maternal body weight gain (229% less than controls; $P < 0.01$) and feed consumption (45% less than controls; $P < 0.01$), which were observed only during the first 3 days of dosing. The high-dose group (10 mg diquat ion/kg bw per day) showed greater decreases in body weight gain (343%; $P < 0.01$) and feed consumption (74%; $P < 0.01$) compared with the mid-dose group during the first 3 dosing days, and the decreases continued throughout the dosing period. Two 10 mg diquat ion/kg bw per day females lost weight throughout dosing and then rapidly gained weight during the post-dosing period. Analyses were done both with and without these two females (Table 14).

Table 14. Intergroup comparison of body weight gain (all females) in a developmental toxicity study in rabbits

Period	Body weight gain (g)				
	0 mg/kg bw per day	1 mg/kg bw per day	3 mg/kg bw per day	10 mg/kg bw per day (all females)	10 mg/kg bw per day (cxcluding two females)
Initial weight (day 1)	3 418.1	3 401.3	3 344.5	3 368.2	3 357.1
Predosing (days 1–7)	130.2	131.0	158.4	171.9	165.0
During dosing (days 7–19)	237.4	309.9	236.4	47.5**	175.1
Days 7–10	42.6	61.4	−54.8**	−103.0**	–
Days 10–13	57.3	66.3	90.8	26.3	–
Days 13–16	109.3	140.3	131.8	94.1	–
Post-dosing (days 19–30)	312.4	387.1	293.3	424.8*	352.4
Overall (days 1–30)	680.1	828.0*	688.2	644.3	692.5

*: $P < 0.05$; **: $P < 0.01$ (Student's *t*-test, two-sided; compared with control group mean)
Source: Hodge (1989b)

There were no treatment-related effects on numbers of corpora lutea, preimplantation or postimplantation loss, litter size, sex distribution, gravid uterus weight or litter weight. Fetal weight was marginally lower at 10 mg diquat ion/kg bw per day. Some evidence of fetotoxicity was observed at 10 mg/kg bw per day (mottled and friable livers and small increase in minor skeletal defects at 3 and 10 mg diquat ion/kg bw per day, in the form of partially ossified sternebrae). The elevation in the proportion of fetuses with minor skeletal defects was significant at 3 and 10 mg diquat ion/kg bw per day; there was a non-significant increase in the lowest-dose group. The incidences were not statistically significant using a litter-based method of analysis (Table 15).

The NOAEL for maternal toxicity was 1 mg diquat ion/kg bw per day, based on reduced weight gain and feed consumption at 3 mg diquat ion/kg bw per day. The NOAEL for embryo and fetal toxicity was 3 mg diquat ion/kg bw per day, based on skeletal anomalies at 10 mg diquat ion/kg bw per day (Hodge, 1989b).

Table 15. Intergroup comparison of selected fetal defects and variants in a developmental toxicity study in rabbits

	0 mg/kg bw per day	1 mg/kg bw per day	3 mg/kg bw per day	10 mg/kg bw per day
No. of litters examined	18	15	20	13
No. of fetuses examined	147	134	129	96
Total incidence of major defects	2	8	2	4
No. of fetuses showing major external/visceral defects	2	8*	2	3
No. of fetuses showing major skeletal defects	1	3	1	1
No. of fetuses showing minor external/visceral defects only	60	56	64	39
No. of fetuses showing minor skeletal defects only	64	65	71*	53*
Liver – friable (fetus/litter)	8/5	7/2	5/2	14*/3
Gall bladder – small blood clot(s) attached (fetus/litter)	18/8	21/9	33**/13	14/8
Vertebral column – 27 presacral vertebrae (fetus/litter)	46/14	54/12	49/15	49**/12
Sternebrae – not ossified 6th (fetus/litter)	0/0	6*/2	3/3	6**/5**
Sternebrae – partially ossified 6th (fetus/litter)	9/6	14/8	19*/8	16**/8
Extra ribs – 13th normal length (fetus/litter)	76/15	61/13	89**/18	71**/12
Mean *manus* score	1.61	1.60	1.62	1.80
Mean *pes* score	1.43	1.45	1.40	1.65*

*: $P < 0.05$; **: $P < 0.01$ (Student's *t*-test, two-sided; compared with control group mean)
Source: Hodge (1989b)

2.6 Special studies

(a) Acute neurotoxicity

Rats

In an acute neurotoxicity study, diquat dibromide (technical grade) was administered in a single gavage dose to 10 male and 10 female Sprague-Dawley rats at a dose of 0, 25, 75 or 150 mg/kg bw, expressed as diquat ion. These rats were assessed for reactions in functional observational battery and motor activity measurements at 6 hours post-dosing and on days 8 and 15.

Evidence for neurotoxicity was observed only during the daily clinical observations. In the 75 mg/kg bw group, females had an increased incidence of diarrhoea (2/10) and staining of the nose (3/10), compared with the controls (0/10). Females in the 150 mg/kg bw group also had piloerection (7/10), urinary incontinence (3/10), mouth staining (3/10), upward curvature of the spine (3/10), tiptoe gait (3/10), hunched posture (2/10), subdued behaviour (2/10) and sides pinched in (1/10). These signs were observed up to day 9 of the study. At 150 mg/kg bw, slightly decreased body weights (4–5%) were observed by day 8 in both sexes, and decreased body weight gains (males 17%; females 23%) were observed during the 1st week only at this dose level in both sexes (Table 16). Feed consumption was decreased in the mid-dose (males 10%) and high-dose (males 22% and females 35%) groups during the 1st week following dosing. One female in the 150 mg/kg bw group, with all of the above signs, was sacrificed in extremis on study day 6. Males were less affected than females. The clinical signs observed in this study may not be due to direct neurotoxicity.

The NOAEL for general toxicity was 75 mg diquat ion/kg bw, based on a range of findings, including clinical signs, at 150 mg diquat ion/kg bw. The NOAEL for neurotoxicity was 150 mg diquat ion/kg bw, the highest dose tested (Horner, 1992a).

Table 16. Intergroup comparison of body weight in an acute neurotoxicity study in rats

	Body weight (g)							
	Males				Females			
	0 mg/kg bw[a]	25 mg/kg bw	75 mg/kg bw	150 mg/kg bw	0 mg/kg bw	25 mg/kg bw	75 mg/kg bw	150 mg/kg bw
Day 1 (post-dosing)	170.6	173.0	169.2	173.2	144.2	144.1	143.5	143.9
Day 8	246.9	251.5	240.1	236.5**	188.4	183.9	185.6	178.3**
Day 15	288.1	295.5	282.5	286.4	207.7	203.6	204.6	203.3

**: $P < 0.01$ (Student's *t*-test, two-sided; compared with control group mean)
[a] Doses expressed as diquat ion.
Source: Horner (1992a)

(b) Subchronic neurotoxicity

Rats

In a subchronic neurotoxicity study, diquat dibromide (purity 20.8%) was administered to 12 Alpk:APfSD rats of each sex per group at a dietary level of 0, 20, 100 or 400 ppm (equal to 0, 1.6, 7.9 and 32.4 mg/kg bw per day for males and 0, 1.9, 9.5 and 38.5 mg/kg bw per day for females, respectively, expressed as the diquat ion) for 13 weeks. Five rats of each sex per dose were used for neuropathological examination at study termination.

There were no treatment-related deaths. Treatment-related clinical signs were observed at the high dose level in both sexes at the end of the study: opaque eyes: 7/12 males and 8/12 females (weeks 13 and 14); eye pallor: 3/12 males and 1/12 females (weeks 12 and 13); and decreased visual placement response: 3/12 males and 1/12 females (week 14). Decreased body weights (9%) were observed throughout the study in both sexes at the high dose level (Table 17); decreased body weight gains (males 20% and females 18%) and decreased feed utilization were also observed at this dose level in both sexes. There was an increased incidence of cataracts and/or posterior lens opacities at the high dose level in both sexes at study termination. Total cataracts were observed in 5/12 high-dose males and 7/12 high-dose females. Posterior opacity of the lens was observed in 8/12 high-dose males and 6/12 high-dose females.

Table 17. Intergroup comparison of body weights (selected time points) in a subchronic neurotoxicity study in rats

Week	Body weight (g)							
	Males				Females			
	0 ppm	20 ppm	100 ppm	400 ppm	0 ppm	20 ppm	100 ppm	400 ppm
1	196.8	201.7	200.9	199.6	159.4	154.8	156.9	152.9
4	318.5	319.9	313.3*	294.5**	218.8	202.8*	210.2	198.7**
8	402.1	398.4	397.7	359.8**	254.4	238.9*	247.1	227.2**
14	477.8	478.3	475.7	420.4**	276.7	271.6	267.3	249.8**
Body weight gain, 0–14 weeks	278.1	274.8	268.5	221.2	116.3	114.8	111.8	95.5
% of controls	–	99	97	80	–	99	96	82

*: $P < 0.05$; **: $P < 0.01$ (Student's *t*-test, two-sided; compared with control group mean)
Source: Horner (1992b)

There was no evidence of neurotoxicity. No treatment-related effects were reported in the functional observational battery (weeks 5, 9 and 14 post-dosing), motor activity assessment (weeks 5, 9 and 14 post-dosing) and gross pathological/histopathological examination at study termination.

The NOAEL for neurotoxicity was 400 ppm (equal to 32.4 mg diquat ion/kg bw per day), the highest dose tested. The NOAEL for systemic toxicity was 100 ppm (equal to 7.9 mg diquat ion/kg bw per day), based on evidence of eye lesions and reductions in body weight gain and feed consumption at 400 ppm (equal to 32.4 mg diquat ion/kg bw per day) (Horner, 1992b).

Karuppagounder et al. (2012) published a high-dose study investigating the chronic neurotoxic effects of diquat. The study involved experiments conducted in young adult male C57Bl6 mice given 10 mg diquat/kg bw via intraperitoneal injection twice a week for 6 weeks (i.e. a total dose of 120 mg/kg bw). The authors reported a "mild degeneration of substantia nigra dopaminergic neurones" and stated that it correlated well with the behavioural and neurochemical results. However, these findings were not observed in the guideline subchronic neurotoxicity study in rats in which doses of up to 38.5 mg diquat ion/kg bw per day were administered in the diet.

(c) Immunotoxicity

In an immunotoxicity study, diquat dibromide (purity 22.0% w/w diquat ion) was administered to female CD-1 mice (10 per dose) in the diet at a concentration of 0, 100, 200 or 350 ppm of diquat ion (equal to 0, 23, 44 and 81 mg diquat ion/kg bw per day, respectively) for 28 days. Animals in the positive control group (10 females) received an oral gavage dose of cyclophosphamide at 10 mg/kg bw per day (dosing volume 5 mL/kg bw) for 28 days. On day 25, animals in all groups were immunized with a suspension of sheep red blood cells (sRBC) by intravenous injection (2×10^8 sRBC/mL, 0.25 mL/animal). Animals were sacrificed on day 29 by carbon dioxide inhalation, and blood samples were collected for analysis of anti-sRBC immunoglobulin M (IgM) titre with an enzyme-linked immunosorbent assay. The natural killer cell activity was not evaluated in this study. All animals were evaluated for mortality, clinical signs, body weight changes, feed consumption and water consumption. Terminal body weight and liver, spleen and thymus weights were measured for all animals at necropsy.

All mice survived the scheduled treatment period, and there were no treatment-related clinical signs. At day 28, animals receiving 200 or 350 ppm of diquat ion had a lower body weight compared with controls receiving only untreated diet. There was also a lower body weight change over the study period in these groups, compared with controls receiving only untreated diet. There were no differences in feed consumed or organ weights that were considered to be related to treatment with diquat dibromide. There were no abnormal findings noted at necropsy. There was no immunosuppressant effect with diquat dibromide.

The systemic toxicity NOAEL was 100 ppm diquat ion (equal to 23 mg diquat ion/kg bw per day), based on decreases in body weights and body weight gains seen at 200 ppm (equal to 44 mg diquat ion/kg bw per day). Under the conditions of the study, after 28 consecutive days of dietary treatment of female CD-1 mice with up to 350 ppm diquat ion (equal to 81 mg diquat ion/kg bw per day), there was no evidence of an immunosuppressant effect on the humoral immune system (Donald & Marr, 2011).

2.7 Studies on metabolites

Diquat monopyridone (6,7-dihydro-4-oxodipyrido(1,2-a:2′,1′-c) pyrazinium) was the major metabolite following oral dosing and could therefore be the toxicologically active species following administration by this route. An acute (single dose) toxicity study and a 2-week repeated-dose toxicity study were conducted in rats to evaluate its toxicity. In the acute toxicity study in Alderley Park rats, six female rats were given a single gavage dose of diquat monopyridone dissolved in deionized water at 0 and 4000 mg/kg bw. No mortality occurred. The oral LD_{50} of this compound in rats was in excess of 4000 mg/kg bw. In a separate study, groups of male and female Alderley Park rats (10 of each sex

per group) were given diquat monopyridone dissolved in deionized water and administered by stomach tube at 0 or 1000 mg/kg bw per day, 5 days/week, for 2 weeks. No clinical, haematological, biochemical or histopathological abnormalities were observed, except for a decreased number of lymphocytes in both males and females (Parkinson, 1974).

The acute toxicity of diquat monopyridone and diquat dipyridone (6,7-dihydro-4,9-dioxodipyrido(1,2-a:2′,1′-c) pyrazine) was evaluated in rats. Preliminary data suggest that diquat monopyridone is less toxic than diquat when administered orally. Diquat monopyridone and diquat were administered at equivalent doses of 90 µmol/kg bw (i.e. 16 mg active ingredient/kg bw) to groups of 10 male Alderley Park rats. The animals in the monopyridone group showed no adverse effects for the entire 14-day period studied, with no deaths occurring. In the diquat group, however, all animals appeared subdued by day 3, and many animals developed abdominal distension. Nine animals of the original group of 10 were dead by the 14th day after dosing, with the remaining animal exhibiting abdominal distension. When diquat dipyridone was administered at a dose of 90 µmol/kg bw (i.e. 16 mg active ingredient/kg bw) to 10 rats, the animals showed no adverse effects for the entire 14-day period studied, with no deaths occurring. Both diquat dipyridone and diquat monopyridone (major metabolites of diquat) are less toxic than diquat dichloride when administered by subcutaneous injection to male rats (Crabtree, 1976).

An acute oral toxicity study was conducted for a plant metabolite, TOPPS (R32245), to determine its toxicity relative to that of diquat. TOPPS was not found in the rat metabolism study, but it has been detected in a livestock metabolism study. In the acute oral toxicity study, groups of five male and five female fasted adult Alpk:AP (Wistar-derived) rats were given a single dose of TOPPS (purity 100%) in deionized water at 2000, 3000 or 5000 mg/kg bw and observed for up to 15 days.

No signs of toxicity or mortality were observed in the animals dosed with 2000 mg/kg bw. At 3000 and 5000 mg/kg bw, the signs of toxicity seen were sedation, tremors, piloerection, upward curvature of the spine, decreased breathing rate and increased breathing depth in surviving animals. All the males were found dead and two females survived following a dose of 3000 mg/kg bw. All animals in the 5000 mg/kg bw group died. All deaths occurred between 1 and 5 hours after dosing.

The acute oral LD_{50} was 2449 mg/kg bw for male rats and 2942 mg/kg bw for female rats (Southwood, 1987).

The mutagenic potential of TOPPS was determined using a deoxyribonucleic acid (DNA) repair test (rec-assay) and reverse mutation assay. TOPPS was found to be negative for mutagenicity in the DNA repair assay using *Bacillus subtilis* strains and in the reverse mutation assay using *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* WP2 uvrA, in the presence and absence of metabolic activation (Ohta, Watanabe & Tsukamoto, 1987).

Plasma corticosteroid concentrations were significantly elevated 15 minutes after intraperitoneal injection of 90 µmol diquat/kg bw into phenobarbitone-anaesthetized rats. Similar effects were noted after subcutaneous dosing. After oral administration, there was a delay of approximately 1 hour before significantly elevated concentrations were observed. The magnitude of the increase after intraperitoneal administration of diquat was dose related up to 26 µmol/kg bw, as was the duration of the response. The increase in plasma corticosteroid concentration in diquat-treated rats could be prevented by pretreating animals with dexamethasone, which also reduced adrenocorticotrophic hormone (ACTH) concentrations in these animals. Experiments in vivo and in vitro indicated that diquat did not increase the steroidogenic response of adrenals to ACTH. It was concluded that the increased adrenal steroid synthesis observed at early times after diquat administration is caused by release of ACTH from the pituitary (Crabtree & Rose, 1976).

Administration of diquat prevents the normal depletion of liver glycogen in starved rats. There is an increase in blood glucose, which returns to normal after approximately 7 hours. Diquat administration increases plasma corticosteroids for at least 24 hours, but plasma ACTH is elevated only for 4 hours. Adrenal cyclic adenosine monophosphate is increased after diquat administration for at least 24 hours. It is suggested that diquat increases the response of the adrenal cortex to ACTH (Crabtree et al., 1973).

Following oral administration of 900 µmol diquat ion/kg bw to male rats, there was a rapid accumulation of water in the lumen of the gastrointestinal tract of all animals, which reached a maximum at 24 hours (about 14 mL water per rat at 24 hours). Blood, liver and muscle showed significant dehydration. Deaths occurred over 9 days, with about 50% occurring within the first 3 days. Animals that did not gain weight 24 hours after the oral dose died within the first 3 days and had significantly greater gastrointestinal water content and more pronounced haemoconcentration than animals that did gain weight. After subcutaneous injection of 90 µmol diquat ion/kg bw, there was only a minimal increase in water content of the gastrointestinal tract at 24 hours. Both mean water content and dry weight of the gastrointestinal tract increased with time. At 14 days, surviving animals could be divided into two distinct groups: those that showed severe abdominal distension and those that appeared normal. Only the abdominally distended animals showed increased water content and dry weight of the gastrointestinal tract. Severe changes were limited to animals that died between 2 and 8 weeks following dosing and had significantly increased tissue water.

It was concluded that diquat has an effect on water distribution within the body following oral dosing, and early death is associated with rapid fluid loss into the gastrointestinal tract. Gastrointestinal distension following subcutaneous administration appeared to be an unrelated phenomenon (Crabtree, Lock & Rose, 1976).

Following the administration to rats of approximately equitoxic doses of paraquat and diquat by intraperitoneal injection, the pattern of mortality was different. After dosing with paraquat, there were no deaths in the first 24 hours, whereas 22% of the rats given diquat died in this period. About 34% of the paraquat-treated rats died on the 3rd day after dosing. The death of paraquat-poisoned rats has been shown to be related to lung damage, as measured by a greatly increased water content. There was no similar increase in the water content of lungs taken from rats poisoned with diquat. Thymidine incorporation into lung DNA was significantly decreased 1 day after dosing with both paraquat and diquat, but became greatly elevated in a proportion of those rats given paraquat, which survived 3 days after dosing. In diquat-poisoned rats, thymidine incorporation into lung DNA remained depressed for at least 8 days.

It was concluded that (1) most rats given paraquat by intraperitoneal injection die from acute lung damage characterized by a dramatic increase in the lung water content; (2) only a small proportion die many days later from lung fibrosis characterized by an increase in thymidine incorporation into DNA; and (3) diquat does not cause lung damage as characterized by increases in either the water content of lung or thymidine incorporation into DNA (Smith & Rose, 1977).

3. Observations in humans

No data were provided by the company, other than the publication by Sechi et al. (1992).

In cases of acute diquat poisoning following oral ingestion, management of the massive fluid losses into the gastrointestinal tract and their potential circulatory and renal consequences is critical, with special attention given to maintaining adequate hydration of the patient. Intestinal paralysis and fluid loss are prominent features of diquat intoxication and may lead to abdominal distension, tissue dehydration, hypotensive shock and severe cerebrovascular complications due to brain stem infarction and/or intracranial haemorrhage (Oreopoulos & McEvoy, 1969; Vanholder et al., 1981; Jones & Vale, 2000; Saeed, Wilks & Coupe, 2001).

Despite the lack of findings indicating neurotoxicity in animals, diquat has aroused interest because its analogue, paraquat, is perceived as being structurally similar to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which is known to elicit a condition similar to Parkinson disease in humans. MPTP and its metabolite 1-methyl-4-phenylpyridine (MPP^+) accumulate in melanin and cause degeneration of the melanin-containing neurons in the substantia nigra. Diquat has therefore also come under scrutiny as a possible environmental factor in the development of Parkinson disease (Sechi et al., 1992).

There is a report in the published literature of a 72-year-old farmer with a history of diabetes and transient right-sided hemiparesis who developed erythema of the skin with hyperkeratosis and conjunctivitis after exposure of the hands to a 10% diquat solution for about 10 minutes. About 10 days later, he developed akathisia with moderate hyperexcitability and insomnia. Over a period of 5 days, he became dysphonic, bradykinetic and rigid. Treatment with carbidopa/levidopa and bromocriptine significantly improved his symptoms. A magnetic resonance imaging scan 4 months after the onset of the illness showed small, multiple, bilateral, symmetric areas of high signal intensity in the caudate nuclei and putamen and in the white matter near the ventricular wall. The authors suggested a causal relationship to diquat exposure. However, there were no clinical signs suggestive of systemic diquat poisoning, nor has there been any other description of Parkinson-like illness following diquat exposures (Sechi et al., 1992).

The results of large epidemiological studies specifically investigating exposure to diquat and the development of Parkinson disease have not shown any evidence of an increased risk (Tanner et al., 2009).

Most cases of diquat poisoning reported to date have resulted from the intentional, usually suicidal, ingestion of concentrated solutions; rarely, accidental ingestion has occurred as a consequence of decanting diquat concentrates into soft drink bottles. Over the period 1968–1999, only 30 cases of diquat poisoning were reported in detail in the literature, of which 13 (43%) were fatal. Local and systemic effects have been reported following diquat exposure, with systemic features being invariably associated with ingestion. In severe and usually fatal cases, gastrointestinal mucosal ulceration, paralytic ileus, hypovolaemic shock, acute renal failure and coma have been reported (Jones & Vale, 2000).

In a report in the published literature, a 52-year-old Caucasian man with a history of alcohol abuse who ingested about 160 mL of "weed killer" (confirmed as diquat ion) with suicidal intent after his wife left him developed aggressive behaviour, oliguric renal failure and intracerebral bleeding. The patient was successfully managed and made a complete recovery. In this paper, special attention was given to the major clinical differences between diquat and paraquat intoxication (Saeed, Wilks & Coupe, 2001).

In a report in the published literature, two cases of diquat poisoning were described. In the first case, a 35-year-old, previously healthy woman ingested a 14% diquat solution. The poisoning had a fulminant course, consisting of severe stomach ache, vomiting, cardiocirculatory shock, respiratory failure and cardiac arrest 20 hours post-ingestion. Autopsy revealed myocardial infarction, bronchopneumonia and incipient renal damage. In the second case, a 64-year-old man developed severe gastroenteritis, corrosive lesions of mucosal surfaces, acute renal injury, arrhythmias, brain stem infarction and bronchopneumonia. The patient died 18 days post-exposure. The most prominent findings on autopsy were pontine haemorrhage and infarction, bronchopneumonia, left ventricle papillary muscle infarction and renal tubular damage. Cardiocirculatory disturbances led to fatal complications, the heart and brain infarction. The study authors pointed out the heart as one of the most severely affected organs in diquat poisoning (Jović-Stošić, Babić & Todorović, 2009).

In a report in the published literature, an 18-year-old man accidentally took a mouthful of undiluted diquat. Although he spat most of it out, he is quite certain that he swallowed a small quantity. Over the next 10 hours, he felt worse and developed diarrhoea (5–6 episodes). The patient reported difficulty in swallowing. There was a small ulcer on the upper surface of the tongue and another on the right side of his pharynx. He was not cyanosed, and there were no abnormal physical signs in the chest. The diquat excretion lasted for about 11 days (Oreopoulos & McEvoy, 1969).

Comments

Biochemical aspects

When administered orally, [^{14}C]diquat is poorly absorbed (< 10%) from the gastrointestinal tract of rats and eliminated mainly via the faeces (about 94%) during the first 24 hours. The small amount absorbed is rapidly eliminated via the urine (about 6%). Biliary excretion represented less than 0.7% of the administered dose. Peak tissue and blood levels were seen at approximately 2 hours, followed by a rapid decline. Liver, kidney, gastrointestinal tract and lung had the highest residues immediately following dosing; at 96 hours, significant residues were seen primarily in the eye lens.

After oral administration of [^{14}C]diquat to rats (45 mg diquat ion/kg bw), the major excreted product was diquat in both urine (about 6% of the dose) and faeces (about 89% of the dose); diquat monopyridone was the main metabolite in the faeces (5% of dose), but a minor one in the urine. In another oral study in rats (100 mg diquat ion/kg bw), small amounts of diquat dipyridone and picolinic acid were found, in addition to the monopyridone, in urine. The biotransformation of diquat is postulated to proceed either by progressive oxidation of the pyridine rings to form diquat monopyridone and diquat dipyridone or by the cleavage of one of the pyridine rings to form picolinic acid, possibly via pyridine-2-carboxamide as an intermediary metabolite, although this was not identified.

Toxicological data

The acute oral LD_{50} in rats was 214 mg diquat ion/kg bw. There were no mortalities or clinical signs of toxicity at 100 mg/kg bw. The acute dermal LD_{50} in rats was greater than 424 mg diquat ion/kg bw. The 4-hour acute inhalation LC_{50} in rats was 0.121 mg/L. Diquat was moderately to severely irritating to rabbit skin and mildly irritating to rabbit eyes. Diquat was a skin sensitizer in the Magnusson and Kligman test.

Unlike paraquat, diquat is not actively taken up by lung slices, and lung toxicity is not characteristic of diquat poisoning. The eye was the main target organ following short-term repeated exposure in rats and dogs. In addition, effects on kidney, liver and haematological parameters were also observed.

In a 90-day toxicity study in rats using dietary concentrations of 0, 20, 100 and 500 ppm (equal to 0, 1.7, 8.5 and 39.5 mg diquat ion/kg bw per day for males and 0, 1.9, 9.2 and 41.5 mg diquat ion/kg bw per day for females, respectively), the NOAEL was 100 ppm (equal to 8.5 mg diquat ion/kg bw per day), based on decreased body weight gain and feed consumption, changes in clinical chemistry parameters, increased urine volume, decreased urinary specific gravity, minor changes in haematological values, erosion of the tongue and oral cavity and ocular changes at 500 ppm (equal to 39.5 mg/kg bw per day). An investigative 90-day toxicity study in rats was conducted at dietary concentrations of 0, 30, 60 and 300 ppm diquat ion (equal to 0, 2.4, 4.7 and 23.2 mg diquat ion/kg bw per day for males and 0, 2.7, 5.0 and 25.3 mg diquat ion/kg bw per day for females, respectively) to determine the NOAEL for cataract formation in rats. The NOAEL was 60 ppm (equal to 4.7 mg diquat ion/kg bw per day) for ocular lesions and lens opacities evident at 300 ppm (equal to 23.2 mg diquat ion/kg bw per day). The overall NOAEL in the 90-day toxicity studies in rats was 100 ppm (equal to 8.5 mg diquat ion/kg bw per day), with an overall LOAEL of 300 ppm (equal to 23.2 mg diquat ion/kg bw per day).

In a 1-year feeding study in dogs, with achieved dietary intakes of 0, 0.46, 2.42 and 11.48 mg diquat ion/kg bw per day for males and 0, 0.53, 2.53 and 13.21 mg diquat ion/kg bw per day for females, the NOAEL was 0.53 mg diquat ion/kg bw per day, based on lens opacity (cataracts) in females at 2.53 mg diquat ion/kg bw per day.

In a 2-year toxicity and carcinogenicity study in mice using dietary concentrations of 0, 30, 100 and 300 ppm (equal to 0, 3.56, 11.96 and 37.83 mg diquat ion/kg bw per day for males and 0, 4.78, 16.03 and 48.27 mg diquat ion/kg bw per day for females, respectively), the NOAEL was 30 ppm (equal to 3.56 mg diquat ion/kg bw per day), based on reduction in body weight gain, increased

relative kidney weights and ocular discharges at 100 ppm (equal to 11.96 mg diquat ion/kg bw per day) and above. There was no evidence of carcinogenicity in mice at doses up to and including 300 ppm (equal to 37.83 mg diquat ion/kg bw per day), the highest dose tested.

A 2-year study of toxicity and carcinogenicity in rats was conducted using dietary concentrations of 0, 5, 15, 75 and 375 ppm (equal to 0, 0.19, 0.58, 2.91 and 14.88 mg diquat ion/kg bw per day for males and 0, 0.24, 0.72, 3.64 and 19.44 mg diquat ion/kg bw per day for females, respectively). There were no effects on survival, changes in haematology, clinical chemistry or urine analysis parameters or neoplastic changes that were considered to be of toxicological significance at any dose level. A treatment-related incidence of opacities of the lens was found in male and female animals receiving 75 and 375 ppm. Among rats receiving 375 ppm, these lesions progressed to total opacification of the lens, affecting all surviving rats receiving 375 ppm when examined at week 104. A low, but slightly higher than in controls, incidence of rats with opacities was seen in the 15 ppm group at 104 weeks only. Cataractous change was observed in the lenses of rats at 75 and 375 ppm. The NOAEL was concluded to be 5 ppm (equal to 0.19 mg diquat ion/kg bw per day) in the 1993 JMPR evaluation. Since that evaluation, the cataract data at 15 ppm have been re-evaluated by the original pathologists. Cataracts were seen in controls and all dose groups, and the incidence and severity were dose related. The present Meeting noted the conclusion of the re-evaluation of the cataract data: that, in contrast to the observations at 75 and 375 ppm, there was no evidence of progression of the disease at 15 ppm that differed from the controls. The present Meeting identified the NOAEL as 15 ppm (equal to 0.58 mg diquat ion/kg bw per day), as the incidence and severity of cataracts were comparable with those of the controls. There was no evidence of carcinogenicity in this study.

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

Diquat was tested for genotoxicity in vitro and in vivo in an adequate range of assays. It gave an equivocal response in the absence of metabolic activation and a positive response in the presence of metabolic activation in the mammalian cell cytogenetic assay; however, it was negative in the in vivo mouse micronucleus assay and dominant lethal assay.

The Meeting concluded that diquat is unlikely to be genotoxic in vivo.

On the basis of the lack of genotoxicity in vivo and the absence of carcinogenicity in mice and rats, the Meeting concluded that diquat is unlikely to pose a carcinogenic risk to humans from the diet.

In a two-generation study of reproductive toxicity in rats using dietary concentrations of 0, 16, 80 and 400/240 ppm (equal to 0, 1.6, 7.9 and 38.7 mg diquat ion/kg bw per day for males and 0, 1.7, 8.4 and 40.4 mg diquat ion/kg bw per day for females, respectively), the NOAEL for parental systemic toxicity was 16 ppm (equal to 1.6 mg diquat ion/kg bw per day), based on a low incidence of mouth lesions in both generations and a slightly increased incidence of cataracts at 80 ppm (equal to 7.9 mg diquat ion/kg bw per day). The NOAEL for reproductive toxicity was 400/240 ppm (equal to 38.7 mg diquat ion/kg bw per day), the highest dose tested. The NOAEL for offspring toxicity was 80 ppm (equal to 7.9 mg diquat ion/kg bw per day), based on decreased pup body weights seen in both generations at 400/240 ppm (equal to 38.7 mg diquat ion/kg bw per day).

In a developmental toxicity study in rats that tested gavage doses of 0, 4, 12 and 40 mg diquat ion/kg bw per day, the NOAEL for maternal toxicity was 12 mg diquat ion/kg bw per day, based on reduced body weights and feed consumption seen at 40 mg diquat ion/kg bw per day. The NOAEL for embryo and fetal toxicity was 12 mg diquat ion/kg bw per day, based on reduced fetal weight, haemorrhagic kidney and increased incidence of soft tissue and minor skeletal anomalies at 40 mg diquat ion/kg bw per day.

In a developmental toxicity study in rabbits that tested gavage doses of 0, 1, 3 and 10 mg diquat ion/kg bw per day, the NOAEL for maternal toxicity was 1 mg diquat ion/kg bw per day, based on reduced weight gain and feed consumption at 3 mg diquat ion/kg bw per day. The NOAEL for embryo and fetal toxicity was 3 mg diquat ion/kg bw per day, based on skeletal anomalies at 10 mg diquat ion/kg bw per day.

The Meeting concluded that diquat is not teratogenic in rats or rabbits.

Diquat has been tested for neurotoxicity in acute and repeated-dose studies in rats. In neither study was there any indication of neurotoxicity. In an acute neurotoxicity study in rats that tested gavage doses of 0, 25, 75 and 150 mg diquat ion/kg bw per day, the NOAEL for general toxicity was 75 mg diquat ion/kg bw, based on a range of findings, including clinical signs, at 150 mg diquat ion/kg bw. In the 90-day study of neurotoxicity in rats using dietary concentrations of 0, 20, 100 and 400 ppm (equal to 0, 1.6, 7.9 and 32.4 mg/kg bw per day for males and 0, 1.9, 9.5 and 38.5 mg/kg bw per day for females, respectively), the NOAEL for systemic toxicity was 100 ppm (equal to 7.9 mg diquat ion/kg bw per day), based on evidence of eye lesions and reductions in body weight gain and feed consumption at 400 ppm (equal to 32.4 mg diquat ion/kg bw per day).

The Meeting concluded that diquat is not neurotoxic.

In a 28-day study of immunotoxicity in female mice using dietary concentrations of 0, 100, 200 and 350 ppm (equal to 0, 23, 44 and 81 mg diquat ion/kg bw per day), there was no evidence of immunotoxicity at any dose tested.

Toxicological data on metabolites and/or degradates

Diquat monopyridone, the major metabolite following oral dosing, was less toxic than the parent compound. The oral LD_{50} for diquat monopyridone was greater than 4000 mg/kg bw. Male and female rats orally administered diquat monopyridone at a dose of 1000 mg/kg bw per day, 5 days/week, for 2 weeks showed no clinical, haematological, biochemical or histopathological abnormalities, except for a decreased number of lymphocytes in both males and females.

TOPPS, a metabolite in livestock but not in rats, was less toxic than the parent compound. The acute oral LD_{50} of TOPPS in rats was 2449 mg/kg bw. There was no evidence of genotoxicity in the DNA repair test.

Human data

No reports of adverse health effects in manufacturing plant personnel were provided. In humans, intestinal paralysis and fluid loss are prominent features of diquat intoxication and may lead to abdominal distension, tissue dehydration, hypotensive shock and severe cerebrovascular complications due to brain stem infarction and/or intracranial haemorrhage.

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

Toxicological evaluation

The Meeting established an ADI of 0–0.006 mg diquat ion/kg bw on the basis of a NOAEL of 0.58 mg diquat ion/kg bw per day in the 2-year carcinogenicity study in rats on the basis of cataracts seen at 2.9 mg diquat ion/kg bw per day. A safety factor of 100 was applied. This ADI is supported by the NOAEL of 0.46 mg diquat ion/kg bw per day observed in a 1-year toxicity study in dogs on the basis of cataracts seen at the LOAEL of 2.53 mg diquat ion/kg bw per day. The present ADI is based on the same study and end-point selected by the 1993 JMPR, but using a different NOAEL value. The current Meeting identified the NOAEL as 0.58 mg diquat ion/kg bw per day, as the incidence, progression with time and severity of cataracts at this dose were comparable with those in the controls.

An ARfD of 0.8 mg/kg bw was established on the basis of a NOAEL of 75 mg diquat ion/kg bw in an acute neurotoxicity study in rats, based on clinical signs and decreased body weight gains in the 1st week and decreased feed consumption seen at the LOAEL of 150 mg diquat ion/kg bw. A safety factor of 100 was applied. This ARfD is further supported by the acute oral toxicity study in

rats (LD_{50} = 214 mg/kg bw) in which no mortality or clinical signs of toxicity were observed at 100 mg/kg bw. The Meeting considered reductions in body weight gain seen at the start of gavage dosing in several studies (e.g. developmental toxicity studies) to be secondary to gastrointestinal irritation and not relevant to establishing an ARfD. The Meeting concluded that the critical effects in the longer-term studies of eye lesions were not likely to be produced following a single dose, as the eye lesions were normally evident only after several weeks of continuous dosing.

Levels relevant to risk assessment of diquat

Species	Study	Effect	NOAEL	LOAEL
Mouse	Two-year study of toxicity and carcinogenicity[a]	Toxicity	30 ppm, equal to 3.6 mg diquat ion/kg bw per day	100 ppm, equal to 12.0 mg diquat ion/kg bw per day
		Carcinogenicity	300 ppm, equal to 37.8 mg diquat ion/kg bw per day[b]	–
Rat	Acute neurotoxicity study[c]	Toxicity	75 mg diquat ion/kg bw	150 mg diquat ion/kg bw
	Ninety-day studies of toxicity[a,d]	Toxicity	100 ppm, equal to 8.5 mg diquat ion/kg bw per day	300 ppm, equal to 23.2 mg diquat ion/kg bw per day
	Two-year study of toxicity and carcinogenicity[a]	Toxicity	15 ppm, equal to 0.58 mg diquat ion/kg bw per day	75 ppm, equal to 2.9 mg diquat ion/kg bw per day
		Carcinogenicity	375 ppm, equal to 14.9 mg diquat ion/kg bw per day[b]	–
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	400/240 ppm, equal to 38.7 mg diquat ion/kg bw per day[b]	–
		Parental toxicity	16 ppm, equal to 1.6 mg diquat ion/kg bw per day	80 ppm, equal to 7.9 mg diquat ion/kg bw per day
		Offspring toxicity	80 ppm, equal to 7.9 mg diquat ion/kg bw per day	400/240 ppm, equal to 38.7 mg diquat ion/kg bw per day
	Developmental toxicity study[c]	Maternal toxicity	12 mg diquat ion/kg bw per day	40 mg diquat ion/kg bw per day
		Embryo and fetal toxicity	12 mg diquat ion/kg bw per day	40 mg diquat ion/kg bw per day
Rabbit	Developmental toxicity study[c]	Maternal toxicity	1 mg diquat ion/kg bw per day	3 mg diquat/kg bw per day
		Embryo and fetal toxicity	3 mg diquat ion/kg bw per day	10 mg diquat ion/kg bw per day
Dog	One-year study of toxicity[a]	Toxicity	0.53 mg diquat ion/kg bw per day	2.53 mg diquat ion/kg bw per day

LOAEL: lowest-observed-adverse-effect level; NOAEL: no-observed-adverse-effect level
[a] Dietary administration.
[b] Highest dose tested.
[c] Gavage administration.
[d] Two or more studies combined.

Estimate of acceptable daily intake

0–0.006 mg/kg bw

Estimate of acute reference dose

0.8 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 diquat

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Poor, ~10% in rat
Dermal absorption	About 6% in 24 h, rat skin
Distribution	Widely distributed, highest levels in eye lens
Potential for accumulation	Potential accumulation only in eye lens
Rate and extent of excretion	Rapid, absorbed dose extensively excreted (> 90%) in urine and bile within 96 h
Metabolism in animals	Metabolism was limited, with < 20% of the urinary residues (< 1% of the administered dose) consisting of metabolites
Toxicologically significant compounds in animals, plants and the environment	Diquat ion
Acute toxicity	
Rat, LD_{50}, oral	214 mg diquat ion/kg bw
Rat, LD_{50}, dermal	> 424 mg diquat ion/kg bw
Rat, LC_{50}, inhalation	0.121 mg diquat ion/L
Rabbit, dermal irritation	Moderately to severely irritating
Rabbit, ocular irritation	Mildly irritating
Guinea-pig, dermal sensitization	Sensitizing (Magnusson and Kligman)
Short-term studies of toxicity	
Target/critical effect	Cataract, body weight (rat, dog)
Lowest relevant oral NOAEL	0.53 mg diquat ion/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	Cataract (rat), kidney lesions (mouse)
Lowest relevant NOAEL	0.58 mg diquat ion/kg bw per day (rat)
Carcinogenicity	Unlikely to pose a carcinogenic risk to humans from the diet
Genotoxicity	
	Not genotoxic in vivo
Reproductive toxicity	
Target/critical effect	No reproductive toxicity
Lowest relevant parental NOAEL	1.6 mg diquat ion/kg bw per day

Lowest relevant offspring NOAEL	7.9 mg diquat ion/kg bw per day
Lowest relevant reproductive NOAEL	38.7 mg diquat ion/kg bw per day, the highest dose tested
Developmental toxicity	
Target/critical effect	Reduced body weight, delayed ossification, skeletal anomalies at maternally toxic doses
Lowest relevant maternal NOAEL	1 mg diquat ion/kg bw per day (rabbit)
Lowest relevant embryo and fetal NOAEL	3 mg diquat ion/kg bw per day (rabbit)
Neurotoxicity	
Acute and subchronic neurotoxicity	Not neurotoxic
Other toxicological studies	
Immunotoxicity	Not immunotoxic
Studies on metabolites	Diquat monopyridone, diquat dipyridone and TOPPS were less toxic than parent diquat
Medical data	
	No reports submitted; characteristics of intoxication obtained from published literature

LC_{50}: median lethal concentration; LD_{50}: median lethal dose; NOAEC: no-observed-adverse-effect concentration; NOAEL: no-observed-adverse-effect level; TOPPS: 1,2,3,4-tetrahydro-1-oxopyrido-(1,2-a)-5-pyrazinium salt

Summary

	Value	Study	Safety factor
ADI	0–0.006 mg/kg bw	Two-year study of carcinogenicity in rats	100
ARfD	0.8 mg/kg bw	Acute study of neurotoxicity in rats	100

ADI: acceptable daily intake; ARfD: acute reference dose

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DITHIANON (addendum)

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Explanation

Dithianon (Chemical Abstracts Service No. 3347-22-6) was evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 2010, when an acceptable daily intake (ADI) of 0–0.01 mg/kg body weight (bw) and an acute reference dose (ARfD) of 0.1 mg/kg bw were established. During the 2013 JMPR, concerns were raised because of the presence of degradation products of dithianon: Reg. No. 31062 and Reg. No. 4110904. Genotoxicity studies were made available during the meeting for these two degradation products.

Evaluation for acceptable daily intake

1. Genotoxicity of dithianon metabolites

Genotoxicity data were submitted for two metabolites of dithianon: Reg. No. 31062 and Reg. No. 4110904. The data are summarized in Table 1. Neither metabolite was found to be genotoxic.

Table 1. Summary of genotoxicity studies on two dithianon metabolites

Study	Strain/species	Substance; concentration/dose	Purity (%)	Result	Reference
Reg. No. 31062					
In vitro					
Bacterial reverse mutation assay (Ames test)	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537; *Escherichia coli* WP2 uvrA	Standard plate test: 0, 20, 100, 500, 2 500 and 5 000 µg/plate Preincubation test: 0, 20, 100, 500, 2 500 and 5 000 µg/plate	99	Negative (±S9)	Schulz & Landsiedel (2007)
In vivo					
Micronucleus assay	NMRI mouse bone marrow cells	24 h preparation interval: 500, 1 000 and 2 000 mg/kg bw 48 h preparation interval: 2 000 mg/kg bw	97.2	Negative	Dony (2013a)

Table 1 (continued)

Study	Strain/species	Substance; concentration/dose	Purity (%)	Result	Reference
Reg. No. 4110904					
In vitro					
Bacterial reverse mutation assay (Ames test)	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2 uvrA	Standard plate test: 0, 33, 100, 333, 1 000, 3 250 and 6 500 µg/plate Preincubation test: 0, 10, 33, 100, 333, 1 000 and 3 250 µg/plate (*Salmonella* strains) Preincubation test: 0, 33, 100, 333, 1 000, 3 250 and 6 500 µg/plate (*E. coli*)	77.4	Negative (±S9)	Woitkowiak (2013)
Gene mutation test (*HPRT* locus assay)	CHO cells	1st experiment +S9: 6.3, 12.5, 25.0, 50.0, 100.0 and 200.0 µg/mL 2nd experiment −S9: 3.1, 6.3, 12.5, 25.0, 50.0, 100.0 and 150.0 µg/mL +S9: 6.3, 12.5, 25.0, 50.0, 100.0, 150.0 and 200.0 µg/mL 3rd experiment +S9: 1.6, 3.1, 6.3, 12.5, 25.0, 50.0, 100.0 and 150.0 µg/mL 4th experiment +S9: 3.1, 6.3, 12.5, 25.0, 50.0, 100.0, 150.0 and 200.0 µg/mL	77.4	Negative (±S9)	Schulz & Landsiedel (2012)
Chromosomal aberration assay	V79 cells	1st experiment −S9: 3.13, 6.25, 12.50, 25.00, 50.00, 100.00, 200.00 and 400.00 µg/mL 2nd experiment ±S9: 1.56, 3.13, 6.25,12.50, 25.00, 50.00, 100.00 and 200.00 µg/mL	77.4	Positive	Schulz & Landsiedel (2013)
Chromosomal aberration assay	Human lymphocytes	26.8–4 130.0 µg/mL	75.6	Negative (±S9)	Bohnerberger (2013)
In vivo					
Micronucleus assay	NMRI mouse bone marrow cells	Females: 24 h preparation interval: 250, 500 and 1 000 mg/kg bw 48 h preparation interval: 1 000 mg/kg bw Males: 24 h preparation interval: 500, 1 000 and 2 000 mg/kg bw 48 h preparation interval: 2 000 mg/kg bw	75.6	Negative	Dony (2013b)

Study	Strain/species	Substance; concentration/dose	Purity (%)	Result	Reference
Comet assay	Liver and duodenum cells of Wistar (Hsd:WI) rats	250, 500 and 1 000 mg/kg bw per day	75.6	Non-DNA damaging	Pant & Celestin (2013)

CHO: Chinese hamster ovary; DNA: deoxyribonucleic acid; HPRT: hypoxanthine–guanine phosphoribosyl-transferase; S9: 9000 × *g* supernatant fraction of rat liver homogenate

Comments

Reg. No. 31062 gave negative results in the Ames test and in the in vivo micronucleus assay in mice.

Reg. No. 4110904 gave negative results in the Ames test, the in vitro gene mutation (*HRPT* locus) test in Chinese hamster ovary cells and a test for chromosomal aberration in human lymphocytes. It gave positive results in a test for chromosomal aberration in V79 cells. However, the in vivo micronucleus assay in mice and the comet assay in liver and duodenum in rats gave negative results.

The Meeting concluded that these degradates of dithianon are unlikely to be genotoxic in vivo.

Toxicological evaluation

Conservative estimates of exposure were 0.08 µg/kg bw per day for chronic exposure and 1.92 µg/kg bw for acute exposure for both Reg. No. 31062 and Reg. No. 4110904. All estimates of exposure were below the threshold of toxicological concern for compounds with no evidence of genotoxicity (for Cramer class III, 1.5 µg/kg bw per day for chronic exposure; 5 µg/kg bw for acute exposure). The Meeting concluded that there is no concern for dietary exposure to these degradates.

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FENAMIDONE

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Explanation

Fenamidone is the International Organization for Standardization–approved common name for (5*S*)-5-methyl-2-(methylthio)-5-phenyl-3-(phenylamino)-3,5-dihydro-4*H*-imidazol-4-one (International Union of Pure and Applied Chemistry [IUPAC]), with Chemical Abstracts Service No. 161326-34-7 (Fig. 1). There is no conversion to the *R*-enantiomer in biological systems. Fenamidone is a foliar fungicide used on vegetables and ornamentals.

Fig. 1. Structure of fenamidone (RPA 407213)

CH_3 * N CH_3 O N S N H

* Denotes the position of the chiral centre

Fenamidone 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.

Unless otherwise stated, the contemporary unpublished 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. As these guidelines specify the clinical pathology tests normally performed and the tissues normally examined, only significant exceptions to these guidelines are reported here, to avoid repetitive listing of study parameters.

Evaluation for acceptable daily intake

1. Biochemical aspects

Two different radiolabelled forms of [^{14}C]fenamidone were used in the pivotal rat metabolism study: [C-phenyl-U-^{14}C]- and [N-phenyl-U-^{14}C]-labelled preparations (Fig. 2).

Fig. 2. Position of the radiolabel in ^{14}C-labelled fenamidone used in rat metabolism studies

[C-Phenyl-U-^{14}C]-labelled fenamidone　　[N-Phenyl-U-^{14}C]-labelled fenamidone

* Denotes the position of the radiolabel

1.1 Absorption, distribution and excretion

Rats

Fenamidone was administered to Sprague-Dawley (SD) rats (five of each sex per dose) as a single gavage dose of [C-phenyl-^{14}C-U]fenamidone (radiochemical purity 99.5%) in aqueous methyl cellulose (0.75% weight per volume [w/v]) at 3 mg/kg body weight (bw), a single gavage dose of radiolabelled fenamidone at 300 mg/kg bw or 14 daily gavage doses of unlabelled fenamidone (purity > 98.9%) at 3 mg/kg bw followed by a single gavage dose of radiolabelled fenamidone at 3 mg/kg bw. Separate groups of rats were dosed similarly with [N-phenyl-U-^{14}C]fenamidone (98.7% purity) as either a single or repeated gavage dose of 3 mg/kg bw. Urine and faeces were collected at 24-hour intervals to 168 hours. Following termination at 168 hours, a range of tissues was sampled. Radioactivity was quantified in urine, faeces and tissues by liquid scintillation counting. Metabolites were analysed by thin-layer chromatography, high-performance liquid chromatography and liquid chromatography–mass spectrometry.

In a pharmacokinetics experiment, [C-phenyl-U-^{14}C]fenamidone (radiochemical purity 99.5%) was administered to SD rats (five of each sex per dose) as a single gavage dose of 3 or 300 mg/kg bw. A separate group of five rats received a single gavage dose of [N-phenyl-U-^{14}C]fenamidone at 3 mg/kg bw. Blood was sampled at 0.5, 1, 2, 3, 4, 5, 6, 8, 24, 48, 72, 96, 120, 144 and 168 hours for the analysis of standard pharmacokinetic parameters.

In a tissue kinetics experiment, [C-phenyl-U-^{14}C]fenamidone (radiochemical purity 99.5%) was administered to SD rats (16 of each sex per dose) as a single gavage dose of 3 or 300 mg/kg bw. Four rats of each sex per dose were killed at 8, 56, 104 or 200 hours (males) or 24, 94, 168 or 292 hours (females). Radioactivity was analysed in various tissues.

The excretion of radioactivity was analysed in groups of bile duct–cannulated rats (five of each sex) that received a single gavage dose of [C-phenyl-U-^{14}C]- or [N-phenyl-U-^{14}C]fenamidone at 3 mg/kg bw. Bile and urine were collected at 0–6, 6–24 and 24–48 hours, whereas faeces were collected at 0–24 and 24–48 hours. Following termination at 48 hours, blood, the intestinal tract and its contents and the stomach and its contents were sampled for radiochemical analysis.

No signs of toxicity were reported.

The results of the excretion/mass balance phase of the study are summarized in Table 1. Mean recoveries of radioactivity across the five dose groups were greater than 95% of the administered dose. The majority of radioactivity was detected in the faeces (up to about 90% of the administered dose of [C-phenyl-U-^{14}C]fenamidone and up to about 64% with [N-phenyl-U-^{14}C]fenamidone, with the remainder mainly detected in urine (up to about 40% of the administered dose). Proportionally higher levels of radioactivity were detected in the urine following dosing with [N-phenyl-U-^{14}C]fenamidone. The majority of radioactivity (> 80%) was eliminated in urine and faeces within 48 hours of dosing. The level of radioactivity remaining in tissues 168 hours after dosing was less than 0.7% of the administered dose. Following administration of [N-phenyl-U-^{14}C]fenamidone as a single oral or repeated dose, the highest concentrations of radioactivity were detected in blood, liver, kidney, spleen and thyroid (all < 0.11 µg equivalents [eq]/g). In rats dosed with [C-phenyl-U-^{14}C]fenamidone, the highest concentrations of radioactivity were detected in the thyroid, blood, skin and fur, and liver, with the thyroid having the highest tissue to plasma ratios (approximately 80–500, depending on the dosing regimen) (Table 2).

Plasma kinetic parameters are summarized in Table 3. At the low oral dose, the time (T_{max}) to reach the maximum plasma concentration of radioactivity (C_{max}) was approximately 2–4 hours, whereas it was approximately 14 hours in males and 26 hours in females at the high oral dose, suggesting saturation of binding at the higher dose. The plasma elimination half-life ($t_{½}$) was at least 60 hours. At the low oral dose, systemic exposure (as shown by the area under the plasma concentration–time curve [AUC]) was comparable between males and females. However, at the high dose, males were less exposed than females; non-linear kinetics were evident in males, as shown by the less than proportional increase in AUC from 3 to 300 mg/kg bw.

The results of the study on the excretion/mass balance of radioactivity in bile duct–cannulated rats are summarized in Table 4. Recovery of radioactivity was greater than 92%. The majority of radioactivity was detected in bile (approximately 70–80% of the administered dose), followed by urine (approximately 6–17% of the administered dose). Based on the level of radioactivity in urine, bile, cage wash and tissues, the level of gastrointestinal absorption is estimated to be greater than 80% of the administered dose (range of 81–96%). Comparison of the levels of radioactivity recovered in bile kinetics and absorption, distribution, metabolism and excretion (ADME) studies suggested that a part of the radioactivity excreted via the bile underwent enterohepatic circulation and was subsequently re-excreted via the urine.

In the tissue kinetics experiment, the T_{max} was approximately the same time as that for plasma. The highest levels of radioactivity were detected in the gastrointestinal tract, stomach, thyroid, liver, pancreas and blood (Totis, 1999).

1.2 Biotransformation

Rats

In the above-described study, the analysis of metabolites in pooled urine, faeces and bile detected up to 22, 24 and 22 radioactive species, respectively, indicating that fenamidone was extensively metabolized. The major metabolites (> 10% of the administered dose) of fenamidone were free RPA 409352, RPA 409361, RPA 412708 and RPA 412636 (Table 5). In rats receiving the

low oral dose, conjugated metabolites of RPA 409352 or RPA 409361 were detected in urine (2.58% and 14.40% of the administered dose in males and females, respectively) and bile (45.67% and 54.56% of the administered dose in males and females, respectively). A sulfo conjugate of RPA 408056 was detected in bile (1.99% and 3.10% of the administered dose in males and females, respectively). An examination of the chirality of selected metabolites by the author found no interconversion from the *S*- to *R*-enantiomer.

Table 1. Mass balance in rats following oral dosing with [C-phenyl-U-^{14}C]- or [N-phenyl-U-^{14}C]fenamidone

Sample	Mean % of administered radioactivity					
	3 mg/kg bw (single dose)		300 mg/kg bw (single dose)		3 mg/kg bw (repeated dose)	
	Males	Females	Males	Females	Males	Females
[C-phenyl-U-^{14}C]Fenamidone						
Urine						
0–24 h	6.74	27.45	4.65	5.66	6.34	23.17
0–48 h	10.48	36.21	8.65	10.86	9.10	28.66
0–72 h	11.94	38.64	10.02	12.39	10.53	30.44
0–168 h	12.79	39.94	10.56	13.01	11.39	31.31
Faeces						
0–24 h	47.84	30.78	54.54	72.00	32.92	44.52
0–48 h	69.90	47.33	76.53	87.95	72.32	56.91
0–72 h	77.70	50.94	82.38	90.39	81.35	59.58
0–168 h	80.69	52.11	83.65	90.99	84.68	60.45
Cage wash	2.35	5.68	2.53	2.47	3.98	10.49
Tissues	0.65	0.45	0.29	0.27	0.54	0.52
Recovery	96.47	98.27	97.02	106.74	100.59	102.76
[N-phenyl-U-^{14}C]Fenamidone						
Urine						
0–24 h	15.99	26.47	–	–	25.91	33.04
0–48 h	23.48	36.74	–	–	35.48	41.56
0–72 h	25.57	39.28	–	–	38.72	44.73
0–168 h	26.59	40.45	–	–	40.58	46.49
Faeces						
0–24 h	41.56	32.53	–	–	13.82	28.94
0–48 h	57.57	44.80	–	–	31.94	39.45
0–72 h	63.08	47.85	–	–	38.87	41.68
0–168 h	64.28	49.55	–	–	52.03	44.71
Cage wash	4.09	5.44	–	–	4.68	7.82
Tissues	0.53	0.62	–	–	0.66	0.65
Recovery	95.49	96.05	–	–	97.95	99.66

Source: Totis (1999)

Table 2. Tissue to plasma ratios of radioactivity following oral dosing with [C-phenyl-U-^{14}C]- or [N-phenyl-U-^{14}C]fenamidone

Sample	Tissue : plasma ratio					
	3 mg/kg bw (single dose)		300 mg/kg bw (single dose)		3 mg/kg bw (repeated dose)	
	Males	Females	Males	Females	Males	Females
[C-phenyl-U-^{14}C]Fenamidone						
Liver	8.34	8.03	7.20	4.67	6.92	10.94
Kidneys	3.97	2.50	2.96	2.32	3.90	3.20
Lungs	2.57	2.89	3.64	4.66	2.75	4.04
Blood	7.61	10.62	11.74	13.95	7.94	9.73
Skin and fur	6.65	3.99	4.02	3.42	3.17	4.86
Thyroid	494.01	394.79	105.29	83.51	523.51	467.88
[N-phenyl-U-^{14}C]Fenamidone						
Liver	29.30	27.38	–	–	14.14	15.02
Kidneys	15.90	9.95	–	–	11.09	6.37
Heart	6.44	5.73	–	–	3.33	3.19
Lungs	6.78	10.02	–	–	3.94	5.21
Spleen	10.44	13.07	–	–	6.49	11.34
Blood	34.94	38.78	–	–	14.61	18.18
Skin and fur	7.17	11.73	–	–	3.39	6.00
Thyroid	NA	16.47	–	–	10.71	15.24

NA: not available
Source: Totis (1999)

Table 3. Kinetic parameters (expressed as means) in rats following a single oral dose of [C-phenyl-U-^{14}C]- or [N-phenyl-U-^{14}C]fenamidone

Parameter	3 mg/kg bw		300 mg/kg bw	
	Males	Females	Males	Females
[C-phenyl-U-^{14}C]Fenamidone				
C_{max} (µg eq/g)	0.29	0.31	12.16	17.70
T_{max} (min)	4.29	3.71	14.64	25.68
$t_{½}$ (h)	61.5	72.8	72	84
$AUC_{(0–n)}$ (µg eq·h/g)	12.79	16.61	775.66	1 674.82
[N-phenyl-U-^{14}C]Fenamidone				
C_{max} (µg eq/g)	0.34	0.31	–	–
T_{max} (min)	3.02	2.63	–	–
$t_{½}$ (h)	109.24	129.60	–	–
$AUC_{(0–n)}$ (µg eq·h/g)	15.37	17.59	–	–

AUC: area under the plasma concentration–time curve; C_{max}: maximum concentration in plasma; $t_{½}$: half-life; T_{max}: time to reach C_{max}
Source: Totis (1999)

Table 4. Mass balance of radioactivity in bile duct–cannulated rats following a single oral dose (3 mg/kg bw) of [C-phenyl-U-^{14}C]- or [N-phenyl-U-^{14}C]fenamidone

Sample	Mean % of administered radioactivity			
	[C-phenyl-U-^{14}C]Fenamidone		[N-phenyl-U-^{14}C]Fenamidone	
	Males	Females	Males	Females
Urine				
0–6 h	4.39	4.46	3.18	7.04
0–24 h	9.91	10.75	6.07	15.76
0–48 h	10.67	11.69	6.33	17.62
Faeces				
0–24 h	2.73	0.21	2.46	0.11
0–48 h	4.11	1.08	4.46	1.36
Bile				
0–6 h	70.71	49.64	76.76	35.95
0–24 h	79.44	68.05	83.15	61.01
0–48 h	79.72	72.62	83.41	71.30
Cage wash	1.13	1.90	0.92	1.50
Tissues	0.68	5.37	1.01	5.43
Total	96.31	92.66	96.13	97.21

Source: Totis (1999)

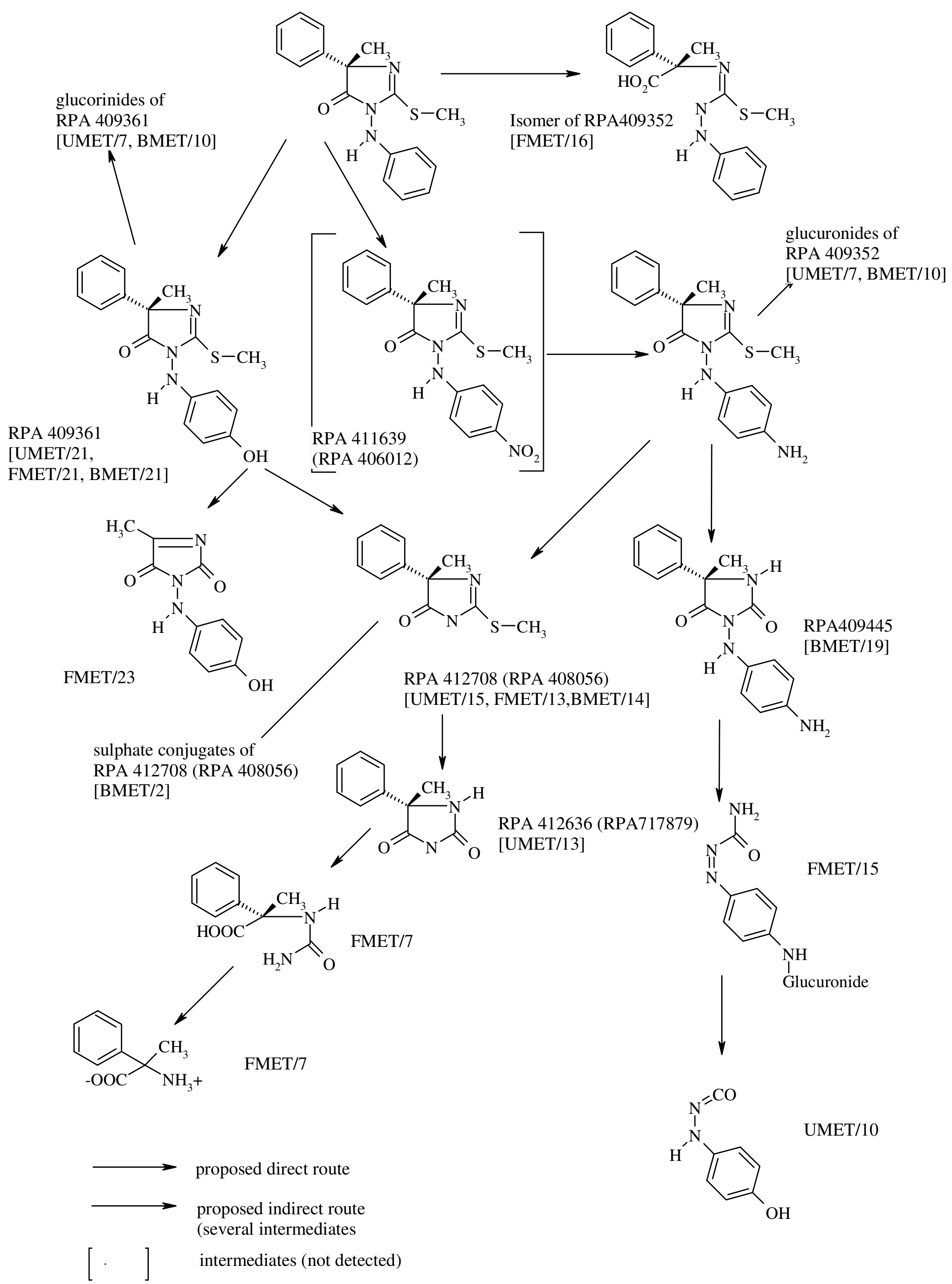
Table 5. Main fenamidone metabolites detected in rat excreta

Metabolite	% of administered dose					
	3 mg/kg bw (single dose)		300 mg/kg bw (single dose)		3 mg/kg bw (repeated dose)	
	Males	Females	Males	Females	Males	Females
RPA 407213: Fenamidone						
Urine	–	–	0.13	0.25	–	–
Faeces	–	–	49.92	67.78	–	–
Bile	–	–	–	–	–	–
RPA 409352: 3-(4-Aminophenylamino)-5-methyl-2-methylthio-5-phenyl-3,5-dihydroimidazol-4-one						
Urine	0.24	1.91	0	0	0.21	1.32
Faeces	7.20	4.19	5.55	7.53	3.44	5.69
Bile	0.18	0	–	–	–	–
RPA 409361: 1-(4-Hydroxyphenylamino)-4-methyl-2-methylthio-4-phenylimidazolin-5-one						
Urine	0.64	4.76	0.52	3.06	0.47	2.17
Faeces	7.67	12.88	6.18	6.90	5.41	14.87
Bile	0.47	0.24	–	–	–	–
RPA 412708: (5*S*)-5-Methyl-2-(methylthio)-5-phenyl-3,5-dihydro-4*H*-imidazol-4-one						
Urine	0.05	0.25	0.10	0.09	0.27	0.34
Faeces	10.47	8.40	1.99	0.84	10.33	13.01
Bile	15.34	2.10	–	–	–	–
RPA 412636: (*S*)-5-Methyl-5-phenyl-2,4-imidazolidine-dione						
Urine	0.53	2.94	0.27	0.84	0.70	0.75
Faeces	0	0	0	0	0	0
Bile	0	0	–	–	–	–

Source: Totis (1999)

The proposed metabolic pathway for fenamidone in rats is given in Fig. 3 (Totis, 1999).

Fig. 3. Proposed metabolic pathway of [14C]fenamidone in rats



In a subsequent discussion on the formation of the metabolite designated RPA 409352, Totis (2000) proposed that fenamidone was metabolized by gut bacteria to the 4-nitrophenyl metabolite RPA 406012, which was rapidly reduced to the amide derivative RPA 409352, which is then reabsorbed and re-excreted via the bile. This is based on the observation that free RPA 409352 is predominantly detected in faeces, with the conjugated form detected in bile.

2. Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

The results of acute toxicity tests on fenamidone in rats are summarized in Table 6. Clinical signs observed for up to 2 days after an acute oral dose greater than or equal to 1000 mg/kg bw included reduced motor activity, hunched posture, staggering gait, prostration, bradypnoea and piloerection (Dange & Foulon, 1997). No clinical signs of toxicity were observed following an acute dermal exposure to 2000 mg/kg bw lasting 24 hours (Dange, 1997). Clinical signs observed for up to 2 days after an acute inhalational exposure (4 hours, nose only) included lethargy, uncoordinated limb movements, wet fur and brown staining around the snout, jaw, body and eyes (Coombs, Horrell & Bannerman, 1998). Additional clinical signs observed in females included gasping and noisy respiration. All treated rats lost body weight over the 1st day after exposure.

Table 6. Results of studies of acute toxicity on fenamidone

Species	Strain	Sex	Route	Purity (%)	Vehicle	LD_{50} (mg/kg bw) or LC_{50} (mg/L)	Reference
Rat	SD Ico:OFA-SD (IOPS Caw)	Male and female	Oral	99.8	0.5% (w/v) aqueous CMC	> 5 000 (males) 2 028 (females)	Dange & Foulon (1997)
Rat	SD Ico:OFA-SD (IOPS Caw)	Male and female	Dermal	99.8	Powder moistened in 0.9% (w/v) saline	> 2 000 (24 h exposure)	Dange (1997)
Rat	SD	Male and female	Inhalation MMAD = 2.5 µm	99.8	Acetone	> 2.1 mg/L (4 h, nose-only exposure)	Coombs, Horrell & Bannerman (1998)

CMC: carboxymethyl cellulose; LC_{50}: median lethal concentration; LD_{50}: median lethal dose; MMAD: mass median aerodynamic diameter; SD: Sprague-Dawley; w/v: weight per volume

(b) Dermal and ocular irritation

The results of skin and eye irritation tests on fenamidone conducted in rabbits are summarized in Table 7.

Table 7. Results of studies of dermal and ocular irritation on fenamidone in rabbits

Strain	Sex	Purity (%)	Application site	Exposure period	Result	Reference
NZW	Male	99.8	6 cm^2, non-abraded skin	4 h, semi-occluded	Not a skin irritant	Chuzel (1997a)
NZW	Male	99.8	Left eye, right eye control	Eye unwashed for up to 72 h	Not an eye irritant	Chuzel (1997b)

NZW: New Zealand White

(c) Dermal sensitization

Fenamidone (purity 99.8%) was analysed for its skin sensitization potential in the guinea-pig maximization test. Fifteen male and 15 female guinea-pigs (Dunkin Hartley strain) were included in the test group, and five of each sex were included in the control group. In the induction phase (day 1), animals were injected intradermally on either side of the dorsal midline with 1% (w/v) fenamidone in paraffin oil and Freund's complete adjuvant (1 : 1). On day 7, the same region received a topical application of sodium lauryl sulfate in petrolatum (10% weight per weight [w/w]) in order to induce local irritation. On day 8, this same test site was treated by topical application of undiluted fenamidone or the vehicle (control group) and was covered by an occlusive dressing for 48 h. On day 22, all animals were challenged by a topical application of undiluted fenamidone under an occlusive dressing, with the left flank serving as the vehicle control. No skin reactions were observed at 24 and 48 hours. On the basis of these findings, fenamidone is not a skin sensitizer (de Jouffrey, 1997).

2.2 Short-term studies of toxicity

Mice

A 14-day range-finding study was conducted in groups of CD-1 mice (five of each sex) that were gavaged with fenamidone (purity 99.9%) in 0.5% (w/v) carboxymethyl cellulose at a dose of 0, 10, 100, 300 or 1000 mg/kg bw per day. There were no deaths or treatment-related clinical signs. There was no treatment-related effect on body weight, feed consumption or clinical chemistry parameters. The absolute liver weight of males at 300 and 1000 mg/kg bw per day was 23% ($P < 0.05$) and 29% ($P < 0.01$) higher than the control values, respectively. The absolute liver weight of high-dose females was 45% higher ($P < 0.01$) than the control value. Relative liver weight was approximately 18% and 30% higher than the control values ($P < 0.05$) in high-dose males and females, respectively. At necropsy, mild liver enlargement was observed in one high-dose female, with a pale-yellowish discoloration (graded as moderate) observed in 0/5, 0/5, 1/5, 0/5 and 2/5 males and 0/5, 0/5, 0/5, 2/5 and 1/5 females at 0, 30, 100, 300 and 1000 mg/kg bw per day, respectively. Histopathological examination revealed centrilobular hypertrophy (graded as slight to mild) in three males each at 300 and 1000 mg/kg bw per day and in three females at 1000 mg/kg bw per day (Dange, 1994).

In a non-guideline study, fenamidone (purity 98.4%) was admixed in the diet at a concentration of 0, 70, 700 or 7000 ppm and fed ad libitum to groups of 10 CD-1 mice of each sex for 90 days. The achieved doses were 0, 11.4, 110 and 1102 mg/kg bw per day for males and 0, 14.5, 146 and 1468 mg/kg bw per day for females at 0, 70, 700 and 7000 ppm, respectively. Observations for mortalities and clinical signs were made daily. Body weight and feed consumption were recorded weekly. Blood was sampled on the last day of dosing for the analysis of limited haematology and clinical chemistry parameters. Following termination, all mice were necropsied, organs weighed and selected tissues examined histopathologically.

Two high-dose males were killed in a moribund condition on days 12 and 41, with a further two found dead on days 51 and 53. There were no other deaths. Clinical signs were observed in five high-dose males (including the four that died or were killed) and included pallor, reduced motor activity, staggering gait, prostration and dyspnoea. No treatment-related clinical signs were observed in females. There was no treatment-related effect on body weight gain or feed consumption. Haematology parameters were unremarkable. Mean total bilirubin was significantly higher ($P < 0.05$) than the control values in females at 700 and 7000 ppm (Table 8). In males, total bilirubin was elevated across all treated groups, but the differences were not statistically significant and did not follow a dose–response relationship. Given the inconsistent findings between males and females and that similar increases in total bilirubin did not occur in the preceding 14-day study by Dange (1994) at comparable doses, the significant increase in total bilirubin in females is not clearly treatment related.

At the highest dose (both sexes), absolute and relative liver weights were significantly higher ($P < 0.01$ or 0.05) than the control values (21% and 15%, respectively, in males and 14% and 18%,

respectively, in females) (Table 8). In the four high-dose male decedents, pale livers (four) and hepatomegaly (two) were observed macroscopically, with these findings also observed across the majority of groups following scheduled termination (Table 8). However, in the absence of a clear dose–response relationship and the occurrence of a relatively high background incidence in the control groups, these macroscopic findings (in survivors) cannot definitively be attributed to treatment. Histopathology revealed centrilobular hypertrophy of hepatocytes (graded as mild to moderate) in all treated groups of males and in females at 700 and 7000 ppm (Table 8). The occurrence of liver hypertrophy without accompanying evidence of hepatotoxicity is considered to be an adaptive response to treatment.

Table 8. Liver findings in mice following 13 weeks of dietary exposure to fenamidone

Parameter	0 ppm		70 ppm		700 ppm		7 000 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
Clinical chemistry[a]								
Total bilirubin (µmol/L)	1.4	1.2	2.1	1.1	2.0	2.1*	2.1	2.2*
Liver weights[a]								
Absolute (g)	1.4	1.4	1.5	1.3	1.5	1.3	1.7*	1.6*
Relative[b] (%)	3.9	4.4	4.1	4.2	4.1	4.5	4.5**	5.2**
Macroscopic liver findings[b]								
N	10	10	10	10	10	10	6	10
Enlargement	0	4	1	6	1	7	2	4
Pale colour	4	2	0	8	1	5	4	7
Histopathological liver findings[c]								
Centrilobular hypertrophy	0	0	2	0	3	4	5	6

*: $P < 0.05$; **: $P < 0.01$

[a] Results expressed as the mean.

[b] Relative to body weight.

[c] Results expressed as the absolute number of mice with the finding.

Source: Berthe (1995)

The no-observed-adverse-effect level (NOAEL) was 700 ppm (equal to 110 mg/kg bw per day), based on clinical signs and deaths in males at 7000 ppm (equal to 1102 mg/kg bw per day) (Berthe, 1995).

In a subsequent 90-day study, fenamidone (purity 98.9%) was admixed in the diet at a concentration of 0, 50, 200, 1000 or 5000 ppm and fed ad libitum to groups of 10 C57 Black 10J mice of each sex. The mean achieved doses were 0, 11.3, 44.5, 220 and 1064 mg/kg bw per day for males and 0, 13.7, 54, 274 and 1375 mg/kg bw per day for females at 0, 50, 200, 1000 and 5000 ppm, respectively. Observations for mortalities and clinical signs were made daily. Body weight and feed consumption were recorded weekly. Blood was sampled 1 day prior to study termination for the analysis of limited clinical chemistry parameters. Following termination, all mice were necropsied, organs weighed and selected tissues examined histopathologically.

There were no treatment-related deaths, clinical signs or effects on body weight gain or feed consumption. In females, plasma cholesterol was significantly lower than the control value (0.88 mmol/L) at 1000 ppm (0.63 mmol/L; $P < 0.05$) and 5000 ppm (0.47 mmol/L; $P < 0.01$). Selected

organ weight, macroscopic and histopathological findings are summarized in Table 9. At the highest dose, absolute and relative liver weights of males were approximately 15% higher than the control values ($P < 0.01$ or 0.05). In high-dose females, only relative liver weight was significantly ($P < 0.05$) higher than the control value (by about 12%). At necropsy, there was a slight increase in the incidence of pale liver in high-dose males; however, the background incidence in the control was relatively high, and the dose–response relationship was limited. Histopathological examination indicated a slight increase in hepatocellular microvacuolation in males, but again there was a high background occurrence in the control group.

Table 9. Liver findings in mice following 90 days of dietary exposure to fenamidone

Parameter	0 ppm		50 ppm		200 ppm		1 000 ppm		5 000 ppm	
	M	F	M	F	M	F	M	F	M	F
Liver weight[a]										
Absolute (g)	1.02	0.87	1.04	0.80	1.04	0.87	1.09	0.91	1.16**	0.95
Relative[b] (%)	4.22	4.22	4.24	4.03	4.36	4.29	4.43	4.41	4.85*	4.72*
Macroscopic liver findings[c]										
N	8	10	10	10	9	10	10	10	10	9
Pale appearance	3	0	3	2	5	3	5	2	7	2
Histopathological liver findings[c]										
Hepatocellular microvacuolation	4	1	5	1	6	2	8	5	8	2

F: female; M: male; *: $P < 0.05$; **: $P < 0.01$
[a] Results expressed as the mean.
[b] Relative to body weight.
[c] Results expressed as the absolute number of mice with the finding.
Source: Bigot & Tassel (1997)

The NOAEL was 1000 ppm (equal to 220 mg/kg bw per day) for equivocal histopathological findings in the liver at 5000 ppm (equal to 1064 mg/kg bw per day) (Bigot & Tassel, 1997).

Rats

In a 14-day range-finding study, groups of SD rats (five of each sex) were administered fenamidone (purity 99.9%) in 0.5% (w/v) carboxymethyl cellulose by gavage at a dose of 0, 100, 300 or 1000 mg/kg bw per day (males) or 0, 30, 100 or 300 mg/kg bw per day (females). The dose selection was based on differences in acute toxicity between males and females. Satellite groups of three rats of each sex were added to the high-dose and control groups for the analysis of hepatic cellular proliferation and liver histology after 3 days of treatment. Rats were observed daily for mortality and clinical signs. Body weight was recorded on days 1, 7 and 14, with feed consumption recorded weekly. Blood was sampled prior to terminal sacrifice for the analysis of haematology and clinical chemistry parameters. All rats were necropsied, and various organs were weighed and examined histopathologically.

There were no deaths or treatment-related clinical signs. Body weight, feed consumption and haematology parameters were unaffected by treatment. In high-dose males, significantly elevated total bilirubin (approximately 78%, $P < 0.01$), total protein (approximately 6%, $P < 0.05$) and cholesterol (approximately 31%, $P < 0.05$) were determined. In high-dose male rats killed after 3 days, absolute and relative liver weights were approximately 22% higher ($P < 0.01$) than the control values. At terminal sacrifice, a 31% increase ($P < 0.01$) in relative liver weight was noted in high-dose males. In high-dose females killed after 3 days, relative liver weight was about 15% higher ($P < 0.05$) than the

control value. No macroscopic abnormalities were observed at the interim sacrifice, whereas liver enlargement was noted in 0/5, 1/5, 1/5 and 3/5 males at 0, 100, 300 and 1000 mg/kg bw per day, respectively, in rats killed after 14 days. Centrilobular hepatocytic hypertrophy was observed histopathologically in 2/3 high-dose males killed after 3 days and in 3/5 high-dose males killed after 14 days. Thyroid follicular cell hyperplasia occurred in 2/5 and 3/5 female rats at 100 and 300 mg/kg bw per day, respectively.

Analysis of the cytochrome P450 isozyme profile by western blotting revealed a dose-related increase in isoform IIB, which was not detected in controls or low-dose females. Benzoxyresorufin *O*-debenzylation (BROD) was increased by 2.7-, 8.5- and 17.1-fold in males and 1.3-, 4.9- and 25.4-fold in females at the low, middle and high doses, respectively. In comparison, treatment with 75 mg/kg bw phenobarbital resulted in increases of 127.4- and 31.2-fold in males and females, respectively. Pentoxyresorufin *O*-depentylation (PROD) was increased by 1.4-, 2.8- and 4.5-fold in males and 1.1-, 4.3- and 15.7-fold in females at the low, middle and high-doses, respectively. Treatment with 75 mg/kg bw phenobarbital resulted in increases of 89.7- and 37.9-fold in males and females, respectively. There was no treatment-related effect on ethoxyresorufin *O*-deethylation (EROD).

In rats killed after 3 days, the numbers of proliferating cell nuclear antigen (PCNA) positive cells in the liver were increased by about 2.9-fold in males and 22.5-fold in females at 100 and 300 mg/kg bw per day, respectively. In rats killed at the end of the study, the number of PCNA positive cells was increased by 2.2- and 4.1-fold in high-dose males and females, respectively (Dange, 1995a).

Fenamidone (purity > 99%) was admixed in the diet at a concentration of 0, 500, 5000 or 15 000 ppm and fed ad libitum to groups of 10 SD rats of each sex for 28 days. The achieved doses were 0, 39, 389 and 1203 mg/kg bw per day for males and 0, 42, 405 and 1194 mg/kg bw per day for females at 0, 500, 5000 and 15 000 ppm, respectively. Rats were observed daily for deaths and clinical signs. Body weight and feed consumption were recorded weekly. Ophthalmoscopy was performed pretreatment and during weeks 2 and 4 of treatment. Blood and urine were collected at the end of the study for the analysis of standard haematology, clinical chemistry or urine analysis parameters. Following sacrifice, rats were necropsied, and organs were weighed and examined histopathologically.

There were no treatment-related deaths or clinical signs. Ophthalmoscopy was unremarkable. In males, mean body weight gain was significantly lower ($P < 0.01$ or 0.05) than the control values at 5000 ppm (days 0–8 and 15–22) and 15 000 ppm (days 0–8), with no significant intergroup differences in females (Table 10). Feed consumption was significantly lower ($P < 0.01$ or 0.05) than the control values in males at every dose and in females at 5000 and 15 000 ppm (Table 10).

Selected haematology, clinical chemistry, organ weight, and macroscopic and histopathological findings are also summarized in Table 10. There were no treatment-related urine analysis findings. At 5000 ppm (males) and 15 000 ppm (both sexes), red cell parameters were significantly lower ($P < 0.01$) than the control values. At 5000 and 15 000 ppm, significantly reduced plasma glucose (both sexes; $P < 0.01$ or 0.05) and increased cholesterol (males; $P < 0.01$) occurred. Additional clinical chemistry findings in high-dose males included reduced urea and increased total bilirubin ($P < 0.01$ or 0.05).

There were a number of organ weights that were significantly different from the control values at and above 5000 ppm (Table 10), but only the increases in liver and spleen weights correlated with increases in their respective relative weights. In addition, both of these organs had treatment-related macroscopic and/or histopathological abnormalities. At necropsy, enlargement and a dark abnormal colour of the spleen were noted at 5000 ppm (males) and 15 000 ppm (both sexes), with mild to marked hyperplasia of the germinative follicle of the white pulp observed microscopically in both sexes at these same dietary concentrations. There were no macroscopic liver abnormalities, whereas histopathology revealed mild to moderate diffuse hepatocellular hypertrophy at 5000 and 15 000 ppm in both sexes. The only other treatment-related histopathological finding was an increase

Table 10. Treatment-related findings in rats following 28 days of dietary exposure to fenamidone[a]

Parameter	0 ppm		500 ppm		5 000 ppm		15 000 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
Body weight gain (g)								
Days 0–8	7.62	2.88	7.30	2.57	5.11**	2.41	−0.07**	1.90
Days 8–15	6.42	2.47	4.67	2.92	5.20	2.20	5.62	2.30
Days 15–22	5.27	1.30	5.17	2.45	3.92*	2.55	4.35	1.91
Days 22–28	2.94	1.30	2.26	0.33	2.03	0.55	1.33	0.86
Feed consumption (g/day)								
Days 0–8	28.5	20.9	27.2	20.4	25.7**	18.8*	19.3**	17.1**
Days 8–15	28.1	21.9	26.9	21.1	26.5	20.1	26.3	20.2
Days 15–22	28.6	21.0	26.3*	20.8	24.8**	20.1	25.9*	19.1
Days 22–28	26.5	18.4	24.2*	18.1	23.3**	17.7	21.8**	17.3
Haematology								
RBC ($\times 10^{12}$)	8.53	8.32	8.61	8.46	8.53	8.34	7.76**	7.62**
Hb (g/100 mL)	15.9	15.3	15.7	15.6	14.8**	15.4	14.6**	14.2**
Hct (%)	47.8	45.9	47.1	46.7	45.2**	46.5	43.9**	43.1**
Clinical chemistry								
Glucose (mmol/L)	8.34	7.79	8.48	7.33	7.02*	6.56**	5.93**	6.33**
Urea (mmol/L)	4.29	4.56	4.36	4.56	4.08	4.91	3.42*	4.52
Cholesterol (mmol/L)	0.69	0.74	0.75	0.81	1.06**	0.86	1.53**	0.86
Bilirubin (µmol/L)	1.7	2.1	1.8	1.8	2.2	2.0	2.6**	1.7
Organ weights								
Brain								
Absolute (g)	1.96	1.83	1.94	1.89	1.89	1.84	1.85*	1.81
Relative[b] (%)	0.52	0.77	0.54	0.77	0.57**	0.79	0.62**	0.79
Heart								
Absolute (g)	1.27	0.88	1.18	0.87	1.13*	0.84	1.05**	0.85
Relative (%)	0.33	0.37	0.33	0.35	0.33	0.36	0.35	0.37
Liver								
Absolute (g)	10	6.6	10	6.8	11.1*	7.4**	11.4**	7.9**
Relative (%)	2.6	2.7	2.8	2.8	3.3**	3.2**	3.8**	3.4**
Pituitary								
Absolute (g)	0.011	0.011	0.011	0.012	0.009	0.010	**0.009***	0.010
Relative (%)	0.002	0.004	0.003	0.005	0.002	0.004	0.003	0.004
Spleen								
Absolute (g)	0.80	0.57	0.79	0.62	0.84	0.61	1.09**	0.67
Relative (%)	0.21	0.24	0.22	0.25	0.25	0.26	0.36**	0.29**
Kidney								
Absolute (g)	2.92	1.87	2.87	2.10	2.62	2.00	2.43**	1.96
Relative (%)	0.78	0.78	0.80	0.86	0.78	0.85	0.81	0.86

Table 10 (continued)

Parameter	0 ppm		500 ppm		5 000 ppm		15 000 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
Epididymis								
Absolute (g)	1.20	–	1.11	–	1.10*	–	1.06**	–
Relative (%)	0.32	–	0.31	–	0.32	–	0.35	–
Prostate								
Absolute (g)	0.58	–	0.51	–	0.44**	–	0.41**	–
Relative (%)	0.15	–	0.14	–	0.13	–	0.13	–
Macroscopic findings[c]								
Spleen								
Enlarged	0/9	0/10	0/10	0/10	2/10	0/10	6/10	3/10
Dark abnormal colour	0/9	0/10	0/10	1/10	3/10	1/10	9/10	5/10
Microscopic findings[c]								
Liver: Diffuse hypertrophy	0/9	0/10	0/10	0/10	6/10	5/9	10/10	10/10
Spleen: Follicular hyperplasia	0/9	0/10	0/10	0/10	10/10	2/9	9/10	5/10
Pituitary gland: Increased chromophobe cells	0/9	0/10	5/10	0/10	7/10	0/9	7/10	0/10

Hb: haemoglobin; Hct: haematocrit; RBC: red blood cells; *: $P < 0.05$; **: $P < 0.01$

[a] Results expressed as the mean unless otherwise specified.

[b] Relative to body weight.

[c] Absolute number of rats with the finding.

Source: Dange (1995b)

in the number of chromophobe cells in males only at every dietary concentration; the toxicological significance of this finding is unclear.

The NOAEL was 500 ppm (equal to 39 mg/kg bw per day), based on decreased red cell parameters and increased spleen weights coincident with hyperplasia of the germinative follicle of the white pulp at and above 5000 ppm (equal to 389 mg/kg bw per day) (Dange, 1995b).

In a second 28-day study, fenamidone (purity > 99%) was admixed in the diet at a concentration of 0, 60, 150, 1000 or 5000 ppm and fed ad libitum to groups of 10 SD rats of each sex. The achieved doses were 0, 4.9, 12.3, 82.7 and 418.5 mg/kg bw per day for males and 0, 5.3, 13.9, 90.6 and 450.1 mg/kg bw per day for females at 0, 60, 150, 1000 and 5000 ppm, respectively. Observations for mortalities and clinical signs were made daily. Body weight and feed consumption were recorded weekly. Blood was sampled during weeks 1, 2 and 4 for the analysis of thyroid stimulating hormone (TSH), triiodothyronine (T_3) and thyroxine (T_4). No haematology, clinical chemistry or urine analysis parameters were analysed. At the end of the study, all rats were necropsied; the brain, liver and thyroid were weighed and examined histopathologically.

There were no treatment-related mortalities or clinical signs. There were no significant intergroup differences in body weight, body weight gain or feed consumption. There was no treatment-related effect on TSH, T_3 or T_4. Absolute and relative liver weights were significantly higher ($P < 0.01$) than the control values in high-dose males (29% and 39%, respectively) and females

(21% and 28%, respectively) (Table 11). There were no significant intergroup differences in thyroid weights. At necropsy, mild to marked liver enlargement was noted predominantly at the highest dose, whereas histopathology revealed slight to moderate diffuse hypertrophy of hepatocytes at 1000 ppm (males) and 5000 ppm (both sexes) (Table 11). The increase in liver weight and hypertrophy were considered to be an adaptive response to treatment. In high-dose males, slight to mild thyroid follicular cell hypertrophy was also observed (Table 11).

Table 11. Liver and thyroid findings in rats exposed to fenamidone in the diet for 28 days[a]

Parameter	0 ppm		60 ppm		150 ppm		1 000 ppm		5 000 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females
Organ weights										
Liver										
Absolute (g)	8.13	5.48	8.20	5.37	8.77	5.70	8.67	5.66	10.53**	6.61**
Relative[b] (%)	2.71	2.84	2.72	2.82	2.85	3.02	2.92	2.93	3.77**	3.63**
Macroscopic findings[c]										
Enlarged liver	1	0	1	0	2	1	3	0	7	3
Microscopic findings[c]										
Liver: Diffuse hypertrophy	0	0	0	0	0	0	4	0	10	4
Thyroid: Follicular cell hypertrophy	0	0	0	0	0	0	0	0	3	0

*: $P < 0.05$; **: $P < 0.01$
[a] Results expressed as the mean unless otherwise specified.
[b] Relative to body weight.
[c] Absolute number of rats with the finding ($n = 10$).
Source: Dange (1999a)

The NOAEL was 1000 ppm (equal to 82.7 mg/kg bw per day) for follicular cell hypertrophy in males at 5000 ppm (equal to 418.5 mg/kg bw per day) (Dange, 1999a).

Fenamidone (purity 98.4%) was admixed in the diet at a concentration of 0, 50, 150, 500 or 5000 ppm and fed ad libitum to groups of 10 SD rats of each sex for up to 90 days. The achieved doses were 0, 3, 9, 30 and 305 mg/kg bw per day for males and 0, 3, 11, 35 and 337 mg/kg bw per day for females at 0, 50, 150, 500 and 5000 ppm, respectively. Clinical signs were recorded daily. Body weight and feed consumption were recorded weekly. An ophthalmological examination was performed on control and high-dose rats prior to the commencement of dosing and then during week 13. Blood was collected 1 week prior to terminal sacrifice for the analysis of standard haematology and clinical chemistry parameters. Urine was collected overnight prior to necropsy for urine analysis. Selected organs were weighed and tissues examined histopathologically.

There were no deaths or treatment-related clinical signs. At the highest dose, mean absolute body weight was approximately 10% lower ($P < 0.01$ or 0.05) than the control values throughout the exposure period in males and from approximately week 9 in females. Body weight gain was also reduced at the highest dose throughout the study (by up to about 10% in both sexes), reaching

statistical significance in males over days 1–8 ($P < 0.01$), 78–84 ($P < 0.01$) and 84–90 ($P < 0.05$) and in females over days 1–8 ($P < 0.05$) and 78–84 ($P < 0.05$). Feed consumption (week 1 in males, approximately 20% lower; weeks 1, 3, 8, 9, 10 and 12 in females, approximately 10–20% lower) was also significantly lower ($P < 0.01$ or 0.05) than the control values. There were no treatment-related ophthalmological abnormalities. At the highest dose, red blood cells and haemoglobin were significantly lower than the control values (red blood cells: 6% [$P < 0.05$] and 8% [$P < 0.01$] lower in males and females, respectively; haemoglobin: 6% and 7% lower [$P < 0.01$] in males and females, respectively). Also at the highest dose, blood glucose was approximately 20% lower ($P < 0.05$) than the control values in both sexes.

At the highest dose, a range of absolute and/or relative organ weights were significantly ($P <$ 0.01 or 0.05) different from the control values. In males, absolute and relative thymus weights were approximately 34% and 26% lower than the control values, respectively; similar differences did not occur in females, and there was no corroborating histopathology. Absolute (males) and relative (both sexes) thyroid weights were approximately 37% and 50% higher than the control values, but there were also no corroborating microscopic findings. The author stated that all organ weights were within the normal range for age- and sex-matched SD rats.

There were no treatment-related macroscopic findings. Histopathology revealed a slight increase in the incidence of bile duct hyperplasia in high-dose males (5/10 versus 1/10 in the control; graded as slight) and prominent germinal centres of the white pulp of the spleen, also in high-dose males (8/10 versus 2/10 in the control). However, these findings were considered by the author to represent normal background findings.

The NOAEL was 500 ppm (equal to 30 mg/kg bw per day) for reduced body weight gain, reduced feed consumption and changes in red cell parameters at 5000 ppm (equal to 305 mg/kg bw per day) (Dange, 1995c).

In a subsequent 90-day dietary study in rats, fenamidone (purity 99.8%) was admixed in the diet at a concentration of 0, 60, 150, 1000 or 5000 ppm and fed ad libitum to groups of 10 SD rats of each sex. The achieved doses were 0, 4.1, 10.4, 68.3 and 344 mg/kg bw per day for males and 0, 4.8, 12.0, 83.3 and 381 mg/kg bw per day for females at 0, 60, 150, 1000 and 5000 ppm, respectively. Observations for deaths and clinical signs were made daily. Body weight and feed consumption were recorded weekly. Ophthalmological examinations were made on all rats prior to the commencement of dosing and then in the control and high-dose groups during week 12. Blood was collected during week 12 for the analysis of haematology and clinical chemistry parameters. One day prior to sacrifice, overnight urine was collected from all rats for the analysis of standard urinary parameters. Following termination, all rats were necropsied, organs weighed and tissues examined histopathologically.

There were no deaths or treatment-related clinical signs. Ophthalmological examinations were unremarkable. In high-dose males, mean absolute body weight was significantly lower ($P < 0.01$ or 0.05) than the control values throughout most of the exposure period (approximately 10% lower than the control values). Body weight gain of high-dose males was also significantly lower ($P < 0.01$ or 0.05) than the control values mainly during the first 5 weeks of exposure (20–30% lower). There were no significant intergroup differences in body weight or body weight gain in females. Feed consumption was inconsistently significantly lower ($P < 0.01$ or 0.05) than the control values in high-dose males (up to about 10% lower) and females (approximately 11–17% lower).

Treatment-related haematological findings were confined to high-dose males and included reduced haemoglobin (approximately 6% lower than the control, $P < 0.05$) and mean corpuscular haemoglobin concentration (MCHC) (approximately 4% lower than the control value, $P < 0.01$). Clinical chemistry findings included increased cholesterol in high-dose males ($P < 0.01$) and females ($P < 0.05$) (about 30% above the control), reduced glucose (approximately 26% lower than the control in high-dose males, $P < 0.01$) and increased phosphorus (approximately 20% higher than the control in females, $P < 0.05$). There were no treatment-related urine analysis findings.

Necropsy revealed a dark liver in four high-dose males and two high-dose females. Selected organ weights and histopathological findings are presented in Table 12. In high-dose females, absolute and relative liver weights were significantly higher ($P < 0.01$ or 0.05) than the control values (13% and 22%, respectively), concomitant with ground glass cytoplasm in hepatocytes (graded as mild) attributable to the proliferation of smooth endoplasmic reticulum. In males, relative but not absolute liver weights were significantly higher ($P < 0.01$ or 0.05) than the control values at 1000 and 5000 ppm (12% and 23%, respectively), with the difference at the highest dose most likely reflecting the lower terminal body weight of this group. Histopathology revealed ground glass cytoplasm in hepatocytes at these same dietary concentrations, a finding consistent with liver enzyme induction. Absolute and relative thyroid weights were significantly higher ($P < 0.01$) than the control values in high-dose males, but there was no supporting histopathology.

Table 12. Organ weights and microscopic findings in rats following 13 weeks of dietary exposure to fenamidone

Parameter	0 ppm		60 ppm		150 ppm		1 000 ppm		5 000 ppm	
	M	F	M	F	M	F	M	F	M	F
Terminal body weight (g)	430	270	477*	277	468	267	435	254	383*	250
Organ weights[a]										
Liver										
Absolute (g)	10.36	6.55	11.82	6.69	1.77	6.52	11.70	6.43	11.49	7.39*
Relative[b] (%)	2.40	2.43	2.51	2.40	2.53	2.43	2.69**	2.54	3.00**	2.96**
Thyroid										
Absolute (g)	0.020	0.022	0.023	0.016	0.023	0.016	0.024	0.019	0.029**	0.019
Relative (g)	0.004 7	0.007 8	0.004 9	0.005 6*	0.004 9	0.005 6*	0.005 5	0.007 3	0.007 5**	0.007 5
Histopathology[c]										
N	10	10	10	10	10	10	10	10	10	10
Hepatocytes – ground glass cytoplasm	0	0	0	0	0	0	2	0	10	7

*: $P < 0.05$; **: $P < 0.01$

[a] Results expressed as means.

[b] Relative to body weight.

[c] Results expressed as the absolute number of rats with the finding.

Source: Bigot (1997)

The NOAEL was 1000 ppm (equal to 68.3 mg/kg bw per day) for reduced body weight gain at 5000 ppm (equal to 344 mg/kg bw per day) (Bigot, 1997).

Dogs

In a non-guideline study, groups of two Beagle dogs of each sex were offered 300 g diet containing fenamidone (purity > 99%) at a concentration of 0, 280, 2800 or 28 000 ppm for 28 days. The achieved doses were 0, 9.1, 89.2 and 595.3 mg/kg bw per day for males and 0, 9.7, 92.7 and 428.8 mg/kg bw per day for females at 0, 280, 2800 and 28 000 ppm, respectively. Observations for mortalities and clinical signs were made daily. Feed consumption was recorded daily, and body weight twice weekly. At the end of the treatment period, an ophthalmological examination was performed on all dogs. Blood and urine were sampled for the analysis of haematology, clinical chemistry or urine analysis parameters. Following terminal sacrifice, dogs were necropsied, selected organs weighed and tissues examined histopathologically.

There were no deaths. Clinical signs were observed at the highest dose and included emaciation (two females, one male), diarrhoea and/or a reduction in faeces (all dogs), vomiting (one male) and ocular discharge (two males). All high-dose dogs lost up to 15–25% of their pretreatment body weight, coincident with reduced feed intake (approximately 50–70% lower than the respective pretreatment value). Ophthalmoscopy confirmed the discharge in the two high-dose males. Treatment-related haematological findings were confined to the highest dose and included (relative to the respective pretreatment value) reduced red blood cells (approximately 15% in males and 28% in one female), haemoglobin (approximately 18% in two males and 30% in one female), haematocrit (approximately 11% in two males and 26% in one female), platelet counts (approximately 48% in one female), neutrophil counts (approximately 52% in one female) and increased prothrombin time and activated partial thromboplastin time (approximately 25% in one female). Effects on clinical chemistry parameters also occurred at the highest dose and included reduced total protein (up to approximately 10% in two males and one female), albumin (up to approximately 20% in two males and one female), cholesterol (approximately 40% in two males and one female), inorganic phosphate (approximately 36% in one female) and calcium (approximately 9% in two females) and increased bilirubin (twice the pretreatment value in one male, 4 times higher in one female) and alanine aminotransferase (ALAT) (twice the pretreatment value in one male). There was no treatment-related effect on any urine analysis parameters.

As a result of high intragroup variation, there were no clear treatment-related effects on organ weights. Necropsy revealed atrophy of the thymus in all high-dose dogs and one female at each of 280 and 2800 ppm. Histopathology revealed abnormalities in the liver (slight to moderate centrilobular sinusoidal dilatation; slight to mild bile duct hyperplasia) and thymus (mild to marked cortical atrophy) (Table 13).

Table 13. Histopathological findings in dogs exposed to fenamidone in the diet for 28 days

Parameter	Incidence of finding							
	0 ppm		280 ppm		2 800 ppm		28 000 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
N	*2*	*2*	*2*	*2*	*2*	*2*	*2*	*2*
Liver								
Centrilobular sinusoidal dilatation	1	0	1	2	1	1	2	2
Bile duct hyperplasia	0	0	2	1	2	1	1	2
Thymus								
Cortical atrophy	1	0	1	1	0	1	2	2

Source: Dange & Katchadourian (1995)

No NOAEL was identified. The lowest-observed-adverse-effect level (LOAEL) was 280 ppm (equal to 9.1 mg/kg bw per day) for bile duct hyperplasia at all dietary concentrations tested (Dange & Katchadourian, 1995).

In a second 28-day range-finding study, groups of three Beagle dogs of each sex received fenamidone (purity 98.3%) via gelatine capsules at a dose of 0, 3, 10 or 100 mg/kg bw per day. The NOAEL was 100 mg/kg bw per day, the highest tested dose (Fisch, 1999a).

Fenamidone (purity > 98%) was administered to groups of four Beagle dogs of each sex in gelatine capsules at a dose of 0, 10, 100 or 500 mg/kg bw per day for 13 weeks. Observations for mortalities and clinical signs were made daily. Feed consumption was recorded daily, and body

weight weekly. An ophthalmological examination was performed on all dogs prior to the commencement of dosing and during week 13. Blood was collected pretreatment and during weeks 5, 8 and 13 for the analysis of standard haematology and clinical chemistry parameters. Urine was collected pretreatment and in week 13 for the analysis of standard urinary parameters. At the end of the study, dogs were killed and necropsied, their organs were weighed and their tissues were examined histopathologically.

All dogs survived to scheduled termination. Treatment-related clinical signs included reddish coloration of the tongue (all high-dose dogs) and ptyalism (three high-dose males and four high-dose females); neither of these findings was considered toxicologically significant. There was no treatment-related effect on body weight gain, feed consumption or ophthalmological findings. Haematology and urine analysis were unremarkable. Plasma cholesterol of high-dose females (4.5 mmol/L) was higher than the control value (3.4 mmol/L) at week 13, but at no other sampling times or in males; on this basis, it was considered an incidental finding. There were no treatment-related organ weight, macroscopic or histopathological findings.

The NOAEL was 500 mg/kg bw per day, the highest dose tested (Fisch, 1999b).

In a 52-week study, fenamidone (purity > 99%) was administered to groups of four Beagle dogs of each sex in gelatine capsules at a dose of 0, 10, 100 or 1000 mg/kg bw. Recorded end-points were consistent with the preceding study, with the exception that ophthalmological examination, blood sampling and urine sampling occurred pretreatment and at weeks 13, 26 and 52.

Treatment-related findings are summarized in Table 14. There were no deaths. Hypersalivation and vomiting were observed at 100 mg/kg bw per day (females) and 1000 mg/kg bw per day (both sexes), with a dose-related increase in their frequency and severity. The hypersalivation that occurred at 100 mg/kg bw per day was slight and occurred prior to dosing, with the vomiting occurring only on two or three occasions. In contrast, these clinical signs occurred frequently at the highest dose. With the exception of one high-dose male that began vomiting from 3 days after the commencement of dosing, vomiting began approximately 3–15 weeks after the commencement of dosing in all other dogs. There was no treatment-related effect on body weight gain, feed consumption, ophthalmological findings, urine analysis parameters or macroscopic findings. At the highest dose, mean haemoglobin and MCHC were significantly lower ($P < 0.01$ or 0.05) than the control values on occasion, but the levels showed no consistency over time or between sexes. Further, the author stated that the results were within the historical control range (data not provided). Significantly elevated ($P < 0.01$ or 0.05) alkaline phosphatase (AP) activity occurred at the highest dose (2.3-fold higher than the control value). Absolute liver weights were significantly higher ($P < 0.05$) than the control values at 100 and 1000 mg/kg bw per day in females (approximately 40% and 32%, respectively) and at 1000 mg/kg bw per day in males (approximately 33%). Corresponding relative liver weights were approximately 23% and 29% higher than the control values in males and females, respectively, but the increases were not statistically significant. In high-dose males, the absolute weight of spleen was approximately 43% higher ($P < 0.05$) than the control value, with the relative spleen weight approximately 38% higher, but not significantly so; in the absence of any supporting microscopic changes, this finding is not considered treatment related. Histopathologically, the incidence of biliary proliferation was increased in high-dose males.

The NOAEL was 100 mg/kg bw per day for clinical signs (hypersalivation and vomiting in both sexes), increased AP activity (both sexes), increased liver weight (males) and increased bile duct hyperplasia (males) at 1000 mg/kg bw per day (Fisch, 1999c).

2.3 Long-term studies of toxicity and carcinogenicity

Mice

In an 80-week oncogenicity study, C57BL/10J_fCD-1 Alpk mice (55 of each sex per group) were exposed to fenamidone (purity 99.8%) in the diet at a concentration of 0, 70, 350, 3500 or 7000

Table 14. Findings in dogs over 52 weeks of oral exposure (capsules) to fenamidone

Parameter	0 mg/kg bw per day		10 mg/kg bw per day		100 mg/kg bw per day		1 000 mg/kg bw per day	
	Males	Females	Males	Females	Males	Females	Males	Females
N	*4*	*4*	*4*	*4*	*4*	*4*	*4*	*4*
Clinical signs[a]								
Vomiting	0	1	0	0	0	2	4	4
Hypersalivation	0	0	0	0	0	1	4	4
Haematology[b]								
Hb (g/dL)								
- Pretreatment	14.0	14.8	13.6	13.7	14.3	14.5	14.8	14.6
- Week 13	16.2	16.9	15.3	15.8	15.0	16.4	14.4*	15.5
- Week 26	16.4	17.5	16.1	16.4	15.3	15.8	14.9	13.9**
- Week 52	16.3	18.1	16.5	17.7	15.7	17.8	16.5	16.0*
MCHC (g/dL)								
- Pretreatment	32.6	32.8	32.3	32.6	32.7	32.8	33.0	32.8
- Week 13	33.0	32.7	32.6	32.7	32.8	33.2	31.8*	32.3
- Week 26	32.8	32.8	32.8	32.7	32.3	32.2	30.7*	31.6
- Week 52	32.3	32.3	32.1	32.6	32.1	31.9	31.5	31.9
Clinical chemistry[b]								
AP (IU/L)								
- Pretreatment	267	298	315	325	400	318	368	315
- Week 13	159	149	170	194	257	214	261	316*
- Week 26	105	116	121	151	203	188	266**	262*
- Week 52	96	102	96	131	176	158	262**	284*
Organ weights[b]								
Liver								
Absolute (g)	235.4	174	243.5	225.6	263.3	244.3*	313.6*	228.9*
Relative[c] (%)	2.25	1.95	2.22	2.13	2.46	2.39	2.76	2.51
Spleen								
Absolute (g)	28.03	27.89	29.46	27.05	29.89	28.79	40.05*	31.27
Relative (%)	0.26	0.32	0.27	0.26	0.28	0.28	0.36	0.35
Histopathology[a]								
Bile duct hyperplasia	**1**	0	0	1	0	0	4	1

AP: alkaline phosphatase; Hb: haemoglobin; IU: International Units; MCHC: mean corpuscular haemoglobin concentration; *: $P < 0.05$; **: $P < 0.01$

[a] Results expressed as the absolute number of dogs with the finding.

[b] Results expressed as means.

[c] Relative to body weight.

Source: Fisch (1999c)

ppm. The mean achieved doses were 0, 9.5, 47.5, 525.5 and 1100 mg/kg bw per day for males and 0, 12.6, 63.8, 690.5 and 1393 mg/kg bw per day for females at 0, 70, 350, 3500 and 7000 ppm, respectively. Additional groups of 10 mice of each sex were similarly exposed for 52 weeks. Observations for mortalities and clinical signs were made daily. Body weight and feed consumption were recorded weekly for the first 14 weeks and every 4 weeks thereafter. Blood was sampled during

week 53 and at the end of the study for the analysis of haematology parameters. Decedents and mice killed at the end of the designated exposure period were necropsied, organs weighed and tissues examined histopathologically.

There were no treatment-related mortalities or clinical signs. Mean body weight was significantly lower ($P < 0.01$) than the control values at 3500 and 7000 ppm in both sexes throughout the dosing period (up to about 8–12% lower in both sexes). Body weight gain was not determined. Mean feed consumption was significantly higher ($P < 0.01$ or 0.05) than the control values at 3500 and 7000 ppm in both sexes (up to about 15%), mainly over the first 40–50 weeks. Overall feed conversion efficiency (weeks 1–12) was significantly lower ($P < 0.01$) than the control values at 3500 and 7000 ppm in both sexes (approximately 15% and 48% lower in males and 14% and 23% lower in females, respectively).

There were a number of haematology parameters that were slightly, albeit significantly, different from the control values, but these showed little consistency over time or between sexes. In females, increased platelet counts occurred at 3500 and 7000 ppm (+23% and +29% during week 53 and +27% and +40% during week 81, respectively).

There were no treatment-related gross lesions. Selected liver findings are presented in Table 15. Absolute liver weights were significantly ($P < 0.01$) higher than the control values at 3500 and 7000 ppm (approximately 10–30% higher than the controls) in mice killed after 53 weeks and at and above 350 ppm in mice killed after 80 weeks (approximately 10–20% higher than the controls). Although relative liver weights were increased at these same dietary concentrations, they were not significantly different from the control values. The increase in liver weight was corroborated histopathologically at 3500 and 7000 ppm by an increase in nuclear pleomorphism with or without increased cytoplasmic eosinophilia plus occasional giant cells and eosinophilic globules in the cytoplasm. At these same dietary concentrations, there was an increase in clear cell foci and/or basophilic foci in the liver. There were no treatment-related neoplastic findings. There was an increase in the incidence of hepatocellular adenocarcinoma in 3500 ppm males (4/65 versus 0/65 in the controls), which the author noted was above the historical control incidence. However, as this increase was not statistically significant and did not occur at 7000 ppm, it was not considered treatment related.

The NOAEL for chronic toxicity was 350 ppm (equal to 47.5 mg/kg bw per day) for lower body weight, increased liver weight and histopathological findings in the liver (nuclear pleomorphism, eosinophilia and giant cells) at 3500 ppm (equal to 525.5 mg/kg bw per day). The NOAEL for carcinogenicity was 7000 ppm (equal to 1100 mg/kg bw per day), the highest dietary concentration tested (Milburn, 1999).

Rats

Fenamidone (purity 98.9% or 99.8%) was admixed in the diet at a concentration of 0, 60, 150, 1000 or 8000 ppm and fed ad libitum to groups of SD rats for 52 weeks (10 rats of each sex per dose) or 104 weeks (60 rats of each sex per dose). Separate groups of 15 rats of each sex in the control and high-dose groups were treated similarly for 52 weeks followed by a 3-month recovery phase. The concentration of fenamidone in the high-dose diet was decreased to 4000 ppm from day 17 because of marked body weight loss. However, the study author (Bigot, 1999a) removed all rats from the reversibility phase on day 20. An additional high-dose group (5000 ppm) and control group formed part of a second study (Bigot, 1999b). Observations for mortality and clinical signs were made daily. Body weight and feed consumption were recorded weekly for the first 14 weeks and every 4 weeks thereafter. Ophthalmological examinations were performed prior to the commencement of dosing and at 1 and 2 years. Blood and urine were collected at approximately 6-month intervals for the analysis of standard haematology, clinical chemistry or urine analysis parameters. Decedents and rats killed at the end of the designated exposure period were necropsied, organs weighed and tissues examined histopathologically.

Table 15. Liver findings in mice following 80 weeks of dietary exposure to fenamidone

Parameter	0 ppm		70 ppm		350 ppm		3 500 ppm		7 000 ppm	
	M	F	M	F	M	F	M	F	M	F
Liver weight[a]										
Week 53										
Absolute (g)	1.54	1.26	1.58	1.34	1.55	1.32	1.70**	1.45**	1.71**	1.60**
Relative[b] (%)	4.35	4.65	4.39	4.76	4.78	4.71	5.22	5.68	5.50	6.11
Week 81										
Absolute (g)	1.57	1.37	1.59	1.32	1.70**	1.52**	1.81**	1.52**	1.83**	1.57**
Relative (%)	4.47	4.69	4.65	4.72	4.90	5.34	5.61	5.76	5.91	6.09
Liver histopathology[c]										
N	*65*	*55*	*65*	*65*	*65*	*65*	*65*	*65*	*65*	*65*
Nuclear pleomorphism ± cytoplasmic eosinophilia ± giant cells ± eosinophilic droplets	0	0	0	0	0	0	43	50	57	59
Altered hepatocytes: Clear cell foci	1	0	1	0	2	0	1	3	3	5
Altered hepatocytes: Basophilic foci	0	0	1	0	0	0	3	1	0	3

F: female; M: male; *: $P < 0.05$; **: $P < 0.01$
[a] Results expressed as means.
[b] Relative to body weight.
[c] Results expressed as the absolute number of mice with the findings.
Source: Milburn (1999)

The achieved doses from weeks 1 to 49 were 0, 3.4, 8.6, 54.4 and 309 mg/kg bw per day for males and 0, 4.3, 10.9, 70.5 and 380 mg/kg bw per day for females at 0, 60, 150, 1000 and 5000 ppm, respectively. The achieved doses from weeks 1 to 101 were 0, 2.8, 7.1, 47.7 and 260 mg/kg bw per day for males and 0, 3.6, 9.2, 60.9 and 335 mg/kg bw per day for females at 0, 60, 150, 1000 and 5000 ppm, respectively.

There were no treatment-related mortalities or clinical signs. There were no treatment-related effects on body weight, body weight gain or feed consumption at and below 1000 ppm. During weeks 1 and 2 of exposure to the diet containing 8000 ppm fenamidone, mean body weight was, respectively, about 14% and 26% lower than the control values in males and about 5% and 6% lower than the control values in females; these differences were statistically significant ($P < 0.01$). This lower body weight gain was concomitant with a decrease in feed consumption (approximately 16% lower than the controls; $P < 0.01$). In the additional study, reduced body weight gain occurred at 5000 ppm (up to 10% lower than the controls in the 1st year and about 7% lower in the 2nd year in males; up to about 14% lower than the controls in the 1st year and up to about 18% lower in the 2nd year in females). These effects on body weight gain were reversible after the 1st year of treatment. Feed consumption was also reduced at 5000 ppm (up to about 10% lower than the controls in males and 13% lower in females).

There were no treatment-related ophthalmological abnormalities or effects on haematology, clinical chemistry or urine analysis parameters.

In rats killed after 52 or 104 weeks of treatment, absolute and relative kidney weights were significantly higher ($P < 0.01$ or 0.05) than the control values at and above 150 ppm, whereas liver weights were significantly increased at and above 1000 ppm (Table 16). Absolute and relative thyroid weights were significantly higher than the control values ($P < 0.01$) at 5000 ppm (Table 16). In rats killed after 52 weeks, no treatment-related macroscopic abnormalities occurred at or below 1000 ppm. In rats dying or killed in a moribund condition, the incidence of enlarged livers and enlarged thyroid glands in 1000 ppm males was higher than the control values (20/36 versus 14/38 and 15/36 versus 7/38, respectively). In rats killed after 104 weeks, the incidence of liver enlargement was increased in 1000 ppm males (16/24 versus 8/22 in the controls). In 150 and 1000 ppm males, the incidence of kidneys with a granular appearance was increased relative to the controls (10/32 and 9/24, respectively, versus 2/22 in the controls). Also at 1000 ppm, there was an increase in males with enlarged kidneys (5/24 versus 1/22 in the controls), consistent with chronic progressive nephropathy. In the second study, large thyroid glands were observed in 5000 ppm males (3/10), but not in the controls.

In rats killed after 52 weeks, there was a dose-related increase in the incidence of slight diffuse thyroid follicular cell hypertrophy and/or hyperplasia at and above 150 ppm (Table 16). At 5000 ppm in the second study, the incidence of slight centrilobular hepatocyte hypertrophy was increased in females (5/10 versus 0/10 in the controls); in males, the incidences of foamy cytoplasm (4/10 mild, 2/10 moderate) and eosinophilic inclusions (2/10 slight, 1/10 mild and 2/10 moderate) in periportal hepatocytes were higher than the control incidences (0/10); these differences were not statistically significant. Similar histopathological changes in the liver were not evident after 104 weeks of treatment.

In rats killed after 104 weeks, hypertrophy of centrilobular hepatocytes (graded as slight or moderate) was significantly higher ($P < 0.05$) than the control incidence in 5000 ppm females (14/60 versus 0/60, respectively). Vacuolation of centrilobular hepatocytes (graded as slight to moderate) was also significantly higher ($P < 0.05$) than the control incidence in 5000 ppm females (12/60 versus 0/60, respectively). In the thyroid, the incidence of focal follicular cell hyperplasia was significantly higher ($P < 0.01$) than the control incidence in males at 1000 and 5000 ppm; no such differences were noted in females (Table 16). The thyroid follicular cell hyperplasia observed in males at 60 and 150 ppm was not statistically different from the control and on this basis was not considered treatment related. Colloid basophilia was significantly increased ($P < 0.001$) in both sexes at 1000 and 5000 ppm. While there was a slight increase in the severity of C-cell hyperplasia at 1000 and 5000 ppm in males and at and above 150 ppm in females, this increase was not statistically significant, nor was there any difference in the total number of rats with C-cell hyperplasia.

The NOAEL was 60 ppm (equal to 2.8 mg/kg bw per day), based on a dose-related increase in follicular cell hypertrophy and/or hyperplasia in both sexes after 52 weeks at 150 ppm (equal to 7.1 mg/kg bw per day). The NOAEL for carcinogenicity was 5000 ppm (equal to 260 mg/kg bw per day in), the highest dietary concentration tested (Bigot, 1999a, 1999b).

2.4 Genotoxicity

The results of genotoxicity assays on fenamidone are summarized in Table 17. In the majority of experimental systems, fenamidone tested negative for genotoxicity. The two in vitro studies in which positive responses were noted are described more fully below. In the mouse lymphoma forward mutation assay, fenamidone tested positive in the presence of an exogenous source of metabolic activation. In a chromosomal aberration assay conducted on human peripheral lymphocytes, fenamidone tested positive in both the presence and the absence of metabolic activation.

Table 16. Organ weight, macroscopic and histopathological findings in rats exposed to fenamidone for up to 2 years in the diet

Parameter	0 ppm		60 ppm		150 ppm		1 000 ppm		0 ppm		5 000 ppm	
	M	F	M	F	M	F	M	F	M	F	M	F
Organ weights[a] (chronic phase – 52 weeks on treatment)												
Kidney												
Absolute (g)	3.28	2.13	3.69	2.12	3.62	2.38	3.78*	2.48*	3.70	2.20	4.40*	2.54**
	(0)	(0)	(+13)	(0)	(+10)	(+12)	(+14)	(+16)	(0)	(0)	(+19)	(+15)
Relative[b] (%)	0.53	0.58	0.55	0.59	0.55	0.66*	0.60*	0.68**	0.56	0.61	0.69**	0.77**
	(0)	(0)	(+4)	(0)	(+4)	(+14)	(+13)	(+17)	(0)	(0)	(+23)	(+26)
Liver												
Absolute (g)	13.42	7.94	15.30	7.78	15.47	8.13	16.07*	8.40	14.98	7.87	18.99*	9.83**
	(0)	(0)	(+14)	(−2)	(+15)	(+2)	(+20)	(+6)	(0)	(0)	(+27)	(+25)
Relative (%)	2.14	2.14	2.28	2.13	2.36	2.23	2.53*	2.29	2.29	2.17	2.98**	2.98*
	(0)	(0)	(+7)	(0)	(+10)	(+4)	(+18)	(+7)	(0)	(0)	(+30)	(+37)
Thyroid												
Absolute (g)	0.030	0.021	0.031	0.020	0.031	0.022	0.034	0.024	0.033	0.024	0.046**	0.023
	(0)	(0)	(+3)	(+5)	(+3)	(+5)	(+13)	(+14)	(0)	(0)	(+39)	(−4)
Relative (%)	0.005	0.006	0.005	0.006	0.005	0.006	0.005	0.007	0.005	0.006	0.007**	0.007
	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(+17)	(0)	(0)	(+40)	(+17)
Organ weights[a] (carcinogenicity phase – 104 weeks on treatment)												
Kidney												
Absolute (g)	4.07	2.77	4.18	3.01	4.51*	3.17**	4.58*	3.26**	4.48	2.83	4.86	2.97
	(0)	(0)	(+3)	(+9)	(+11)	(+14)	(+13)	(+18)	(0)	(0)	(+8)	(+13)
Relative (%)	0.63	0.57	0.62	0.63	0.72*	0.66**	0.73	0.71**	0.74	0.58	0.82	0.74**
	(0)	(0)	(0)	(+10)	(+14)	(+16)	(+16)	(+25)	(0)	(0)	(+11)	(+28)
Liver												
Absolute (g)	14.03	12.95	14.52	12.39	14.34	12.42	15.81*	12.95	14.76	13.66	17.86**	13.47
	(0)	(0)	(+3)	(−4)	(+2)	(−4)	(+13)	(0)	(0)	(0)	(+21)	(0)

Parameter	0 ppm		60 ppm		150 ppm		1 000 ppm		0 ppm		5 000 ppm	
	M	F	M	F	M	F	M	F	M	F	M	F
Relative (%)	2.16 (0)	2.59 (0)	2.14 (−1)	2.56 (−1)	2.27 (+5)	2.57 (−1)	2.46* (+14)	2.75 (+6)	2.38 (0)	2.75 (0)	2.97** (+25)	3.28** (+19)
Thyroid												
Absolute (g)	0.049 (0)	0.038 (0)	0.043 (−12)	0.031 (−18)	0.047 (−4)	0.036 (−5)	0.057 (+16)	0.036 (−5)	0.051 (0)	0.056 (0)	0.067* (+31)	0.039 (−30)
Relative (%)	0.008 (0)	0.008 (0)	0.006 (−25)	0.007 (−12)	0.008 (0)	0.008 (0)	0.009 (+13)	0.008 (0)	0.009 (0)	0.011 (0)	0.011 (+22)	0.01 (+10)
Histopathological findings[c] – thyroid (chronic phase – 52 weeks on treatment)												
Follicular cell hypertrophy and/or hyperplasia												
- Slight	0/9	0/10	0/10	0/10	2/9	2/10	3/10	5/10	0/10	0/10	9/10	9/10
Histopathological findings[c] – thyroid (carcinogenicity phase – 104 weeks on treatment)												
Focal follicular cell hyperplasia												
- Slight	0/59	0/59	3/58	0/59	2/59	0/60	6/60	1/60	0/60	1/60	8/60	3/60
- Mild	0/59	0/59	0/58	0/59	0/59	0/60	0/60	0/60	0/60	0/60	5/60	0/60
- Moderate	0/59	0/59	0/58	0/59	0/59	0/60	2/60	0/60	0/60	0/60	0/60	0/60
- Marked	0/59	0/59	0/58	0/59	0/59	0/60	1/60	0/60	0/60	0/60	0/60	0/60
- Total	0/59	0/59	3/58	0/59	2/59	0/60	9/60**	1/60	0/60	1/60	13/60**	3/60
Basophilia, colloid												
- Slight	0/59	0/59	0/58	0/59	0/59	0/60	19/60	2/60	0/60	0/60	24/60	16/60
- Mild	0/59	0/59	0/58	0/59	0/59	0/60	6/60	0/60	0/60	0/60	20/60	6/60
- Total	0/59	0/59	0/58	0/59	0/59	0/60	25/60***	2/60***	0/60	0/60	44/60***	22/60***
Diffuse follicular cell hypertrophy												
- Slight	0/59	0/59	0/58	0/59	0/59	0/60	6/60	0/60	0/60	0/60	16/60	16/60
- Mild	0/59	0/59	0/58	0/59	0/59	0/60	0/60	0/60	0/60	0/60	1/60	1/60
- Total	0/59	0/59	0/58	0/59	0/59	0/60	6/60	1/60	0/60	0/60	17/60	17/60

Table 16 (continued)

Parameter	0 ppm		60 ppm		150 ppm		1 000 ppm		0 ppm		5 000 ppm	
	M	F	M	F	M	F		M	F	M	F	M
Increased follicular diameter												
- Slight	0/59	0/59	0/58	0/59	0/59	0/60	3/60	0/60	0/60	0/60	12/60	2/60
C-cell hyperplasia												
- Slight	20/59	23/59	17/58	19/59	22/59	22/60	15/60	18/60	16/60	23/60	26/60	19/60
- Mild	0/59	1/59	0/58	3/59	1/59	5/60	8/60	7/60	2/60	0/60	12/60	4/60
- Moderate	0/59	0/59	0/58	0/59	0/59	1/60	0/60	2/60	0/60	1/60	0/60	0/60
- Total	20/59	24/59	17/58	22/59	23/59	28/60	23/60	27/60	18/60	24/60	38/60	23/60

F: females; M: males; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$

[a] Results expressed as means.

[b] Relative to body weight.

[c] Results expressed as the number of rats with the finding/total number of rats sampled.

Source: Bigot (1999a, 1999b)

Table 17. Results of genotoxicity assays on fenamidone

End-point	Test object	Concentration	Purity (%)	Results	Reference
In vitro studies					
Gene mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537	*Cytotoxicity test*: 1–5 000 µg/plate (±S9) *Mutagenicity test 1*: 50–2 500 µg/plate (±S9) *Mutagenicity test 2*: 10–1 000 µg/plate (−S9); 50–2 500 µg/plate (+S9) DMSO vehicle	98.9	Negative[a]	Katchadourian, Berniaud & Fourmer (1996)
Gene mutation	Mouse lymphoma cells (L5178Y)	*Cytotoxicity test*: 18.75–600 µg/mL (±S9) *Experiment 1*: 12.5–150 µg/mL (−S9); 3.125–50 µg/L (+S9) *Experiment 2*: 50–200 µg/mL (−S9); 6.25–43.75 µg/mL (+S9) DMSO vehicle	99.8	Positive[b]	Fellows (1999a)
Chromosomal aberrations	Human lymphocytes	*Experiment 1*: 2.91–5.93 µg/mL (−S9); 147–300 µg/mL; 3 or 20 h incubation *Experiment 2*: 22.53–40.05 µg/mL (−S9); 168.8–300 µg/mL (+S9); 3 or 20 h incubation DMSO vehicle	99.8	Positive[b]	Marshall (1999a)
Unscheduled DNA synthesis	Rat hepatocytes	*Experiment 1*: 0.064–40 µg/mL *Experiment 2*: 1.25–30 µg/mL DMSO vehicle	99.8	Negative[c]	Fellows (1999b)
In vivo studies					
Micronucleus	Swiss CD-1 mice, bone marrow (5/sex/group)	0, 500, 1 000 or 2 000 mg/kg bw, ip; killed 24 or 48 h after dosing 0.5% (w/v) CMC vehicle	99.8	Negative[d]	Marshall (1999b)
Unscheduled DNA synthesis	Male Wistar rats, hepatocytes (5/group)	0, 80 or 2 000 mg/kg bw, po; killed after 12–14 h (Experiment 1) or 2–4 h (Experiment 2) after dosing 0.5% (w/v) CMC vehicle	99.8	Negative	Fellows (1999c)

CMC: carboxymethyl cellulose; DMSO: dimethyl sulfoxide; DNA: deoxyribonucleic acid; ip: intraperitoneal; po: per os (by mouth); S9: 9000 × *g* supernatant fraction of rat liver homogenate; w/v: weight per volume

[a] Cytotoxicity at and above 1000 µg/plate (standard plate incorporation) or 250 µg/plate (liquid preincubation); precipitation at and above 1000 µg/plate.

[b] See evaluation in text.

[c] Cytotoxicity at ≥ 30 µg/mL.

[d] The majority of mice showed signs of toxicity at the highest dose (lethargy, hunched appearance, eye closure and distended abdomen).

Table 18. Effect of fenamidone on survival of mouse lymphoma L5178Y cells (cytotoxicity test)

Concentration (µg/mL)	Mean relative survival (%)	
	−S9	+S9
0	100	100
18.75	120.8	58.4
37.5	106.3	5.5
75	91.8	0
150	0	1
300	0	0
600	0	0

S9: 9000 × *g* supernatant fraction of rat liver homogenate
Source: Fellows (1999a)

Fellows (1999a) analysed the potential of fenamidone (purity 99.8%) to cause mutations at the *tk* locus in mouse lymphoma L5178Y cells. 4-Nitroquinoline 1-oxide (NQO) and benzo(*a*)pyrene (BP) in dimethyl sulfoxide (DMSO) were used as positive controls. Experiments were conducted in the presence and absence of an exogenous source of metabolic activation (S9 post-mitochondrial liver fraction from Aroclor-induced rats). In a cytotoxicity/range-finding experiment, fenamidone in DMSO was tested at concentrations from 18.75 to 600 µg/mL. The highest concentration was the limit of solubility for the test material. Toxicity occurred at and above 150 µg/mL in the absence of S9 and at and above 37.5 µg/mL in the presence of S9 (Table 18). Based on the results of the range-finding experiment, two mutagenicity experiments were conducted, which tested concentrations of 3.125–200 µg/mL (±S9). In both experiments, fenamidone increased the mutation frequency only in the presence of S9 (Table 19). Although there were increases in both small and large colonies, the increase in the frequency of small colony mutants was more pronounced.

Fenamidone (99.8% purity) in DMSO was tested for its ability to cause chromosomal aberrations in peripheral human lymphocytes in the presence and absence of S9. In Experiment 1, concentrations ranging from 2.9 to 300 µg/mL were tested. Cells were exposed for 20 hours in the absence of S9 and 3 hours (with a 17-hour recovery phase) in the presence of S9. Concentrations tested in Experiment 2 ranged from 0.95 to 300 µg/mL. NQO and cyclophosphamide (CPA) were used as positive controls. There was a significant ($P < 0.05$ or 0.01) treatment-related increase in cells with chromosomal aberrations in both experiments following 3 or 20 hours of exposure (Table 20). The effect was more pronounced in the presence of S9 (Marshall, 1999a).

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

Rats

Fenamidone (purity 99.8%) was admixed in the diet at a concentration of 0, 60, 1000 or 5000 ppm and fed ad libitum to two parental generations of Crl:CD®(SD)BR rats (28 of each sex per group) and their offspring. Achieved doses during the premating period in F_0 rats were 0, 3.9, 63.8 and 328.3 mg/kg bw per day for males and 0, 5.15, 84.4 and 459.6 mg/kg bw per day for females at 0, 60, 1000 and 5000 ppm, respectively. Achieved doses during the premating period in F_1 rats were 0, 4.0, 68.6 and 353.2 mg/kg bw per day for males and 0, 5.45, 89.22 and 438.34 mg/kg bw per day for females at 0, 60, 1000 and 5000 ppm, respectively.

Rats were observed for mortalities and clinical signs 3 times daily. Parental rats were weighed weekly throughout the premating phase. Maternal rats were weighed on gestation days 0, 7, 14, 17 and 21 and on lactation days 0 (day of parturition), 7, 14 and 21. Feed consumption was recorded weekly throughout the premating phase, on days 7, 14 and 21 during gestation and on days 7 and 14 of lactation. Standard reproduction and litter parameters were recorded. Following death or scheduled

Table 19. Mutation frequency in L5178Y cells 2 days after exposure to fenamidone[a]

−S9			+S9		
Concentration (µg/mL)	Relative survival (%)	Mutation frequency	Concentration (µg/mL)	Relative survival (%)	Mutation frequency[b]
Experiment 1					
0	100	78.46	0	100	68.58
12.5	108	–	3.125	90.87	77.61
25	109.4	–	6.25	79	85.68
50	112.9	70.44	12.5	62	118.78*
75	93.6	67.42	25	32.8	240.87*
100	77.6	73.21	37.5	6.2	377.91
125	76	72.93	50	5.1	–
150	45	77.80	–	–	–
NQO = 0.05	116.6	357.48	BP = 2	64.6	596.5
NQO = 0.1	76.3	481.93	BP = 3	41.5	1074
Experiment 2					
0	100	106.54	0	100	75
50	88.8	111.08	6.25	88.8	72
75	75	123.21	12.5	63.8	234.9*
100	60.5	105.66	18.75	37.1	391.8*
125	40.7	97.03	25	4.8	529.6
150	10.6	120.16	31.25	7.2	410.9
175	1.7	–	37.5	6.0	–
200	0.93	–	43.75	7.7	–
NQO = 0.05	84.5	499.20	2	75.1	729.60
NQO = 0.1	77.8	442.46	3	37.1	752.75

BP: benzo(*a*)pyrene; NQO: 4-nitroquinoline 1-oxide; S9: 9000 × *g* supernatant fraction of rat liver homogenate; *: $P < 0.05$

[a] Results expressed as the mean.

[b] Significant ($P < 0.001$) linear trend.

Source: Fellows (1999a)

termination, necropsies were performed on all parental rats and offspring. The following organs from parental rats were weighed: testes, epididymides, seminal vesicles, prostate, uterus, ovaries, brain, pituitary, liver, kidneys, adrenal glands and spleen. The following tissues from control and high-dose parental rats were examined histopathologically: right testis, right epididymis, seminal vesicles, coagulating gland, prostate, pituitary, adrenal glands, ovaries, uterus, oviduct, cervix and vagina.

There were no treatment-related mortalities or clinical signs. At 5000 ppm, mean absolute body weight of F_0 parental rats was significantly lower ($P < 0.01$ or 0.05) than the control values during the premating phase (up to about 7% lower than the controls in males and 9% lower in females). Body weight gain was also significantly lower ($P < 0.01$ or 0.05) than the control values during this same period at 1000 and 5000 ppm in males (up to about 20% and 36% lower, respectively) and at 5000 ppm in females (up to about 36% lower). Mean feed consumption was significantly lower ($P < 0.01$ or 0.05) than the control values at these same doses. On occasion in both sexes, feed conversion efficiency was significantly lower ($P < 0.01$ or 0.05) than the control values during premating (up to about 50% lower than the controls). Throughout gestation and in the first week or two of lactation, the mean absolute body weight of 5000 ppm females was also significantly

Table 20. Proportion of human lymphocytes with chromosomal aberrations following exposure to fenamidone[a]

−S9			+S9		
Treatment (µg/mL)	Proportion of cells with aberrations excluding gaps	Mitotic index	Treatment (µg/mL)	Proportion of cells with aberrations excluding gaps	Mitotic index
Experiment 1					
0	0.030	3.6	0	0.020	5.9
2.907	0.025	3.0	147	0.18**	3.2
4.152	0.050	1.8	210	0.22**	3.1
5.932	0.060*	1.2	300	0.21**	1.6
NQO = 2.5	0.22**	Not reported	CPA = 25	0.56**	Not reported
Experiment 2					
0	0.020	4.1	0	0.025	5.6
22.53	0.040	2.3	168.8	0.13**	3.6
30.03	0.045	2.3	225	0.12**	3.0
40.05	0.065*	1.5	300	0.12**	3.3
NQO = 2.5	0.34**	Not reported	CPA = 25	0.40**	Not reported
			0[b]	0.005	5.7
			300[c]	0.080**	2.7

CPA: cyclophosphamide; NQO: 4-nitroquinoline 1-oxide; S9: 9000 × *g* supernatant fraction of rat liver homogenate; *: $P < 0.05$; **: $P < 0.01$

[a] Results expressed as means.

[b] Additional solvent control group.

[c] Additional fenamidone treatment group.

Source: Marshall (1999a)

lower ($P < 0.01$) than in the controls (up to approximately 10% lower), but there was no difference in body weight gain. No effect on feed consumption occurred during lactation or gestation.

There was no treatment-related effect on reproduction, sperm or litter parameters in any generation.

There were no significant intergroup differences in F_1 pup weights during lactation. Following weaning, the mean absolute body weights of F_1 rats were significantly lower ($P < 0.001$ or 0.01) than the control values at 5000 ppm (weeks 10 and 12 in males, approximately 7% and 10% lower, respectively; weeks 7 and 9 in females, approximately 9% and 8% lower, respectively). During gestation, the mean absolute body weight of F_1 dams was significantly lower ($P < 0.05$; approximately 7% lower) than in the controls at 5000 ppm only during week 3; there was no difference in body weight gain. There were no intergroup differences in maternal body weight or body weight gain during lactation. Feed consumption was generally unremarkable in F_1 parental rats, with a reduction of about 13% ($P < 0.01$ or 0.05) occurring in dams during lactation at 1000 and 5000 ppm. Feed conversion efficiency was significantly higher ($P < 0.01$) at 1000 and 5000 ppm in F_1 males (up to about 2.5 times higher than in the controls).

F_2 pup weights were significantly lower ($P < 0.01$ or 0.05) than in the controls in males at 5000 ppm (days 8 and 21; 17% and 21% lower, respectively) and in females at 1000 and 5000 ppm (day 21; 21% lower, $P < 0.01$ or 0.05).

At 5000 ppm, absolute liver and spleen weights of F_0 maternal rats were significantly higher ($P < 0.01$ or 0.05) than those of the controls (7% and 11% higher, respectively). In F_0 males and females at the same dose, relative liver and spleen weights were significantly higher ($P < 0.01$ or 0.05) than those of the controls (17% and 6% higher, respectively, in males; 15% and 16% higher,

respectively, in females). In F_1 females, absolute liver and spleen weights were significantly higher ($P < 0.01$ or 0.05) than those of the controls at 5000 ppm (16% higher for both), but there were no differences in males. Relative liver and spleen weights were significantly higher ($P < 0.01$) than those of the controls in 5000 ppm F_1 males (10% and 19%, respectively) and females (21% and 26%, respectively). In F_2 male pups, the absolute weights of the spleen and thymus were significantly lower than those of the controls (26% and 22% lower, respectively), but there was no similar effect in females or any difference in relative organ weights. None of the differences in organ weights in this study was accompanied by any histopathological findings.

The NOAEL for reproductive toxicity was 5000 ppm (equal to 328.3 mg/kg bw per day), the highest dietary concentration tested. The NOAEL for parental toxicity was 60 ppm (equal to 3.9 mg/kg bw per day), for lower body weight, reduced body weight gain and reduced feed consumption at 1000 ppm (equal to 63.8 mg/kg bw per day). The NOAEL for offspring toxicity was 1000 ppm (equal to 63.8 mg/kg bw per day) for lower F_1 and F_2 pup weights at 5000 ppm (equal to 328.3 mg/kg bw per day) (Bussi, 1999).

(b) Developmental toxicity

Rats

Fenamidone (99.8% purity) in 0.5% (w/v) carboxymethyl cellulose was administered by gavage to groups of 25 sperm-positive Crl: CD (SD) BR rats at a dose of 0, 25, 150 or 1000 mg/kg bw per day from day 6 to day 15 of gestation. Dams were observed daily throughout gestation for clinical signs of toxicity, with body weight and feed consumption recorded throughout this period. On day 20 of gestation, surviving dams were killed and fetuses examined for external, visceral and skeletal abnormalities.

There were no maternal deaths or treatment-related clinical signs. At 1000 mg/kg bw per day, dams lost a small amount (~8 g) of body weight over the first 2 days of dosing, with mean body weight gain significantly lower ($P < 0.01$) than in the controls from days 6 to 16 of gestation (35% lower). Feed consumption was significantly lower than in the controls from days 6 to 9 and from days 9 to 12 (40% and 15% lower, respectively). There were no treatment-related macroscopic findings in dams. Other than significantly lower ($P < 0.01$) fetal weight at 1000 mg/kg bw per day (5% lower than in the controls in both sexes), there were no treatment-related effects on litter parameters.

There were no treatment-related external abnormalities. Internal examination revealed a slight increase in the incidence of enlarged thymus at 150 and 1000 mg/kg bw per day (4.1% and 4.4%, respectively, compared with the control incidence of 1.1%); however, these values were well below the historical control mean (8.5%) and maximum (14.7%). The slight increase in incomplete ossification at 1000 mg/kg bw per day (Table 21) was not statistically significant and was considered a secondary effect of the reduction in fetal weight.

Table 21. Summary of skeletal fetal findings in rats

Observation	Incidence of finding (%)			
	0 mg/kg bw per day	25 mg/kg bw per day	150 mg/kg bw per day	1 000 mg/kg bw per day
Interparietal bone incompletely ossified				
Fetal incidence	1.5	3.2	3.3	5.6
Litter incidence	12	21.7	18.2	25
Hyoid body incompletely ossified				
Fetal incidence	7	7	8	15
Litter incidence	5	6	5	11

Source: Foulon & Wason (1997)

The NOAEL for maternal toxicity was 150 mg/kg bw per day for reduced body weight gain and feed consumption at 1000 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 150 mg/kg bw per day for lower fetal weight at 1000 mg/kg bw per day (Foulon & Wason, 1997).

Rabbits

Groups of 30 pregnant New Zealand White rabbits were exposed to fenamidone (purity 99.8%) in 0.5% (w/v) aqueous carboxymethyl cellulose by gavage from days 6 to 28 of gestation at a dose of 0, 10, 30 or 100 mg/kg bw per day. Dams were observed daily throughout gestation for clinical signs of toxicity, with body weight and feed consumption recorded regularly. Surviving dams were killed on day 29 of gestation and necropsied, with standard litter parameters recorded. Fetuses were examined for external, visceral and skeletal abnormalities.

There were no treatment-related deaths or clinical signs. Significantly reduced ($P < 0.01$) body weight gain occurred in dams at 100 mg/kg bw per day from days 6 to 10 of gestation (10 g versus 50 g in the control), but not from days 6 to 8 of gestation, when feed consumption was approximately 15% lower ($P < 0.01$) than in the controls. There were no significant intergroup differences in feed consumption at other times or in absolute body weight at any time. Mean absolute liver weight was significantly higher ($P < 0.01$) than in the controls at 30 and 100 mg/kg bw per day (19% and 37% higher, respectively). Litter parameters were unremarkable. There were no treatment-related external, visceral or skeletal abnormalities in fetuses.

The NOAEL for maternal toxicity was 30 mg/kg bw per day for decreased body weight gain at 100 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 100 mg/kg bw per day, the highest dose tested (Foulon, 1999).

2.6 Special studies

(a) Neurotoxicity

In an acute neurotoxicity study, fenamidone (purity 99.8%) in 0.5% (w/v) aqueous carboxymethyl cellulose was administered by gavage to groups of 10 Crl:CD Br rats of each sex at a dose of 0, 125, 500 or 2000 mg/kg bw. Observations for mortalities and clinical signs were made daily. Body weight was recorded prior to dosing, on the day of dosing and weekly thereafter. Feed consumption was recorded weekly. A functional observational battery was performed on all rats pretreatment and at 4 hours, 7 days and 14 days after dosing. Survivors were killed after 14 days, and nervous tissue was examined histopathologically.

There were no deaths. Clinical signs observed 24 hours after dosing included soiling of the anogenital region (2/10 males at 2000 mg/kg bw) or stained/wet anogenital region (1/10 females at 500 mg/kg bw and 2/10 females at 2000 mg/kg bw). Abnormalities observed during the functional observational battery were confined to the 4-hour time point and included increased urination in 2000 mg/kg bw females (6/10 compared with 1/10 in the controls; $P < 0.01$); soiling or staining of the fur around the anogenital region (2/10 males at 2000 mg/kg bw; 4/10 and 5/10 females at 500 and 2000 mg/kg bw, respectively; $P < 0.05$); mucoid faeces at 500 and 2000 mg/kg bw (2/10 males and 3/10 females at both doses); hunched posture (3/10 females at 2000 mg/kg bw; $P < 0.05$); unsteady gait (2/10 and 2/10 females at 500 and 2000 mg/kg bw, respectively); significantly lower ($P < 0.01$) rectal temperature in 2000 mg/kg bw females (0.3 °C lower than the control); and significantly reduced locomotor activity at 2000 mg/kg bw in both sexes ($P < 0.01$ in females and $P < 0.05$ in males) at 4 hours after dosing. There were no treatment-related histopathological abnormalities of the brain or nervous tissues.

The NOAEL was 125 mg/kg bw for nonspecific findings in the functional observational battery at 4 hours after dosing at 500 mg/kg bw (Hughes & Crome, 1999).

In a 13-week neurotoxicity study, groups of 10 Crl:CD BR rats of each sex per group were exposed to fenamidone (purity 98.9%) at a dietary concentration of 0, 150, 1000 or 5000 ppm. The mean achieved doses over 13 weeks were 0, 11.2, 73.5 and 392.3 mg/kg bw per day for males and 0,

12.7, 83.4 and 414.2 mg/kg bw per day for females at 0, 150, 1000 and 5000 ppm, respectively. Mortalities, clinical signs, body weight and feed consumption were recorded regularly throughout the study. A functional observational battery and motor activity assessment were performed prior to the commencement of dosing and then after 4, 8 and 13 weeks. Survivors were killed at the end of the exposure period and their brain weights recorded. Nervous tissue from five rats of each sex per dose was examined histopathologically.

There were no treatment-related deaths or clinical signs. Mean overall body weight gain (weeks 0–13) was significantly lower ($P < 0.01$) than in the controls at 5000 ppm (26% and 21% lower in males and females, respectively). Feed consumption was also significantly lower ($P < 0.05$) than in the controls at 5000 ppm (6% and 9% lower in males and females, respectively). There were no treatment-related functional observational battery findings or effects on locomotor activity. There were no intergroup differences in brain weight and no treatment-related histopathological abnormalities of the brain or nervous tissues.

The NOAEL was 1000 ppm (equal to 73.5 mg/kg bw per day) for reduced body weight gain at 5000 ppm (equal to 392.3 mg/kg bw per day) (Hooks, 2001).

In a developmental neurotoxicity study, fenamidone (purity 99.2%) was admixed in the diet at concentrations of 0, 60, 250, 1000 and 4700 ppm and fed ad libitum to groups of 30 pregnant female Wistar rats from day 6 of gestation to day 21 of lactation. The mean achieved doses in maternal rats over gestation and lactation were 0, 5.5, 23, 92.3 and 429 mg/kg bw per day at 0, 60, 250, 1000 and 4700 ppm, respectively. Observations for deaths and clinical signs were made regularly. Body weight and feed consumption were recorded regularly. Maternal rats were subjected to a functional observational battery during days 13 and 30 of gestation and days 11 and 21 of lactation. Following delivery, pups were examined and weighed. On postnatal days 4, 11, 21, 35, 45 and 60, at least 10 pups of each sex per dose were subjected to a functional observational battery. Pup weights were recorded on postnatal days 0, 4, 11, 17 and 21 and weekly thereafter. Female pups were examined daily for vaginal patency from postnatal day 29, whereas males were examined for preputial separation from postnatal day 38. A motor activity assessment was performed on one male and/or one female from each litter on postnatal days 13, 17, 21 and 60. The acoustic startle response was evaluated on postnatal days 22 and 60 on one male and/or one female from each litter. Ophthalmoscopy was performed on at least 10 rats of each sex per dose at about 50–60 days of age. Following sacrifice, all rats were necropsied, brain morphometry was recorded and brain tissue was examined histopathologically in the control and high-dose groups.

There were no treatment-related maternal deaths or clinical signs. Maternal body weight gain was significantly lower ($P < 0.05$) than in the controls from day 0 to day 20 of gestation at 4700 ppm (8% lower). There were no significant intergroup differences in maternal body weight gain during gestation. Feed consumption was unaffected by treatment. The functional observational battery was unremarkable.

In offspring, there were no treatment-related deaths or clinical signs. At the highest dose, mean absolute body weight was significantly lower ($P < 0.01$) than in the controls on postnatal days 4, 11, 17 and 21 (up to 10% lower than in the controls in both sexes). Mean body weight gain was also significantly lower ($P < 0.01$ or 0.05) than in the controls during lactation (up to 20% lower than in the controls in both sexes). There were no treatment-related functional observational battery findings and no effects on motor activity, startle response, the incidence of gross lesions, brain weight or brain morphometry.

The NOAEL for maternal toxicity was 1000 ppm (equal to 92.3 mg/kg bw per day) for reduced body weight gain at 4700 ppm (equal to 429 mg/kg bw per day). The NOAEL for offspring toxicity was also 1000 ppm (equal to 92.3 mg/kg bw per day), for lower absolute body weight and reduced body weight gain at 4700 ppm (equal to 429 mg/kg bw per day) (Sheets, Gilmore & Hoss, 2005).

(b) Studies on metabolites

Additional toxicity studies were conducted on three fenamidone metabolites: RPA 412636 (*S*-enantiomer of RPA 717879), RPA 410193 (*S*-enantiomer of RPA 405862) and RPA 412708 (*S*-enantiomer of RPA 408056). Details of these metabolites are provided in Table 22.

Table 22. Fenamidone metabolites on which additional toxicity tests were conducted

Code	Chemical name (IUPAC)	Structure	Description
RPA 412636	(*S*)-5-Methyl-5-phenyl-2,4-imidazolidine-dione	CH_3 H N O N O H	Present in rat urine at < 1% of the administered dose
RPA 410193	(5*S*)-5-Methyl-5-phenyl-3-(phenylamino)-2,4-imidazolidine-dione	CH_3 H N O N O N H	Not detected in rat metabolism studies; identified as a metabolite in grapes, grape juice and wine
RPA 412708	(5*S*)-5-Methyl-2-(methylthio)-5-phenyl-3,5-dihydro-4*H*-imidazol-4-one	CH_3 N O N S CH_3 H	Biliary or faecal metabolite in rats (> 10% of the administered dose)

IUPAC: International Union of Pure and Applied Chemistry

Acute toxicity

The results of acute oral (gavage) toxicity tests of fenamidone metabolites in SD rats are summarized in Table 23. Sedation, lateral recumbency, piloerection, dyspnoea, coma, tremors and hypoactivity were observed from 1 to 7 days after dosing with RPA 412636 at 500, 1000 or 2000 mg/kg bw, with recovery in survivors evident from 4 to 6 days after dosing (Manciaux, 1999a). No clinical signs were observed in the acute toxicity study on RPA 410193 (Manciaux, 1999b). A range of clinical signs was observed at and above a single dose of RPA 412708 at 100 mg/kg bw, including hypoactivity or sedation, piloerection, dyspnoea, lateral recumbency and ptosis; these signs resolved within 2 days (Manciaux, 1999c). Reduced body weight gain was also noted at 150 or 200 mg/kg bw.

Table 23. Results of studies of acute oral toxicity of fenamidone metabolites in SD rats

Metabolite/strain	Sex	Purity (%)	Vehicle	LD_{50} (mg/kg bw)	Reference
RPA 412636					
ICO: OFA-SD (IOPS Caw)	Male and female	99.8	0.5% CMC	1 520	Manciaux (1999a)
RPA 410193					
ICO: OFA-SD (IOPS Caw)	Male and female	99.8	0.5% CMC	> 2 000	Manciaux (1999b)
RPA 412708					
ICO: OFA-SD (IOPS Caw)	Male and female	99.8	0.5% CMC	100–200	Manciaux (1999c)

CMC: carboxymethyl cellulose; LD_{50}: median lethal dose

Short-term studies of toxicity

In a non-guideline study, RPA 412636 (purity 99.8%) was admixed in the diet at a concentration of 0, 300, 1200 or 3000 ppm and fed ad libitum to groups of five ICO: OFA-SD (IOPS Caw) rats of each sex per dose for 14 days. The mean achieved doses were 0, 23, 90 and 215 mg/kg bw per day for males and 0, 24.5, 96.7 and 233 mg/kg bw per day for females at 0, 300, 1200 and 3000 ppm, respectively. A satellite group of three rats of each sex was added to the control and high-dose groups and killed after 3 days for the analysis of liver weight, hepatic cellular proliferation and liver histopathology. Rats were observed daily for mortalities and clinical signs. Body weight was recorded on days 1, 7 and 14. Feed consumption was recorded weekly. Blood was sampled prior to terminal sacrifice for the analysis of standard haematology and clinical chemistry parameters. Following necropsy, the following organs were weighed: brain, kidneys, liver, ovaries, spleen, testes and thyroid. The following organs were examined histopathologically: duodenum, kidneys, liver, ovaries, spleen, testes and thyroid gland. Hepatic cellular proliferation was assessed by immunostaining. Microsomes were prepared for the analysis of cytochrome P450 isoenzyme profile.

There were no deaths or clinical signs. During the 1st week of treatment, mean absolute body weight was significantly lower ($P < 0.05$) than that of the controls in 3000 ppm males (4% lower). At 3000 ppm, body weight gain was significantly lower ($P < 0.05$) than that of the controls in both sexes (males: 40% lower than in the controls; females: body weight loss of 0.5 g). Significantly reduced ($P < 0.05$) feed consumption also occurred at this same dose during the 1st week (14% lower in males and 17% lower in females). Haematology parameters were unremarkable. In 3000 ppm females, urea and cholesterol were increased by 26% ($P < 0.05$) and 41% ($P < 0.01$), respectively.

The relative liver weight of high-dose males killed after 3 days of treatment was approximately 18% higher than that of the controls ($P < 0.05$). Mild liver enlargement was noted macroscopically in two males and one female at 3000 ppm. Hepatocytic mitosis was observed histopathologically in all males and two females at 3000 ppm. Mild hepatic (mainly centrilobular) hypertrophy was observed in all 3000 ppm males.

In males killed after 14 days of treatment, mean absolute and relative liver weights were significantly higher ($P < 0.01$) than those of the controls at 1200 and 3000 ppm (absolute: 30% and 34%; and relative: 28% and 40%, respectively). In 3000 ppm females, relative liver weight was significantly higher ($P < 0.05$) than that of the controls (16%). Enlargement of the liver was observed macroscopically in all males at 1200 and 3000 ppm in association with prominent lobulation in two or three males. One 500 ppm male and one female at each of 1200 and 3000 ppm had liver enlargement without prominent lobulation. Histopathology revealed a dose-related increase in the incidence and grade of hepatic hypertrophy in both sexes (Table 24). In the kidneys of all treated groups of males, an increase of eosinophilic droplets occurred in cortical tubular cells (Table 24). Additionally, multifocal cortical tubular hyaline degeneration occurred in all 1200 and 3000 ppm males and in one 500 ppm male. Staining of α_{2u}-globulin was evident in all 3000 ppm males (one graded as moderate, four graded as marked) compared with only one control male (graded as moderate). These kidney findings in males are not considered relevant to humans.

The results of the liver enzyme analysis are summarized in Table 25. There was a slight increase in total cytochrome P450, whereas BROD and PROD were increased, markedly consistent with the effect of phenobarbital. There was no treatment-related effect on EROD or lauric acid hydroxylation. In rats killed after 3 days, the numbers of PCNA positive cells in the liver were increased by 253% in males and by 276% in females at 3000 ppm, relative to the control group. In rats killed at the end of the study, the numbers of PCNA positive cells were increased at and above 300 ppm (6%, 44% and 133% in males and 14%, 0% and 66% in females at 300, 1200 and 3000 ppm, respectively).

The NOAEL was 1200 ppm (equal to 90 mg/kg bw per day) for reduced body weight gain and feed consumption at 3000 ppm (equal to 215 mg/kg bw per day) (Bigot, 1999c).

Table 24. Histopathological observations in rats following 14 days of dietary exposure to the fenamidone metabolite RPA 412636

Parameter	Incidence of finding (n = 5)							
	0 ppm		300 ppm		1 200 ppm		3 000 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
Hepatocytic hypertrophy								
- Slight	0	0	4	0	0	1	0	1
- Mild	0	0	0	0	2	2	0	1
- Moderate	0	0	0	0	2	0	1	3
- Marked	0	0	0	0	1	0	4	0
- Total	0	0	4	0	5	3	5	5
Eosinophilic droplets (kidney)								
- Slight	1	0	2	0	0	0	0	0
- Mild	0	0	1	0	3	0	0	0
- Moderate	0	0	1	0	2	0	5	0
- Total	1	0	4	0	5	0	5	0
Tubular degeneration								
- Slight	1	0	1	0	1	0	2	0
- Mild	0	0	0	0	2	0	2	0
- Moderate	0	0	0	0	2	0	1	0
- Total	1	0	1	0	5	0	5	0

Source: Bigot (1999c)

Table 25. Induction of rat liver enzymes by the fenamidone metabolite RPA 412636

Mean fold increase relative to the solvent control											
RPA 412636						Positive controls					
300 ppm		1 200 ppm		3 000 ppm		PB		β-NF		CLA	
Males	Females	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females
Total cytochrome P450											
1.8	1.25	2.2	1.3	2.3	1.5	2.2	1.8	1.3	1.5	1.2	0
BROD											
35	14	44	18	56	24	43	31	2.1	2.3	–	–
PROD											
20	17	27	22	39	39	28.8	20	3.4	5.1	–	–

β-NF: beta-naphthoflavone at 75 mg/kg bw per day; BROD: benzoxyresorufin *O*-debenzylation; CLA = clofibric acid at 300 mg/kg bw per day; PB: phenobarbital at 75 mg/kg bw per day; PROD: pentoxyresorufin *O*-depentylation
Source: Bigot (1999c)

In a similarly designed 14-day study, RPA 410193 (purity 99.8%) was admixed in the diet at concentrations of 0, 450, 4500 and 15 000 ppm and fed ad libitum to groups of five ICO: OFA-SD (IOPS Caw) rats of each sex per dose. The achieved doses were 0, 30, 299 and 1098 mg/kg bw per

day for males and 0, 37, 374 and 1133 mg/kg bw per day for females at 0, 450, 4500 and 15 000 ppm, respectively.

There were no deaths or treatment-related clinical signs or effects on body weight or feed consumption. At the highest dose, small reductions in red blood cells (males), haemoglobin (females) and haematocrit (females) were considered to be incidental findings. Cholesterol was significantly higher ($P < 0.01$) than in the controls at 4500 and 15 000 ppm (1.4- and 2-fold higher in males, respectively; 1.5- and 1.7-fold higher in females, respectively). In high-dose rats killed on day 4, absolute and relative liver weights were higher than those of the controls (absolute: 13% and 42% in males and females, respectively; relative: 14% and 44% in males and females, respectively), with only the relative weight of males significantly different from that of the controls ($P < 0.05$). No gross pathological changes were observed in these interim-killed rats, whereas slight centrilobular hypertrophy was observed histopathologically (two males and one female).

In rats killed on day 15, both relative and absolute liver weights were significantly higher ($P <$ 0.01 or 0.05) than those of the controls at 4500 and 15 000 ppm. The magnitude of this increase was approximately 35% and 50% for both absolute and relative liver weights in males at 4500 and 15 000 ppm, respectively. In females, the increases were approximately 17% and 30% for both absolute and relative weights at 4500 and 15 000 ppm, respectively. Macroscopically, liver enlargement was noted in all high-dose rats and all but one female at 4500 ppm; prominent lobulation and/or a dark coloration were associated with this enlargement. Histopathological examination revealed centrilobular hypertrophy at 4500 ppm (all males and 3/5 females) and 15 000 ppm (all rats), with the severity of this finding more pronounced in males. Histopathological findings in the thyroid gland comprised follicular cell hypertrophy characterized by a high columnar epithelial lining of the thyroid follicles and the presence of smaller follicles and loss of colloid (1/5, 2/5, 5/5 and 4/5 males and 0/5, 0/5, 4/5 and 4/5 females at 0, 450, 4500 and 15 000 ppm, respectively).

Results of liver enzyme analysis in rats killed on day 4 are given in Table 26. There was a dose-related increase in total cytochrome P450 in males, with a relatively modest increase in high-dose females. There was no treatment-related effect on EROD, whereas BROD and PROD were increased in both sexes. There was no effect on lauric acid hydroxylation. Cell cycling analysis of rats killed on day 4 revealed an approximate 5-fold increase in PCNA positive cells in high-dose rats.

Table 26. Induction of rat liver enzymes by fenamidone metabolite RPA 410193

Mean fold increase relative to the solvent control											
RPA 410193						Positive controls					
450 ppm		4 500 ppm		15 000 ppm		PB		β-NF		CLA	
Males	Females	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females
Total cytochrome P450											
1.4	0	1.9	0	2.2	1.4	2.2	1.8	1.3	1.5	1.2	0
BROD											
6	0	66	24	78	102	43	31	2.1	2.3	–	–
PROD											
1.2	0	16	9.7	15.5	23	28.8	20	3.4	5.1	–	–

β-NF: beta-naphthoflavone at 75 mg/kg bw per day; BROD: benzoxyresorufin *O*-debenzylation; CLA = clofibric acid at 300 mg/kg bw per day; PB: phenobarbital at 75 mg/kg bw per day; PROD: pentoxyresorufin *O*-depentylation
Source: Chuzel (1999a)

The NOAEL was 450 ppm (equal to 30 mg/kg bw per day) for histopathological findings in the thyroid (follicular cell hypertrophy, smaller follicles and loss of colloid) at 4500 ppm (equal to 299 mg/kg bw per day) (Chuzel, 1999a).

In another non-guideline study, RPA 412708 (purity 98%) was admixed in the diet at concentrations of 0, 200, 500 and 2000 ppm and fed ad libitum to groups of five Wistar (AF) rats RJ: WI (IOPS AF) rats of each sex per dose for 14 days. The achieved doses were, respectively, 0, 15.1, 38.5 and 150.5 for males and 0, 16.1, 37.8 and 147.3 mg/kg bw per day for females.

There were no deaths or treatment-related clinical signs. There were no treatment-related effects on body weight, feed consumption, haematology or clinical chemistry parameters. In rats killed at the end of the study, mean absolute liver weight was significantly higher ($P < 0.05$; 18%) at 2000 ppm than in the controls, with relative liver weight significantly increased at 500 ppm ($P < 0.05$; 8%) and 2000 ppm ($P < 0.01$; 22%). There were no treatment-related macroscopic or histopathological findings. There was no treatment-related induction of cytochrome P450 or increase in BROD, PROD or EROD. In rats killed after 3 days of treatment, an approximate 4.5-fold increase in cell proliferation in the liver occurred at 2000 ppm, but no such effect was evident in rats killed at the end of the study.

The NOAEL was 2000 ppm (equal to 147.3 mg/kg bw per day), the highest dietary concentration tested (Chuzel, 1999b).

In a 90-day study, RPA 412636 (purity 99.8%) was admixed in the diet at concentrations of 0, 100, 500 and 2500 ppm and fed ad libitum to groups of 10 ICO: OFA-SD (IOPS Caw) rats of each sex per dose. The mean achieved doses were 0, 6.4, 33 and 162 mg/kg bw per day for males and 0, 7.7, 39 and 196 mg/kg bw per day for females at 0, 100, 500 and 2500 ppm, respectively. Rats were observed daily for mortalities and clinical signs. Body weight and feed consumption were measured weekly. A neurotoxicity assessment was conducted pretreatment and during week 12, comprising a grasping reflex, righting reflex, corneal reflex, pupillary reflex, auditory startle reflex and head shaking reflex. An ophthalmological examination was performed pretreatment and during week 12. Blood was sampled during weeks 2 and 13 for the analysis of T_3, T_4 and TSH. Blood sampled during week 12 was also analysed for the standard range of haematology and clinical chemistry parameters. Urine was collected overnight prior to necropsy for urine analysis. Decedents and rats killed at the end of the designated exposure period were necropsied, organs weighed and tissues examined histopathologically.

There were no treatment-related mortalities, clinical signs, neurotoxicity or ophthalmological findings. Body weight was unaffected by treatment. Feed consumption was significantly lower ($P < 0.05$) than in the controls at 2500 ppm at weeks 1 (both sexes, ~9% lower), 6 (males, 12% lower) and 11 (females, 11% lower).

Selected haematology and clinical chemistry findings are summarized in Table 27. Haematological effects included significantly reduced ($P < 0.01$ or 0.05) haematocrit, mean corpuscular volume and mean corpuscular haemoglobin and increased prothrombin time at 2500 ppm, with reduced haematocrit also occurring in 500 ppm males. Treatment-related clinical chemistry findings at the highest dose included significantly increased ($P < 0.01$ or 0.05) ALAT (34% in males), creatinine (31% in males), cholesterol (30% in both sexes), triglycerides (65% in females), albumin (7% in females,), phosphorus (17% and 25% in males and females, respectively), potassium and calcium (5% in females). Thyroid hormone analysis was unremarkable. Urine analysis revealed an increase in crystals in 2500 ppm females (1/10, 2/10, 6/10 and 9/10 rats at 0, 100, 500 and 2500 ppm, respectively).

Selected organ weight and histopathological findings are presented in Table 28. Absolute and relative liver weights were significantly increased ($P < 0.01$) at 500 and 2500 ppm in males and at 2500 ppm in females. Macroscopically, livers appeared enlarged at 500 (two males) and 2500 ppm (nine males and one female), with prominent lobulation observed in one, two and five males at 100, 500 and 2500 ppm, respectively; four high-dose females also had prominent lobulation. Microscopically, hypertrophy of centrilobular hepatocytes was observed at 500 and 2500 ppm in both sexes, with vacuolation also observed at these doses. In high-dose males, absolute and relative adrenal weights were significantly higher than in the controls. Minimal diffuse hypertrophy of the zona

fasciculata was observed microscopically in three males at 2500 ppm. Thymus weights were significantly lower than in the controls ($P < 0.01$) in high-dose rats, without any accompanying pathology. In the kidneys, there was a slight increase in eosinophilic droplets (graded as slight) in the proximal tubules in high-dose males (1/10, 0/10, 2/10 and 6/10 at 0, 100, 500 and 2500 ppm, respectively). This finding is consistent with α_{2u}-globulin staining, which was observed in the 14-day study by Bigot (1999c).

Table 27. Haematology and clinical chemistry findings in rats exposed to metabolite RPA 412636 for 90 days

Parameter	0 ppm		100 ppm		500 ppm		2 500 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
Haematology[a]								
Hct (L/L)	0.48	0.45	0.47	0.44	0.46*	0.44	0.46*	0.44
MCV (fL)	50.3	52.1	49.3	51.9	49.0	51.8	47.1**	50.0*
MCH (pg)	17.18	17.94	16.81	17.85	16.97	17.81	16.29**	17.34**
PT (s)	12.01	12.55	11.78	12.75	12.42	12.54	14.13**	12.33
Clinical chemistry[a]								
ALAT (IU/L)	21.0	17.8	21.3	17.5	21.6	17.0	28.1*	20.9
Creatinine (µmol/L)	22.2	26.8	24.0	29.2	24.9	25.5	29.0**	26.5
Cholesterol (mmol/L)	2.26	2.40	2.33	2.57	2.36	2.50	2.95**	3.10**
Triglycerides (mmol/L)	0.71	0.40	0.80	0.38	0.71	0.41	0.50	0.66**
Albumin (g/L)	32.7	33.0	32.5	34.6	32.8	34.6	34.3	35.2*
Phosphorus (mmol/L)	1.79	1.39	1.82	1.54	1.90	1.49	2.01**	1.74**
Potassium (mmol/L)	3.97	3.51	3.99	3.72	3.92	3.68	3.97	3.77*
Calcium (mmol/L)	2.66	2.58	2.69	2.64	2.71	2.61	2.73	2.70**

ALAT: alanine aminotransferase; Hct: haematocrit; IU: International Units; MCH: mean corpuscular haemoglobin; MCV: mean corpuscular volume; PT: prothrombin time; *: $P < 0.05$; **: $P < 0.01$

[a] Results expressed as means.

Source: Dange (1999b)

The NOAEL was 100 ppm (equal to 6.4 mg/kg bw per day) for vacuolation of centrilobular hepatocytes in males at 500 ppm (equal to 33 mg/kg bw per day) (Dange, 1999b).

RPA 410193 (purity 99.8%) was admixed in the diet at concentrations of 0, 150, 1500 and 15 000 ppm and fed ad libitum to groups of 10 ICO: OFA-SD (IOPS Caw) rats of each sex per dose for 90 days. The achieved doses were 0, 9.3, 93.3 and 978 mg/kg bw per day for males and 0, 11.5, 115 and 1090 mg/kg bw per day for females at 0, 150, 1500 and 15 000 ppm, respectively. End-points and sampling intervals were consistent with those analysed in the preceding study by Dange (1999b).

There were no deaths, treatment-related clinical signs or effects on body weight or feed consumption. There were no treatment-related ophthalmological or neurotoxicity findings. Selected haematology and clinical chemistry findings are summarized in Table 29. Perturbations in red cell parameters occurred at the highest dose and included significantly lower ($P < 0.01$ or 0.05) red blood cells, haemoglobin, haematocrit and mean corpuscular haemoglobin concentration (both sexes), with

Table 28. Organ weights and histopathological findings in rats exposed to metabolite RPA 412636 for 90 days

Parameter	0 ppm		100 ppm		500 ppm		2 500 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
Organ weights[a]								
Liver								
Absolute (g)	10.98	6.39	12.00	6.63	13.68**	6.70	16.70**	8.00**
Relative[b] (%)	2.45	2.57	2.56	2.53	2.84*	2.70	3.69**	3.30
Adrenals								
Absolute (g)	0.065	0.072	0.066	0.077	0.068	0.078	0.087**	0.085
Relative (%)	0.015	0.029	0.014	0.030	0.014	0.032	0.019**	0.039
Thymus								
Absolute (g)	0.426	0.297	0.388	0.279	0.377	0.276	0.242**	0.194**
Relative (%)	0.096	0.119	0.083	0.107	0.078	0.112	0.054**	0.081**
Liver histopathology[c]								
Hypertrophy of centrilobular hepatocytes								
- Minimal	0	0	0	0	5	3	0	1
- Slight	0	0	0	0	5	0	0	0
- Moderate	0	0	0	0	0	0	10	9
- Total	0	0	0	0	10	3	10	0
Vacuolation of centrilobular hepatocytes								
- Minimal	0	0	0	0	5	0	8	1

*: $P < 0.05$; **: $P < 0.01$

[a] Results expressed as means.

[b] Relative to body weight.

[c] Results expressed as the absolute number of rats with the finding.

Source: Dange (1999b)

increased mean corpuscular volume in females. Cholesterol was significantly increased ($P < 0.01$) in males at 1500 and 15 000 ppm and in females at 15 000 ppm. Triglycerides were significantly elevated ($P < 0.01$) in high-dose males. Total protein was significantly higher ($P < 0.01$ or 0.05) than in the controls at 15 000 ppm, with elevated albumin also noted at 1500 and 15 000 ppm in both sexes.

Mean urine volume was approximately 2.9-fold higher ($P < 0.01$) than in the controls in high-dose females. Hormone analysis conducted in blood sampled during week 2 detected a significant increase (17% higher than in the controls, $P < 0.05$) in T_4 and a significant increase (50%; $P < 0.05$) in TSH in high-dose males; no differences were noted in females, in T_3 or in either sex at week 12.

The only effect on organ weights was a significant increase ($P < 0.01$ or 0.05) in absolute and relative liver weights at 1500 and 15 000 ppm in males and at 1500 ppm in females. In males, absolute liver weights were approximately 15% and 43% higher than in the controls at 1500 and 15 000 ppm, respectively, whereas relative liver weight was approximately 13% and 51% higher than in the controls, respectively. In females, absolute and relative liver weights were approximately 42% and 43% higher than in the controls at 15 000 ppm. At necropsy, liver enlargement was observed macroscopically (1/10, 5/10 and 9/10 males and 0/10, 0/10 and 9/10 females at 150, 1500 and 15 000

Table 29. Haematology and clinical chemistry findings in rats exposed to metabolite RPA 410193 for 90 days

Parameter	0 ppm		150 ppm		1 500 ppm		15 000 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
Haematology[a]								
RBC (1×10^{12})	9.68	8.51	9.30	8.71	9.48	8.52	9.00**	7.70**
Hb (g/100 mL)	15.56	14.91	15.35	15.05	15.58	14.75	14.54**	13.73**
Hct (L/L)	0.47	0.46	0.47	0.45	0.47	0.44	0.45**	0.42*
MCV	48.5	52.3	50.2	51.6	49.6	51.7	50.2	54.4*
MCHC (g/100 mL)	33.21	33.57	32.96	33.55	33.21	33.50	32.31**	32.85*
Clinical chemistry[a]								
Cholesterol (mmol/L)	2.03	2.28	2.13	2.12	2.81**	2.45	4.10**	3.69**
Triglycerides (mmol/L)	0.61	0.37	0.74	0.37	0.88	0.43	1.21**	0.59
Total protein (g/L)	70.2	67.4	69.7	68.1	72.8	70.6	73.4*	74.3**
Albumin (g/L)	30.3	31.5	30.1	32.7	32.5**	33.5*	31.7*	33.7*

Hb: haemoglobin; Hct: haematocrit; MCHC: mean corpuscular haemoglobin concentration; MCV: mean corpuscular volume; RBC: red blood cells; *: $P < 0.05$; **: $P < 0.01$
[a] Results expressed as means.
Source: Dange (1999c)

Table 30. Incidence of hypertrophy of centrilobular hepatocytes in rats exposed to metabolite RPA 410193 for 90 days

Grading	Incidence of hypertrophy of centrilobular hepatocytes (n = 10)[a]							
	0 ppm		150 ppm		1 500 ppm		15 000 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
Minimal	0	0	0	0	9	2	0	0
Slight	0	0	0	0	1	0	8	3
Moderate	0	0	0	0	0	0	2	7
Total	0	0	0	0	10	2	10	10

[a] Results expressed as the absolute number of rats with the finding.
Source: Dange (1999c)

ppm, respectively). Hypertrophy of centrilobular hepatocytes was observed histopathologically at 1500 and 15 000 ppm in both sexes (Table 30).

The NOAEL was 1500 ppm (equal to 93.3 mg/kg bw per day) for perturbations in red cell parameters at 15 000 ppm (equal to 978 mg/kg bw per day) (Dange, 1999c).

Genotoxicity

The results of genotoxicity studies on fenamidone metabolites are summarized in Table 31.

Table 31. Results of genotoxicity assays on fenamidone metabolites

End-point	Test object	Concentration	Purity (%)	Results	Reference
RPA 412636					
In vitro studies					
Gene mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537 *Escherichia coli* WP2uvrA	8–5 000 µg/plate (±S9) DMSO vehicle	99.8	Negative	Beevers (1999a)
Gene mutation	Mouse lymphoma cells (L5178Y)	100–1 600 µg/mL (±S9) DMSO vehicle	99.8	Negative	Fellows (1999d)
In vivo studies					
Micronucleus	CD-1 mice, bone marrow (5 males/group)	0, 75, 150 or 300 mg/kg bw, 2 × ip; killed 24 h after second dose 0.5% (w/v) CMC vehicle	99.8	Negative[a]	Whitewell (1999a)
RPA 410193					
In vitro studies					
Gene mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537 *E. coli* WP2uvrA	8–5 000 µg/plate (±S9) DMSO vehicle	> 99.8	Negative	Beevers (1999b)
Gene mutation	Mouse lymphoma cells (L5178Y)	50–800 µg/mL (±S9) DMSO vehicle	> 99.8	Negative	Fellows (1999e)
In vivo studies					
Micronucleus	CD-1 mice, bone marrow (8 males/group)	0, 500, 1 000 or 2 000 mg/kg bw, 2 × ip; killed 24 h after second dose 0.5% (w/v) CMC vehicle	99.8	Negative	Whitewell (1999b)
RPA 412708					
In vitro studies					
Gene mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537 *E. coli* WP2uvrA	8–5 000 µg/plate (±S9) DMSO vehicle	98	Negative	Beevers (1999c)
In vivo studies					
Micronucleus	CD-1 mice, bone marrow (8 males/group)	0, 37.5, 75 or 150 mg/kg bw, 2 × ip; killed 24 h after second dose 1% (w/v) CMC vehicle	98	Negative[b]	Whitewell (1999c)

CMC: carboxymethyl cellulose; DMSO: dimethyl sulfoxide; ip: intraperitoneal, S9, 9000 × *g* supernatant fraction of rat liver homogenate

[a] Clinical signs observed at highest dose (lethargy and piloerection).

[b] Clinical signs observed at the middle and high doses (lethargy and eye closure at 75 mg/kg bw; prostration, tremors, eye closure, abnormal breathing, lethargy and piloerection at 150 mg/kg bw).

3. Observations in humans

Steffens (2013) examined the occurrence of occupational medical experiences in 15 workers at a fenamidone processing plant in Mumbai, India, from 2008 until present. It was stated that workers wore personal protective equipment, including a protective suit, chemical-resistant gloves, safety glasses and dust mask. Workers were examined annually. Laboratory examinations included a full blood count, urine examination, fasting blood sugar, lipid profile, liver enzymes, creatinine and TSH. Technical examinations included vision testing, ultrasonography, electrocardiogram and lung function test. Additionally, biomonitoring for methaemoglobin and phenol in urine was conducted every 6 months. No adverse events were reported over the 5-year observation period.

Comments

Biochemical aspects

In experiments conducted in rats using [^{14}C]fenamidone labelled at either the C-phenyl or N-phenyl part of the molecule, the time to reach the maximum plasma concentration of radioactivity was 2–4 hours after a single oral dose of 3 mg/kg bw and 14–26 hours after a single oral dose of 300 mg/kg bw. Gastrointestinal absorption was greater than 80%. Radioactivity distributed to most tissues, with no evidence of accumulation. Relatively high concentrations of radioactivity (approximately 400 times higher than that in plasma) were detected in the thyroid following dosing with C-phenyl- but not N-phenyl-labelled fenamidone, suggesting the distribution of a radiolabelled metabolite to the thyroid. Fenamidone undergoes extensive metabolism in the rat by phase I (oxidation, reduction and hydrolysis) and phase II reactions (conjugation). More than 20 metabolites were detected in rat excreta. The plasma elimination half-life was at least 60 hours, with the majority of radioactivity excreted in the faeces (up to approximately 90% of the administered dose) and the remainder in urine. Mass balance data indicated that the majority of radioactivity (> 80%) was eliminated within 48 hours of dosing.

Toxicological data

The oral LD_{50} in rats was greater than 2000 mg/kg bw. The dermal LD_{50} in rats was greater than 2000 mg/kg bw, and the LC_{50} was greater than 2.1 mg/L. Fenamidone was neither a skin irritant nor an eye irritant in rabbits. In a guinea-pig maximization test, no skin sensitization occurred.

The target organs for fenamidone are the liver and thyroid. In rats, fenamidone was an inducer of cytochrome P450. Consistent with studies indicating a relatively high distribution of radiolabel to the rat thyroid, increased thyroid weight, follicular cell hypertrophy and hyperplasia were observed. This did not appear to be a secondary effect of liver enzyme induction, as thyroid hormone levels in plasma were not affected by treatment.

In a non-guideline 90-day toxicity study in mice that tested dietary concentrations of 0, 70, 700 and 7000 ppm (equal to 0, 11.4, 110 and 1102 mg/kg bw per day for males and 0, 14.5, 146 and 1468 mg/kg bw per day for females, respectively), the NOAEL was 700 ppm (equal to 110 mg/kg bw per day), based on clinical signs and deaths in males at 7000 ppm (equal to 1102 mg/kg bw per day). In a second 90-day study in mice that tested dietary concentrations of 0, 50, 200, 1000 and 5000 ppm (equal to 0, 11.3, 44.5, 220 and 1064 mg/kg bw per day for males and 0, 13.7, 54, 274 and 1375 mg/kg bw per day for females, respectively), the NOAEL was 1000 ppm (equal to 220 mg/kg bw per day) for equivocal histopathological findings in the liver at 5000 ppm (equal to 1064 mg/kg bw per day). The overall NOAEL for the two 90-day studies in mice was 1000 ppm (equal to 220 mg/kg bw per day), with an overall LOAEL of 5000 ppm (equal to 1064 mg/kg bw per day).

A 28-day toxicity study in rats tested dietary concentrations of 0, 500, 5000 and 15 000 ppm (equal to 0, 39, 389 and 1203 mg/kg bw per day for males and 0, 42, 405 and 1194 mg/kg bw per day for females, respectively). The NOAEL was 500 ppm (equal to 39 mg/kg bw per day) for reduced red cell parameters and increased spleen weights coincident with hyperplasia of the germinative follicle of the white pulp at and above 5000 ppm (equal to 389 mg/kg bw per day). The NOAEL in a second 28-

day study in rats, which tested dietary concentrations of 0, 60, 150, 1000 and 5000 ppm (equal to 0, 4.9, 12.3, 82.7 and 418.5 mg/kg bw per day for males and 0, 5.3, 13.9, 90.6 and 450.1 mg/kg bw per day for females, respectively), was 1000 ppm (equal to 82.7 mg/kg bw per day) for thyroid follicular cell hypertrophy in males at 5000 ppm (equal to 418.5 mg/kg bw per day).

In a 90-day toxicity study in rats, which tested dietary concentrations of 0, 50, 150, 500 and 5000 ppm (equal to 0, 3, 9, 30 and 305 mg/kg bw per day for males and 0, 3, 11, 35 and 337 mg/kg bw per day for females, respectively), the NOAEL was 500 ppm (equal to 30 mg/kg bw per day) for reduced body weight gain, reduced feed consumption and changes in red cell parameters at 5000 ppm (equal to 305 mg/kg bw per day). The NOAEL in a second 90-day rat study, which tested dietary concentrations of 0, 60, 150, 1000 and 5000 ppm (equal to 0, 4.1, 10.4, 68.3 and 344 mg/kg bw per day for males and 0, 4.8, 12.0, 83.3 and 381 mg/kg bw per day for females, respectively), was 1000 ppm (equal to 68.3 mg/kg bw per day) for reduced body weight gain at 5000 ppm (equal to 344 mg/kg bw per day).

In a 28-day toxicity study in dogs, which tested doses of 0, 3, 10 and 100 mg/kg bw per day given by capsule, the NOAEL was 100 mg/kg bw per day, the highest dose tested. In a 13-week study in dogs, which tested doses of 0, 10, 100 and 500 mg/kg bw per day given by capsule, the NOAEL was 500 mg/kg bw per day, the highest dose tested. In a 52-week study in dogs, which tested doses of 0, 10, 100 and 1000 mg/kg bw per day given by capsule, the NOAEL was 100 mg/kg bw per day for clinical signs (hypersalivation and vomiting), increased AP activity, increased liver weight (males) and increased bile duct hyperplasia (males) at 1000 mg/kg bw per day. The overall NOAEL for the 13- and 52-week studies was 500 mg/kg bw per day, with an overall LOAEL of 1000 mg/kg bw per day.

In a long-term study of toxicity and carcinogenicity in mice, which tested dietary concentrations of 0, 70, 350, 3500 and 7000 ppm (equal to 0, 9.5, 47.5, 525.5 and 1100 mg/kg bw per day for males and 0, 12.6, 63.8, 690.5 and 1393 mg/kg bw per day for females, respectively), the NOAEL was 350 ppm (equal to 47.5 mg/kg bw per day) for lower body weight, increased liver weight and histopathological findings in the liver at 3500 ppm (equal to 525.5 mg/kg bw per day). No treatment-related neoplastic lesions were detected at dietary concentrations up to 7000 ppm (equal to 1100 mg/kg bw per day).

In a long-term toxicity and carcinogenicity study in rats that tested dietary concentrations of 0, 60, 150, 1000 and 5000 ppm (equal to 0, 2.8, 7.1, 47.7 and 260 mg/kg bw per day for males and 0, 3.6, 9.2, 60.9 and 335 mg/kg bw per day for females, respectively), the NOAEL was 60 ppm (equal to 2.8 mg/kg bw per day) for thyroid follicular cell hypertrophy and hyperplasia at 150 ppm (equal to 7.1 mg/kg bw per day). No treatment-related neoplastic lesions were detected at dietary concentrations up to 5000 ppm (equal to 260 mg/kg bw per day).

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

Fenamidone was negative in the Ames test and in an in vitro unscheduled DNA synthesis assay in rat hepatocytes. Fenamidone was positive in a mouse lymphoma forward mutation assay and caused chromosomal aberrations in cultured human peripheral blood lymphocytes in the presence of metabolic activation. Negative responses were obtained in vivo in the mouse micronucleus test and unscheduled DNA synthesis assay in rat hepatocytes.

The Meeting concluded that fenamidone is unlikely to be genotoxic in vivo.

Considering the lack of in vivo genotoxicity potential and the absence of a carcinogenic response in mice and rats, the Meeting concluded that fenamidone is unlikely to pose a carcinogenic risk to humans from the diet.

In a two-generation study in rats, which tested dietary concentrations of 0, 60, 1000 and 5000 ppm (equal to 0, 3.9, 63.8 and 328.3 mg/kg bw per day for males and 0, 5.15, 84.4 and 459.6 mg/kg bw per day for females, respectively), there was no evidence of reproductive toxicity up to the highest tested dietary concentration of 5000 ppm (equal to 328.3 mg/kg bw per day). The NOAEL for parental toxicity was 60 ppm (equal to 3.9 mg/kg bw per day) for lower body weight, reduced body

weight gain and reduced feed consumption at 1000 ppm (equal to 63.8 mg/kg bw per day). The NOAEL for offspring toxicity was 1000 ppm (equal to 63.8 mg/kg bw per day) for lower F_1 and F_2 pup weights at 5000 ppm (equal to 328.3 mg/kg bw per day).

In a rat developmental toxicity study that tested doses of 0, 25, 150 and 1000 mg/kg bw per day, the NOAEL for maternal toxicity was 150 mg/kg bw per day for reduced body weight gain and feed consumption at 1000 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 150 mg/kg bw per day for lower fetal weight at 1000 mg/kg bw per day.

In a rabbit developmental toxicity study that tested doses of 0, 10, 30 and 100 mg/kg bw per day, the NOAEL for maternal toxicity was 30 mg/kg bw per day for decreased body weight gain at 100 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 100 mg/kg bw per day, the highest dose tested.

The Meeting concluded that fenamidone is not teratogenic in rats or rabbits.

Acute and subchronic neurotoxicity studies were conducted in rats. In the acute gavage study, which tested doses of 0, 125, 500 and 2000 mg/kg bw per day, the NOAEL was 125 mg/kg bw for nonspecific findings in the functional observational battery at 4 hours after dosing at 500 mg/kg bw per day and above. In the subchronic study, which tested dietary concentrations of 0, 150, 1000 and 5000 ppm (equal to 0, 11.2, 73.5 and 392.3 mg/kg bw per day for males and 0, 12.7, 83.4 and 414.2 mg/kg bw per day for females, respectively), the NOAEL was 1000 ppm (equal to 73.5 mg/kg bw per day) for reduced body weight gain at 5000 ppm (equal to 392.3 mg/kg bw per day).

There was no evidence of developmental neurotoxicity in a dietary study conducted in female rats, which tested dietary concentrations of 0, 60, 250, 1000 and 4700 ppm (equal to 0, 5.5, 23, 92.3 and 429 mg/kg bw per day, respectively). The NOAEL for maternal toxicity was 1000 ppm (equal to 92.3 mg/kg bw per day) for reduced body weight gain at 4700 ppm (equal to 429 mg/kg bw per day). The NOAEL for offspring toxicity was also 1000 ppm (equal to 92.3 mg/kg bw per day), for lower absolute body weight and reduced body weight gain at 4700 ppm (equal to 429 mg/kg bw per day).

The Meeting concluded that fenamidone is not neurotoxic.

Toxicological data on metabolites and/or degradates

Toxicity studies were conducted on three fenamidone metabolites: (1) (*S*)-5-methyl-5-phenyl-2,4-imidazolidine-dione (RPA 412636), which is a metabolite in rat urine present at less than 1% of an administered dose; (2) (5*S*)-5-methyl-5-phenyl-3-(phenylamino)-2,4-imidazolidine-dione (RPA 410193), which is a novel plant metabolite; and (3) (5*S*)-5-methyl-2-(methylthio)-5-phenyl-3,5-dihydro-4*H*-imidazol-4-one (RPA 412708), which is a metabolite in rat bile present at greater than 10% of an administered dose.

The oral LD_{50} in rats was 1520 mg/kg bw for RPA 412636, greater than 2000 mg/kg bw for RPA 410193 and greater than 100 mg/kg bw for RPA 412708.

Short-term studies of toxicity were performed on RPA 412636, RPA 410193 and RPA 412708 in rats. As with the parent compound, liver enzyme induction and liver hypertrophy occurred with RPA 412636 and RPA 410193, but not RPA 412708. In a non-guideline 14-day study on RPA 412636 that tested dietary concentrations of 0, 300, 1200 and 3000 ppm (equal to 0, 23, 90 and 215 mg/kg bw per day for males and 0, 24.5, 96.7 and 233 mg/kg bw per day for females, respectively), the NOAEL was 1200 ppm (equal to 90 mg/kg bw per day) for reduced body weight gain and feed consumption at 3000 ppm (equal to 215 mg/kg bw per day). In a similar non-guideline study on RPA 410193, which tested dietary concentrations of 0, 450, 4500 and 15 000 ppm (equal to 0, 30, 299 and 1098 mg/kg bw per day for males and 0, 37, 374 and 1133 mg/kg bw per day for females, respectively), the NOAEL was 450 ppm (equal to 30 mg/kg bw per day) for histopathological findings in the thyroid at 4500 ppm (equal to 299 mg/kg bw per day). In the non-guideline 14-day study on RPA 412708, which tested dietary concentrations of 0, 200, 500 and 2000 ppm (equal to 0, 15.1, 38.5 and 150.5 mg/kg bw per day for males and 0, 16.1, 37.8 and 147.3 mg/kg bw per day for

females, respectively), the NOAEL was 2000 ppm (equal to 147.3 mg/kg bw per day), the highest dietary concentration tested.

Ninety-day dietary studies were conducted on RPA 412636 and RPA 410193 in rats. The study on RPA 412636 tested dietary concentrations of 0, 100, 500 and 2500 ppm (equal to 0, 6.4, 33 and 162 mg/kg bw per day for males and 0, 7.7, 39 and 196 mg/kg bw per day for females, respectively); the NOAEL was 100 ppm (equal to 6.4 mg/kg bw per day) for vacuolation of centrilobular hepatocytes at 500 ppm (equal to 33 mg/kg bw per day) in males. In the study conducted on RPA 410193, which tested dietary concentrations of 0, 150, 1500 and 15 000 ppm (equal to 0, 9.3, 93.3 and 978 mg/kg bw per day for males and 0, 11.5, 115 and 1090 mg/kg bw per day for females, respectively), the NOAEL was 1500 ppm (equal to 93.3 mg/kg bw per day), based on perturbations in red cell parameters at 15 000 ppm (equal to 978 mg/kg bw per day).

There was no evidence of genotoxicity in vitro or in vivo for RPA 412636, RPA 410193 and RPA 412708.

Human data

In a cohort of 15 workers involved in the processing of fenamidone over a 5-year period, no adverse medical events were reported, and there were no detectable effects following physical and biochemical examinations.

The Meeting concluded that the database on fenamidone was adequate to characterize the potential hazards to fetuses, infants and children.

Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) of 0–0.03 mg/kg bw per day, based on the NOAEL of 2.8 mg/kg bw per day for a dose-related increase in thyroid follicular cell hypertrophy and hyperplasia in both sexes after 52 weeks in the 2-year rat study. A 100-fold safety factor was applied.

The Meeting established an acute reference dose (ARfD) of 1 mg/kg bw, based on the NOAEL of 125 mg/kg bw per day for nonspecific findings in the functional observational battery in the acute neurotoxicity study in rats, and using a 100-fold safety factor. The Meeting noted that decreased maternal body weight gain in the developmental toxicity study in rabbits with a lower NOAEL of 30 mg/kg bw per day was not an acute effect and was therefore not a suitable basis for establishing an ARfD for fenamidone.

Levels relevant to risk assessment of fenamidone

Species	Study	Effect	NOAEL	LOAEL
Mouse	Ninety-day studies of toxicity[a,b]	Toxicity	1 000 ppm, equal to 220 mg/kg bw per day	5 000 ppm, equal to 1 064 mg/kg bw per day
	Two-year study of toxicity and carcinogenicity[a]	Toxicity	350 ppm, equal to 47.5 mg/kg bw per day	3 500 ppm, equal to 525.5 mg/kg bw per day
		Carcinogenicity	7 000 ppm, equal to 1 100 mg/kg bw per day[c]	–

Species	Study	Effect	NOAEL	LOAEL
Rat	Acute neurotoxicity study[d]	Toxicity	125 mg/kg bw per day	500 mg/kg bw per day
	Thirteen-week studies of toxicity or neurotoxicity[a,b]	Toxicity	1 000 ppm, equal to 73.5 mg/kg bw per day	5 000 ppm, equal to 305 mg/kg bw per day
	Two-year study of toxicity and carcinogenicity[a]	Toxicity	60 ppm, equal to 2.8 mg/kg bw per day	150 ppm, equal to 7.1 mg/kg bw per day
		Carcinogenicity	5 000 ppm, equal to 260 mg/kg bw per day[c]	–
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	5 000 ppm, equal to 328.3 mg/kg bw per day[c]	–
		Parental toxicity	60 ppm, equal to 3.9 mg/kg bw per day	1 000 ppm, equal to 63.8 mg/kg bw per day
		Offspring toxicity	1 000 ppm, equal to 63.8 mg/kg bw per day	5 000 ppm, equal to 328.3 mg/kg bw per day
	Developmental toxicity study[d]	Maternal toxicity	150 mg/kg bw per day	1 000 mg/kg bw per day
		Embryo and fetal toxicity	150 mg/kg bw per day	1 000 mg/kg bw per day
Rabbit	Developmental toxicity study[d]	Maternal toxicity	30 mg/kg bw per day	100 mg/kg bw per day
		Embryo and fetal toxicity	100 mg/kg bw per day[c]	–
Dog	Thirteen- and 52-week studies of toxicity[b,e]	Toxicity	500 mg/kg bw per day	1 000 mg/kg bw per day

LOAEL: lowest-observed-adverse-effect level; NOAEL: no-observed-adverse-effect level

[a] Dietary administration.

[b] Two or more studies combined. For the rat, two 90-day toxicity studies and a subchronic neurotoxicity study were combined.

[c] Highest dose tested.

[d] Gavage administration.

[e] Capsule administration.

Levels relevant to risk assessment of fenamidone metabolites based on studies conducted in rats

Metabolite	Study	Effect	NOAEL	LOAEL
RPA 412636[a]	Ninety-day study of toxicity[b]	Toxicity	100 ppm, equal to 6.4 mg/kg bw per day	500 ppm, equal to 33 mg/kg bw per day
RPA 410193[c]	Ninety-day study of toxicity[b]	Toxicity	1 500 ppm, equal to 93.3 mg/kg bw per day	15 000 ppm, equal to 978 mg/kg bw per day
RPA 412708[d]	Fourteen-day study of toxicity[b]	Toxicity	2 000 ppm, equal to 147.3 mg/kg bw per day[e]	–

LOAEL: lowest-observed-adverse-effect level; NOAEL: no-observed-adverse-effect level

[a] (*S*)-5-Methyl-5-phenyl-2,4-imidazolidine-dione.

[b] Dietary administration.

[c] (5*S*)-5-Methyl-5-phenyl-3-(phenylamino)-2,4-imidazolidine-dione.

[d] (5*S*)-5-methyl-2-(methylthio)-5-phenyl-3,5-dihydro-4*H*-imidazol-4-one.

[e] Highest dose tested.

Estimate of acceptable daily intake

0–0.03 mg/kg bw

Estimate of acute reference dose

1 mg/kg bw

Information that would be useful for the continued evaluation of fenamidone

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

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

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rats: T_{max} = 2–26 h; extensive, > 80%
Distribution	Widespread tissue distribution; preferential distribution of radiolabel to the thyroid
Potential for accumulation	No potential for accumulation
Rate and extent of excretion	Excretion via faeces and urine; half-life > 60 h
Metabolism in animals	Extensive
Toxicologically significant compounds in animals, plants and the environment	Fenamidone, RPA 412636, RPA 410193, RPA 412708
Acute toxicity	
Rat, LD_{50}, oral	> 2 000 mg/kg bw
Rat, LD_{50}, dermal	> 2 000 mg/kg bw
Rat, LC_{50}, inhalation	> 2.1 mg/L
Rabbit, dermal irritation	Non-irritating
Rabbit, ocular irritation	Non-irritating
Dermal sensitization	Non-sensitizing (guinea-pigs)
Short-term studies of toxicity	
Target/critical effect	Liver, thyroid
Lowest relevant oral NOAEL	68.3 mg/kg bw per day (90 days, rat)
Lowest relevant dermal NOAEL	No data
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Thyroid
Lowest relevant NOAEL	2.8 mg/kg bw per day (rat)
Carcinogenicity	Unlikely to pose a carcinogenic risk to humans from the diet
Genotoxicity	
	Not genotoxic in vivo
Reproductive toxicity	
Reproduction target/critical effect	No evidence of reproductive toxicity (rat)
Lowest relevant parental NOAEL	3.9 mg/kg bw per day
Lowest relevant offspring NOAEL	63.8 mg/kg bw per day
Lowest relevant reproduction NOAEL	328.3 mg/kg bw per day, the highest dose tested

Developmental toxicity	
Developmental target/critical effect	Decreased pup weights at maternally toxic doses (rat)
Lowest maternal NOAEL	30 mg/kg bw per day (rabbit)
Lowest embryo/fetal NOAEL	100 mg/kg bw per day, the highest dose tested (rabbit); 150 mg/kg bw per day (rat)
Neurotoxicity	
Neurotoxicity	Not neurotoxic
Developmental neurotoxicity	No developmental neurotoxicity
Toxicological studies on RPA 412636	
Rat, LD_{50}, oral	1 520 mg/kg bw
Lowest relevant short-term oral NOAEL	6.4 mg/kg bw per day (90 days, rat)
Genotoxicity	Not genotoxic
Toxicological studies on RPA 410193	
Rat, LD_{50}, oral	> 2 000 mg/kg bw
Lowest relevant short-term oral NOAEL	93.3 mg/kg bw per day (90 days, rat)
Genotoxicity	Not genotoxic
Toxicological studies on RPA 412708	
Rat, LD_{50}, oral	100 mg/kg bw
Lowest relevant short-term oral NOAEL	147.3 mg/kg bw per day (14 days, rat)
Genotoxicity	Not genotoxic
Medical data	
	No adverse effects in workers involved with manufacturing fenamidone

LC_{50}: median lethal concentration; LD_{50}: median lethal dose; NOAEC: no-observed-adverse-effect concentration; NOAEL: no-observed-adverse-effect level

Summary

	Value	Studies	Safety factor
ADI	0–0.03 mg/kg bw	Two-year dietary study in rats	100
ARfD	1 mg/kg bw	Acute neurotoxicity study in rats	100

ADI: acceptable daily intake; ARfD: acute reference dose

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FLUENSULFONE

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Explanation

Fluensulfone is the International Organization for Standardization–approved name for 5-chloro-2-(3,4,4-trifluorobut-3-en-1-ylsulfonyl)-1,3-thiazole (International Union of Pure and Applied Chemistry) (Chemical Abstracts Service No. 318290-98-1). Fluensulfone is a nematicide for use on a range of vegetable crops. Target pests are root knot, root lesion and cyst nematodes. Fluensulfone's mode of pesticidal activity has not been determined.

Fluensulfone has been known under the development codes MCW-2 and BYI 01921. Most toxicological studies were performed with pilot plant–produced material (batch 36372130-291-PF1), produced by Makhteshim Chemical Works Ltd. A small number of initial toxicological studies were conducted with the test batches produced by Bayer CropScience.

Fluensulfone 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 met the minimum requirements of the applicable Organisation for Economic Co-operation and Development (OECD) or national test guidelines.

Evaluation for acceptable daily intake

1. Biochemical aspects

The metabolism and excretion of [^{14}C]fluensulfone were determined using fluensulfone radiolabelled at the thiazole and butene positions (Fig. 1).

Fig. 1. Structure and radiolabel positions for fluensulfone

[thiazole-^{14}C]fluensulfone

[butene-^{14}C]fluensulfone

* Indicates position of ^{14}C label

1.1 Absorption, distribution and excretion

Rats

The absorption and excretion of [^{14}C]fluensulfone (thiazole and butene labels), prepared as a suspension in polyethylene glycol (PEG) 300, were investigated using single oral low (5 mg/kg body weight [bw]) or high (500 mg/kg bw) doses administered to groups of 4–8 male and 4–8 female Wistar rats. For the excretion segment, rats were housed in individual glass metabolism cages equipped for collection of $^{14}CO_2$, urine and faeces over 120 hours and were terminated thereafter. Kinetic parameters were determined from blood samples obtained from jugular vein cannulae over a period of up to 336 hours.

The recovery of radioactivity was 91.3–97.3% of the administered dose for all groups of rats for the excretion segment. The excretion of radiolabel was generally similar in males and females (Table 1). There was no indication that elimination processes were saturated by the 100-fold increase in dose level, as levels of radiolabel in urine, cage wash, faeces, carcass and gastrointestinal tract were similar. Urine was the major route of excretion (> 70%) for both radiolabel positions. Analysis showed that the metabolites in cage wash and urine were similar, thus indicating that the cage wash mainly derived from urine.

Table 1. Summary of excretion (to 72 hours) for rats administered [^{14}C]fluensulfone

	Excretion (% of administered dose; mean ± standard deviation)							
	[thiazole-^{14}C]fluensulfone				[butene-^{14}C]fluensulfone			
	Low dose (5 mg/kg bw)		High dose (500 mg/kg bw)		Low dose (5 mg/kg bw)		High dose (500 mg/kg bw)	
	Males	Females	Males	Females	Males	Females	Males	Females
CO_2 traps	0.36 ± 0.13	0.55 ± 0.13	0.06 ± 0.03	0.07 ±0.05	4.0 ± 1.8	4.4 ± 1.7	1.9 ± 1.0	1.3 ± 0.7
Urine	77.8 ± 1.6	71.7 ± 9.9	76.0 ± 7.6	76.0 ± 9.6	63.8 ± 4.5	63.2 ± 6.0	71.7 ± 8.1	71.8 ± 4.9
Cage wash	6.6 ± 0.9	14.2 ± 4.0	9.1 ± 3.3	12.2 ± 5.2	11.9 ± 2.2	11.9 ± 6.6	8.9 ± 3.7	10.9 ± 3.2
Faeces	11.0 ± 1.1	8.3 ± 0.7	7.2 ± 0.9	5.1 ± 1.3	13.3 ± 1.2	10.0 ± 1.1	11.4 ± 3.0	6.3 ± 1.5
Carcass	1.5 ± 0.3	1.8 ± 0.1	1.2 ± 0.1	1.2 ± 0.3	2.6 ± 0.8	1.6 ± 0.2	1.4 ± 0.4	0.9 ± 0.4
GI tract	0.07 ± 0.01	0.08 ± 0.02	0.06 ± 0.01	0.08 ± 0.02	0.27 ± 0.21	0.15 ± 0.02	0.09 ± 0.01	0.07 ± 0.02
Total	**97.3**	**96.6**	**93.6**	**94.7**	**95.9**	**91.3**	**95.4**	**91.3**

GI: gastrointestinal

Source: Quistad, Kovatchev & La Mar (2010, 2011a)

Kinetic parameters are presented in Table 2. Fluensulfone was rapidly absorbed at a dose level of 5 mg/kg bw, but the absorption phase was significantly longer at 500 mg/kg bw. Area under the plasma concentration–time curve (AUC) values increased proportionately (> 50-fold) with the administered dose (100-fold). The elimination from whole blood was longer than from plasma, indicating an affinity for blood cells. Investigative work showed that fluensulfone reacts with the free thiol moiety of globin to form a covalent linkage to the thiazole group and displacing butene sulfinic acid. The decline of ^{14}C-thiazole radiolabel from whole blood seems to follow the removal of derivatized haemoglobin, with a time required for the concentration to decline to half the peak concentration of 148–216 hours (6–9 days) for the low dose.

Table 2. Summary of kinetic parameters for rats (males and females combined) administered [^{14}C]fluensulfone

Kinetic parameter	Radiolabel	Dose rate	Whole blood	Plasma
C_{max} (µg eq/g)	Thiazole	Low	1.1–1.4	1.2–1.6
		High	75–92	39–49
	Butene	Low	0.97–0.99	1.2
		High	25–30	29–34
T_{max} (h)	Thiazole	Low	2–8	2
		High	48	1–8
	Butene	Low	4	4
		High	24	24
$t_{½}$ (h)	Thiazole	Low	146–208	14–15
		High	134–162	58–67
	Butene	Low	30–33	20
		High	30–32	24–26
$t_{1/10}$ (h)	Thiazole	Low	485–691	47–50
		High	445–537	193–221
	Butene	Low	100–110	66
		High	100–105	79–85
AUC (h·µg eq/g)	Thiazole	Low	237–306	40–45
		High	16 000 – 21 000	3 200–3 400
	Butene	Low	43–47	37–38
		High	2 000–2 100	1 900–2 000

AUC: area under the plasma concentration–time curve; C_{max}: maximum concentration; eq: equivalents; $t_{½}$: time for decline to half of C_{max}; $t_{1/10}$: time for decline to 1/10 of C_{max}; T_{max}: time to reach C_{max}

Source: Quistad, Kovatchev & La Mar (2010, 2011a)

The distribution of [^{14}C]fluensulfone (thiazole and butene labels) in rat tissues was investigated using a suspension in PEG 300, at single oral low (5 mg/kg bw) or high (500 mg/kg bw) doses administered to groups of three male and three female Wistar rats. The tissue distribution study was carried out with two radiolabels and three sacrifice intervals for each of the low and high doses. Collection intervals in each dose group were based on the previously determined kinetic parameters. The concentrations of fluensulfone-derived ^{14}C were measured in 32 tissues/organs and in the carcass.

Residues in males and females were generally similar. The thiazole label was slowly eliminated from red blood cells and organs associated with their formation/storage (bone, bone marrow, spleen). Highest initial concentrations were seen in liver, kidney and thyroid with the thiazole label and in liver, kidney and lung with the butene label. Two hours after administration at both high and low doses, the thyroid had a high concentration of thiazole-labelled material relative to

whole blood, although levels in thyroid were comparable to the whole blood concentrations by the subsequent time point (51 hours) (Supplementary Tables 1 and 2[1]) (Quistad & Kovatchev, 2011).

The disposition and metabolism of [^{14}C]fluensulfone were studied in male and female Wistar rats after 14 repeated daily oral doses of unlabelled fluensulfone, followed by a single oral dose of [^{14}C-thiazole]fluensulfone at 5 mg/kg bw, 1 day after the last unlabelled dose. Fluensulfone was administered as a suspension in PEG 300 by oral gavage. Rats were terminated 1 or 7 days after administration of the radiolabelled dose. The study determined the excretion pattern and distribution of radiolabel in selected tissues and identified the major metabolites of fluensulfone in urine and faeces.

The recovery of excreted radiolabel was 94–99% for individual rats. Radiolabel was found mainly in urine (71–83%), faeces (9–11%) and the cage wash (6–16%). One day after dosing with radiolabelled fluensulfone, an average of 0.6–0.8% of the administered radiolabel was in liver and 0.1% in kidney, with 1.7–2.1% in the carcass and 4.5–4.7% remaining in the gastrointestinal tract. By 7 days after treatment with radiolabelled fluensulfone, an average of 0.1–0.2% of the administered radiolabel remained in liver, and 0.03% in kidney. There were no significant sex differences in either elimination or tissue residues. The results are broadly in line with those from the equivalent single-dose study (Quistad, Kovatchev & La Mar, 2011b).

1.2 Biotransformation

Rats

Urine, faeces and cage wash samples from the study of Quistad, Kovatchev & La Mar (2011a,b, described above) were analysed for metabolites using radio–high-performance liquid chromatography. Fluensulfone was not a significant residue in either faeces or urine. The majority of the faecal radioactivity was not identified. It consisted of a number of peaks, each representing less than 2% of the administered dose; the only faecal metabolite representing more than 5% of the administered dose was thiazole sulfonic acid. In urine, there were no metabolites common to both radiolabels, which indicates that the initial step is cleavage at the sulfonyl bond. The initial reaction is probably with glutathione to release the trifluorobutene group and conjugation of the thiazole moiety (Table 3). The positioning of the radiolabel in the butene moiety did not permit the fate of the fluorine atoms to be determined. Similar results were seen after 14 daily doses of unlabelled fluensulfone and one dose of [thiazole-^{14}C]fluensulfone, indicating that there is no significant induction of the metabolism of fluensulfone.

A metabolic pathway is presented in Fig. 2.

Fluensulfone has a molecular weight of 272 Da and contains three fluorine atoms. If all the available fluorine is released, this means that a fluensulfone dose of 100 mg/kg bw could provide about 20 mg of free fluoride ion per kilogram body weight. Approximately 50% of an administered dose of fluensulfone is excreted as fluoride-containing compounds.

2. Toxicological studies

2.1 Acute toxicity

Fluensulfone is of moderate acute toxicity following oral exposure and of low acute toxicity following dermal and inhalation exposure (Table 4). It is slightly irritating to the skin of rabbits (Simon, 2009c) and not irritating to rabbit eyes (Simon, 2009d), but it is a skin sensitizer in a maximization test in guinea-pigs (Simon, 2009e).

[1] Supplementary tables are available on the JMPR website.

Table 3. Comparison of urinary and cage wash metabolites in rats administered fluensulfone

Metabolite	% of administered dose							
	[thiazole-^{14}C]Fluensulfone				[butene-^{14}C]Fluensulfone			
	Low dose (5 mg/kg bw)		High dose (500 mg/kg bw)		Low dose (5 mg/kg bw)		High dose (500 mg/kg bw)	
	Males	Females	Males	Females	Males	Females	Males	Females
Thiazole mercapturate	40	45	49	63	–	–	–	–
Thiazole glucuronide MW 327-I	20	18	12	0.6	–	–	–	–
Thiazole glucuronide MW 327-II	7	11	10	21	–	–	–	–
Thiazole sulfonic acid	5	4	3	2	–	–	–	–
Other	3	4	2	3	–	–	–	–
Butene sulfinic acid	–	–	–	–	35	38	53	65
Butene sulfonic acid	–	–	–	–	4	6	4	5
Solvent front	–	–	–	–	8	8	4	3
Other	–	–	–	–	4	5	2	2

Source: Quistad, Kovatchev & La Mar (2011a)

2.2 Short-term studies of toxicity

Repeated-dose oral (dietary) toxicity studies with fluensulfone are available for rats, mice and dogs.

In all species, the liver was a target organ, although often the observed effects were limited to changes typical of those seen in animals responding to high levels of xenobiotics (e.g. hepatocyte hypertrophy concomitant with increased liver weight). Investigations on liver enzyme activities in all species revealed that fluensulfone administration does not result in significant induction of phase I cytochrome P450 (CYP) subfamilies, but that the (conjugating) phase II enzymes were induced to a limited extent.

The Meeting concluded that reductions in serum and hepatic alanine aminotransferase (ALAT) activities were not adverse (see below) and that effects related to the fluoride content of fluensulfone should be considered as part of total fluoride exposure. These effects have been considered as not adverse in the identification of no-observed-adverse-effect levels (NOAELs) for individual studies.

Mice

In a 4-week dietary study, groups of five male and five female Crl:CD1 mice received fluensulfone (purity 97%; batch NLL6692-7-5) in their diet at 0, 100, 500 or 2000 parts per million (ppm). Achieved doses were 0, 30, 101 and 375 mg/kg bw per day for males and 0, 41, 155 and 353 mg/kg bw per day for females, respectively. Clinical observations were performed daily. Body weights and feed and water consumption were determined weekly. Clinical laboratory examinations were performed in week 4, and a gross necropsy (with organ weights and tissue sampling) was performed on all surviving animals at termination. An extensive range of organs and tissues was examined microscopically from control and top-dose animals plus a limited number of animals from the other groups.

Fig. 2. Proposed metabolic pathway of fluensulfone in rats

Source: Quistad, Kovatchev & La Mar (2011a)

Table 4. Summary of acute toxicity data for fluensulfone

Species	Strain	Sex	Route	LD_{50} (mg/kg bw)	LC_{50} (mg/L)	Purity; batch	Vehicle	Reference
Rat	Wistar	F	Oral	671 (OECD TG 423)	–	96.5%; 36372130-291-PF1	0.8% hydroxypropyl methyl cellulose	Haferkorn (2010)
Rat	HanRcc: Wistar	F	Oral	300–2 000 (OECD TG 423)	–	96.2%; 36372130-291-PF1	PEG 300	Simon (2009a)
Rat	HanRcc: Wistar	M/F	Dermal	> 2 000	–	96.2%; 36372130-291-PF1	PEG 300	Simon (2009b)
Rat	HanRcc: Wistar	M/F	Inhalation (4 h, nose only)	–	> 5.1 (MMAD < 2.5 µm)	96.2%; 36372130-291-PF1	None (aerosol)	Pothmann (2009)

F: female; LC_{50}: median lethal concentration; LD_{50}: median lethal dose; M: male; MMAD: mass median aerodynamic diameter; OECD TG: Organisation for Economic Co-operation and Development Test Guideline; PEG: polyethylene glycol

No mortality and no clinical signs occurred. Feed intake was decreased by 25% or more at 500 ppm and above in both sexes. Water consumption was slightly increased at 2000 ppm in females. Body weight loss was observed at 2000 ppm in the 1st week of treatment in both sexes. At the end of treatment, the difference from the controls was −13% in males and −4% in females. Dose-related increases in red blood cell parameters were seen, achieving statistical significance at 2000 ppm in females (increases of erythrocyte and reticulocyte counts, concentration of haemoglobin, haematocrit values), indicative of a response to damage to mature erythrocytes. Increases in activities of plasma aspartate aminotransferase (ASAT) (2-fold) and plasma ALAT (2-fold) were recorded in both sexes at 2000 ppm and considered as correlates of the observed microscopic and organ weight changes in the liver.

The relative kidney weights were decreased slightly at 2000 ppm in males. Liver weights (relative) were increased (approximately 15%) at 500 ppm and above in females. Histopathology revealed treatment-related findings only in the liver at 2000 ppm in both sexes:

- a cytoplasmic alteration in the periportal region, characterized by a dense cytoplasmic structure, eosinophilia (females) or slight basophilia (males);
- degenerative changes of the periportal zone, which also encompassed single-cell necrosis and, in some of the females, mitotic figures; and
- a slight increase in the number and size of bile ducts in the portal regions (bile duct hyperplasia) in one male and three females.

The NOAEL of the study was considered to be 500 ppm for both sexes (equal to 101 mg/kg bw per day), based on body weight loss, changes in erythrocyte parameters (e.g. increased reticulocytes) and hepatotoxicity (altered cytoplasmic structure, single-cell necrosis, bile duct hyperplasia) at 2000 ppm (equal to 353 mg/kg bw per day) (Krötlinger & Rühl-Fehlert, 2003).

In a 90-day dietary toxicity study, groups of 12 male and 12 female Crl:CD1 (ICR)BR mice received diets containing 0, 60, 300 or 1500 ppm fluensulfone (purity > 97%; batch NLL 6692-7-5) for a period of 13 weeks. Achieved intakes were 0, 11, 51 and 229 mg/kg bw per day for males and 0, 18, 68 and 252 mg/kg bw per day for females, respectively. Doses were selected on the basis of a 4-week range-finding study. Additional satellite groups of five mice of each sex were similarly treated at 0 or 1500 ppm for 4 weeks and then terminated for determinations of enzyme levels in liver tissue. Clinical observations were carried out daily, and body weight and feed and water consumption were measured weekly. Clinical laboratory investigations and a gross necropsy (with organ weights and tissue sampling) were performed on all surviving animals at their terminal sacrifice. Organs and tissues were examined microscopically. Biochemical investigations in liver tissue were done on satellite animals at day 28.

There were several mortalities during the study: 2, 2, 4 and 4 males and 3, 0, 0 and 5 females, respectively, for the control and treated groups, in order of ascending doses. A single female from the highest-dose group was the only animal in which clinical signs (piloerection, squatting position, poor general condition and emaciation) were recorded; all other mortalities were related to retro-orbital blood sampling on day 86 or 91.

At 1500 ppm, feed intake (both sexes) and water intake (males) were decreased. At 300 ppm, feed intake was decreased in females. Body weight gain was significantly retarded at 1500 ppm in both sexes during the 1st week of treatment, with a body weight loss recorded for females. At the end of treatment, mean body weights of animals in the high-dose group differed from the control values by approximately −10% for both sexes.

Haematological investigations showed evidence of increased red blood cell synthesis, with increases in erythrocyte counts (10%), haemoglobin (10%) and haematocrit (15%) values at 300 ppm and above in males, increased mean corpuscular volume and decreased mean corpuscular haemoglobin concentration (MCHC) in males at 1500 ppm, and 30% increases in reticulocyte counts in both sexes treated at 1500 ppm. Leukocyte counts were increased in males treated at and above 300

ppm. Clinical chemistry showed increased concentrations of total bilirubin at 300 ppm and above in both sexes and decreased urea concentrations in animals of both sexes treated at 1500 ppm. The activities of ASAT, ALAT and alkaline phosphatase were increased at 1500 ppm in both sexes, often attaining statistical significance.

No treatment-related macroscopic findings were noted at necropsy. At 1500 ppm, liver weights were increased in males (relative) and females (absolute and relative). Histopathology revealed a range of liver findings in both sexes treated at 1500 ppm, and hepatocellular hypertrophy was also observed in females receiving 300 ppm (Table 5). In the spleen, an increased incidence of animals with pigment deposition in the red pulp was recorded in both sexes at 1500 ppm.

Table 5. Incidence of microscopic findings in selected organs

Organ	Incidence of findings in males/females (*n* = 12)			
	0 ppm	60 ppm	300 ppm	1 500 ppm
Liver				
Hepatocyte hypertrophy	– / 1	– / 1	– / 6	12 / 10
- Grade 1	– / 1	– / 1	– / 6	– / –
- Grade 2	– / –	– / –	– / –	3 / –
- Grade 3	– / –	– / –	– / –	9 / 10
Bile duct hyperplasia (grades 1–3)	– / –	– / –	– / –	11 / 10
Oval cell proliferation (grades 1–3)	– / –	– / –	– / –	7 / 2
Degeneration (grades 1–4)	– / –	– / –	– / –	10 / 10
Pigment deposits (grades 1–2)	– / –	– / –	– / –	7 / 9
Spleen				
Pigment deposits (grades 1–3)	5 / 5	5 / 6	6 / 8	12 / 11

Source: Krötlinger (2003)

Determinations in liver tissues in satellite animals sacrificed after 28 days of treatment showed no evidence for toxicologically significant effects on the concentration of cytochrome P450 at 1500 ppm. In male mice, only the monooxygenase 7-ethoxycoumarin deethylase was marginally increased. The phase II enzymes epoxide hydrolase (EH) and glutathione *S*-transferase (GST) were marginally increased, whereas uridine diphosphate (UDP)–glucuronyltransferase (UDPGT) was not affected. In female mice, the monooxygenases were not significantly affected, whereas all phase II enzymes (EH, GST, UDPGT) were significantly induced at 1500 ppm.

The NOAEL of the study was 60 ppm (equal to 11 mg/kg bw per day), based on haematological effects and hepatocyte hypertrophy at 300 ppm (equal to 51 mg/kg bw per day) (Krötlinger, 2003).

Rats

Groups of five male and five female Wistar rats received fluensulfone (purity > 98%; batch NLL6692-3) in their diet at 0, 125, 500 or 2000 ppm for approximately 4 weeks. Achieved doses were 0, 10, 43 and 152 mg/kg bw per day for males and 0, 12, 37 and 166 mg/kg bw per day for females, respectively. Animals were inspected once a day for morbidity and mortality. In the 4th exposure week, sensory reactivity to stimuli of different types (auditory, visual, touch, nociceptive) and grip strength were assessed. Urine and blood samples were taken prior to sacrifice. In addition to the standard parameters, immunoglobulins (Ig) A, G and M, triiodothyronine (T_3), thyroxine (T_4) and thyroid stimulating hormone (TSH) concentrations were also determined. At terminal sacrifice, selected organs were weighed, and an extensive range of tissues from top-dose and control animals was examined histopathologically; lung, liver and kidney were examined in other groups. Liver

specimens were taken to investigate liver enzymes and substrates. Other liver specimens were excised and frozen to analyse tissue for cell proliferation potential. Blood (by cardiac puncture), left femur and half of the spleen were taken and immediately placed in chilled test tubes containing sterile physiological saline for immunotoxicity investigations.

There were no mortalities, clinical signs or effects in open-field investigations or on sensory reactivity. Mean feed consumption was slightly decreased in animals receiving 2000 ppm during the first few days of treatment, whereas water consumption was slightly increased in males of this group, but not in females. Body weight gain was retarded (~30%) in male and female rats receiving 2000 ppm and, to a lesser extent, in males receiving 500 ppm (Table 6). No treatment-related changes were noted in haematology. Clinical chemistry revealed dose-related decreases in the activity of ALAT in all treatment groups of both sexes (Table 7). In 2000 ppm males, cholesterol and total protein were slightly, but statistically significantly, increased, and triglyceride concentration was decreased (Table 7). Urine analysis did not indicate test compound–related effects.

Table 6. Body weight and selected relative organ weights in rats fed diets containing fluensulfone for 28 days

Organ	Relative organ weight (mg/100 g)			
	0 ppm	125 ppm	500 ppm	2 000 ppm
Male rats				
Terminal body weight (g)	*294*	*291*	*267*	*263*
Liver	4 552	4 887	4 856	5 205**
Kidneys	685	722	765	827**
Spleen	201	191	194	210
Female rats				
Terminal body weight (g)	*203*	*194*	*200*	*181***
Liver	4 211	4 186	4 470	4 467
Kidneys	704	692	701	756
Spleen	223	212	225	257*

*: $P < 0.05$; **: $P < 0.01$
Source: Temerowski & Rühl-Fehlert (2002)

Table 7. Selected clinical chemistry parameters in rats fed diets containing fluensulfone for 28 days

Parameter	0 ppm	125 ppm	500 ppm	2 000 ppm
Male rats				
ALAT (U/L)	91.6	63.0**	35.2**	11.8**
Cholesterol (nmol/L)	1.55	1.64	1.82	2.19*
Triglycerides (nmol/L)	1.73	1.84	1.30	0.82**
Total protein (g/L)	60.9	62.9	60.8	64.6*
Female rats				
ALAT (U/L)	74.2	54.6**	31.9**	11.0**
Cholesterol (nmol/L)	2.06	1.93	1.89	2.34
Triglycerides (nmol/L)	1.10	1.02	1.02	1.19
Total protein (g/L)	64.7	64.7	64.8	62.6

ALAT: alanine aminotransferase; U: units; *: $P < 0.05$; **: $P < 0.01$
Source: Temerowski & Rühl-Fehlert (2002)

Analysis of organ weight data showed statistically significant increases in mean relative liver (15%) and kidney weights (20%) in male rats treated at 2000 ppm and in relative spleen weight (10%) in females of the same group. Gross pathology revealed surface changes in the kidneys of males dosed at 500 ppm (1/5 animals) and 2000 ppm (5/5 animals). Histopathology revealed degeneration and hyaline inclusions in the proximal tubules. At 2000 ppm, focal tubular necrosis was observed in one male rat (Table 8). Histopathology of the liver, spleen and female kidneys revealed no toxicological effects. Immunotoxicity examinations showed no notable effects on the splenic and bone marrow cell counts, in the composition of the splenic subpopulations or in antibody titres (although splenic samples from top-dose females were lost).

Table 8. Histopathological findings in kidneys of male rats fed fluensulfone for 28 days

Lesion	Incidence of finding (*n* = 5)			
	0 ppm	125 ppm	500 ppm	2 000 ppm
Basophilic tubules	5	5	5	4
- Grade 1	4	5	3	1
- Grade 2	1	–	2	3
Hyaline droplets	–	–	1	5
- Grade 2	–	–	1	4
- Grade 3	–	–	–	1
Degeneration of proximal tubules	–	–	2	5
- Grade 1	–	–	2	–
- Grade 2	–	–	–	3
- Grade 3	–	–	–	2
Mononuclear infiltration	1	1	–	2
- Grade 1	1	1	–	2
Intratubular protein	–	–	2	1
- Grade 1	–	–	2	–
- Grade 2	–	–	–	1
Tubular necrosis	–	–	–	1
- Grade 1	–	–	–	1

Source: Temerowski & Rühl-Fehlert (2002)

Monooxygenases were markedly decreased in females at 500 ppm and above and, to a lesser extent, in males at 2000 ppm. Phase II enzymes (GST, UDPGT and EH) were induced in males at 500 ppm and above and in females at 2000 ppm (by 40–170%).

The NOAEL of the study was 125 ppm (equal to 10 mg/kg bw per day), based on reductions in body weight gain and kidney lesions in male rats at 500 ppm (equal to 43 mg/kg bw per day) (Temerowski & Rühl-Fehlert, 2002).

In a 90-day dietary toxicity study, groups of 10 male and 10 female Wistar rats received diets containing 0, 60, 120, 500 or 2000 ppm fluensulfone (purity 97%; batch NLL 6692-7-5) for a period of 3 months. Doses were chosen on the basis of a 28-day range-finding study. Additional recovery groups were similarly treated at 0 or 2000 ppm and then maintained without treatment for a recovery period of 28 days. Additional groups of five males were treated for 1 week at 0 or 2000 ppm for investigation of α_{2u}-globulin accumulation in kidneys. Mean daily intakes were 0, 4, 8, 35 and 139 mg/kg bw per day for males and 0, 5, 12, 53 and 149 mg/kg bw per day for females of the main groups, respectively. Clinical observations were carried out daily, and body weight and feed and water

consumption were measured weekly. A detailed clinical observation and an open-field observation were performed weekly. A functional observational battery was performed in week 12 on both main and recovery groups and in week 16 on recovery groups. Motor activity assessments were carried out in week 12 on main groups and in weeks 13 and 16 on recovery groups. At the end of both the treatment and recovery periods, haematology, clinical chemistry and urine analysis examinations were performed. Animals were necropsied, organ weights were determined, and organs and tissues were subjected to a histopathological examination. The liver, teeth and left femur were retained at both necropsies from all animals; the total fluoride concentrations in teeth and left femur ashes were determined, and enzyme activities and cytochrome P450 content were determined in liver tissue homogenates.

No mortality occurred during the study. Clinical observations of animals revealed no treatment-related findings, and detailed behavioural investigations (functional observational battery, motor activity) gave no evidence of any neurotoxicity. No abnormalities were detected in ophthalmological investigations or urine analysis. Body weight gains were reduced (10%) in males and females treated at 2000 ppm and slightly reduced in males receiving 500 ppm (7%). During the recovery period, animals of the 2000 ppm group gained slightly more body weight than the respective control groups. Feed consumption of females was decreased at 2000 ppm during the treatment period and was not affected by the treatment in the other groups. Water intake was increased in males at 500 ppm (10%) and in males (23%) and females (15%) at 2000 ppm. During the recovery period, mean daily water intake remained similarly increased, compared with the control groups.

At 2000 ppm, decreases in haemoglobin concentrations and MCHC and increased reticulocytes were recorded, most of which were reversible during the recovery period. Significant reductions in serum ALAT activities were seen in both sexes treated at 500 or 2000 ppm, with values returning to normal during the recovery phase. Clinical chemistry tests gave evidence for effects on liver function (significantly increased triglycerides) at 500 ppm and above during treatment, with subsequent recovery. At the end of the treatment period, concentrations of urea were significantly increased at 2000 ppm in males.

At 2000 ppm, relative weights of livers were increased 9% in females and 17% in males; relative kidney weights were significantly increased in both sexes (males: 21%, females: 14%), but not at the end of the recovery period. In this dose group, changes of the surface of the kidneys were observed in five males at necropsy at the end of the treatment period. Histopathological investigations revealed hepatocyte hypertrophy in both sexes at 2000 ppm and hyaline droplet nephropathy accumulation in males at 500 and 2000 ppm, associated with increased α_{2u}-globulin. Hyaline droplet accumulation was reversible within the recovery period, whereas other tubular changes observed at 500 ppm and above after the treatment period were still discernible in 2000 ppm males after the recovery period. In females, a slightly increased grade of Schmorl-positive pigment was seen in renal tubules at 2000 ppm. This change was reversible within the recovery period.

In the forestomach, a diffuse basal cell hyperplasia was observed in both sexes at 500 ppm (40% of rats) and 2000 ppm (80% of males and all females). At the end of the recovery period, this change partly regressed, but was still detectable in a few animals (30% of males and 20% of females) at 2000 ppm (500 ppm animals did not form part of the recovery phase).

At necropsy at the end of the treatment period, discoloration of teeth was observed in 6/10 males at 2000 ppm. Treatment with the test substance increased the content of fluoride in ashes from bones and teeth of treated male and female rats in a dose-related manner (Supplementary Table 3). Statistically significant increases in fluoride concentrations were observed at 120 ppm and above. At the end of the recovery period, fluoride concentrations, although still significantly increased in the top-dose groups, were of a smaller magnitude than at the end of dosing, indicating a possible reversibility of this effect. Histopathological examinations revealed no abnormalities in teeth and investigated bones. There were no investigations of bone density, bending strength or compression resistance.

The NOAEL was 120 ppm (equal to 8 mg/kg bw per day for males and 12 mg/kg bw per day for females), based on forestomach hyperplasia, increased triglycerides and increased water

consumption in females and and decreased body weight/body weight gains in males at 500 ppm (equal to 35 mg/kg bw per day for males and 53 mg/kg bw per day for females) (Schladt & Rühl-Fehlert, 2003).

[The authors of the study report identified the NOAEL as 60 ppm (equal to 4 mg/kg bw per day), based on the increased content of fluoride in ashes from bones and teeth of rats treated at 120 ppm (equal to 8 mg/kg bw per day) and above.]

Dogs

In a 4-week dietary study, groups of two male and two female Beagle dogs received fluensulfone (purity 96.7%; batch 36372130-291-PF1) in their diet at 0, 50, 200 or 900 ppm for 30 days. Mean intakes were 0, 1.9, 7 and 31 mg/kg bw per day for males and 0, 2, 8 and 30 mg/kg bw per day for females, respectively. All males and females were sacrificed on day 30 and subjected to standard investigations. In addition, thyroid hormone levels in blood, ALAT activity in liver tissue and metabolic enzyme activity in liver were also determined. Animals were examined regularly for clinical signs, body weight and feed consumption. Ophthalmoscopic and veterinary examinations plus blood and urine sampling were performed pretest and during the last week of treatment. At necropsy, a number of organs were weighed, and histological examination was performed on 14 organs and tissues as well as on all gross lesions from all animals.

No mortality occurred and no clinical signs were noted during the study. A slight reduction in mean feed consumption and corresponding body weight stasis in males and body weight loss in females were noted at 900 ppm during the treatment period when compared with the pretreatment values or control group. No treatment-related changes were recorded in ophthalmological examination or in haematological or urine analysis parameters. Decreased ALAT activity in the blood was observed in all treated groups, and decreased protein and albumin levels and albumin to globulin ratio were noted in the females at 900 ppm. TSH values were increased 4- to 5-fold in male and female dogs at 900 ppm when compared with the control values. However, no differences in T_3 or T_4 levels were observed in the treated groups when compared with the control values.

Marked increased absolute liver (> 20%), relative liver (30%) and relative thyroid (> 50%) weights were recorded in the males at 900 ppm; organ weights in females were unaffected. There was no associated macroscopic or microscopic histopathology. Fluensulfone caused a reduction to 55–65% of control values in liver ALAT activity in both sexes at 900 ppm. The results of liver enzyme determinations showed some increases (up to 4-fold) in some total cytochrome P450 and specific phase I and phase II hepatic enzyme activities.

The NOAEL was 200 ppm (equal to 7 mg/kg bw per day), based on reductions in feed consumption and body weight gain and increases in organ weights (liver and thyroid) at 900 ppm (equal to 30 mg/kg bw per day) (Braun, 2009a).

In a 90-day dietary study, groups of four male and four female Beagle dogs received fluensulfone (purity 96.7%; batch 36372130-291-PF1) in their diets at 0, 5, 50 or 500 ppm for 13 consecutive weeks. Doses were set on the basis of a range-finding study. Achieved intakes were 0, 0.2, 1.7 and 17 mg/kg bw per day for males and 0, 0.2, 1.8 and 18 mg/kg bw per day for females, respectively. Similar-sized recovery groups for the control and the high dose were treated for 13 consecutive weeks and sacrificed after an additional 28-day period without treatment. Throughout the study, all animals were observed daily for viability and clinical signs. In addition, body weight and feed consumption were recorded. Blood and urine samples were collected pretest, in week 6 and prior to sacrifice for routine examinations and thyroid hormone levels. Ophthalmoscopy examinations were performed pretest and prior to sacrifice. At scheduled necropsy, a part of the liver was taken from all animals for liver enzyme activity determinations. Additionally, blood samples were drawn from the jugular vein. Organ weights were recorded, and samples from an extensive range of organs were subjected to histopathological examination.

There were no mortalities, clinical signs or changes observed in ophthalmological or veterinary examinations or urine analysis. There were no effects on feed consumption, but female animals receiving 500 ppm gained less weight than the controls. Increases in reticulocyte counts and MCHC were seen at 500 ppm in weeks 6 and 13. Clinical chemistry investigation revealed statistically significant decreases in ALAT after 6 weeks in males and females at 50 and 500 ppm; however, values were within historical control ranges for the performing laboratory, and the decreases were not evident at 13 weeks. Decreased total protein and albumin levels and albumin to globulin ratios were recorded after 6 and 13 weeks at 500 ppm. A tendency to higher TSH levels was seen in male and female dogs treated at 500 ppm, but the results were driven by high values in one dog of each sex; no notable differences in T_3 and T_4 levels were observed

Increased liver weights were seen in males and females at 500 ppm, but were not apparent at the end of the recovery period (Table 9). Slight diffuse hepatocellular hypertrophy was seen in one main study female at 500 ppm. There was some evidence of enzyme induction in the livers of 500 ppm dogs, but the increases in activity were approximately 2-fold. Hepatic ALAT activity was reduced at 500 ppm, to 40% and 70% of control values in males and females, respectively.

Table 9. Body and liver weights in dogs (n = 4) exposed to fluensulfone

	Control[a]	5 ppm	50 ppm	500 ppm[a]
Males				
Body weight (kg)	10.6	–	–	10.0
	(12.0)			*(10.8)*
Liver weight				
- Absolute (g)	286.0 ± 63.8	295.0 ± 68.3	280.6 ± 22.5	315.0 ± 50.8
	(337.3 ± 80.8)			*(309.8 ± 24.7)*
- Relative to body weight (%)	2.7 ± 0.3	2.6 ± 0.7	2.6 ± 0.2	3.2 ± 0.2
	(2.8 ± 0.6)			*(2.9 ± 0.2)*
Females				
Body weight (kg)	10.5	9.8	9.3	8.7
	(10.0)			*(9.0)*
Liver weight				
- Absolute (g)	318.2 ± 47.3	304.7 ± 30.5	299.1 ± 63.8	343.6 ± 19.1
	(267.8 ± 9.3)			*(254.3 ± 21.5)*
- Relative to body weight (%)	3.1 ± 0.4	3.2 ± 0.4	3.2 ± 0.3	4.0 ± 0.3**
	(2.7 ± 0.4)			*(2.9 ± 0.1)*

**: $P < 0.01$
[a] Recovery group shown in parentheses.
Source: Braun (2009b)

The NOAEL was 50 ppm (equal to 1.7 mg/kg bw per day), based on alterations in reticulocytes and marked increases in relative and absolute liver weights at 500 ppm (equal to 17 mg/kg bw per day) (Braun, 2009b).

In a 1-year oral toxicity study, fluensulfone (purity 96.7%; batch 36372130-291-PF1) was administered in the diet at 0, 5, 50, 100 or 500 ppm to purebred Beagle dogs (four of each sex per group) for a period of 52 weeks, with selected animals retained for an 8-week recovery period. Achieved intakes were 0, 0.1, 1.5, 3 and 16 mg/kg bw per day, respectively. Throughout the study, all animals were observed daily for viability and clinical signs. Body weight was recorded at selected intervals, and feed consumption was recorded daily. Ophthalmoscopic and veterinary examinations

were performed pretest, during weeks 13, 26 and 52 and during week 8 of recovery. Blood and urine samples were collected for clinical laboratory investigations pretest, during weeks 6, 13, 26 and 52 and during week 8 of recovery. Additional blood samples were collected for liver parameter determinations during weeks 1, 2, 4, 8, 10, 17 and 39 and during week 4 of recovery as well as for hormone analyses pretest and during week 52 and week 8 of recovery. Following completion of the treatment or recovery period, a detailed necropsy was performed on all animals, and selected organs were weighed. The collected tissues were placed in fixative, processed and examined microscopically. Additional samples of liver and bone were collected and frozen for specific analyses.

Body weight gain was reduced at 500 ppm (Table 10). Alterations in red blood cell parameters were seen at 500 ppm (Tables 11 and 12). Marked decreases in plasma ALAT activity were observed at 50, 100 and 500 ppm. Increased relative liver and kidney weights were seen at 100 ppm and above (Table 13), with brown pigment deposition and diffuse hypertrophy observed in the liver of the majority of dogs at 500 ppm. There were no treatment-related histopathological findings in the kidneys or increases in plasma urea levels. Increases in hepatic activities of EH and GST were seen at 500 ppm, together with decreased ALAT activity at 50 ppm and above. A dose-related increase in fluoride content was evident at doses of 50, 100 and 500 ppm in the ashes from bone and teeth, attaining statistical significance only at the high dose level (Supplementary Table 4). No signs of dental fluorosis were observed during the study, and no abnormality in bone structure was observed at microscopic examination. With the exception of the pigment deposition in the sinusoidal cells of the liver and fluoride levels, all the findings showed extensive resolution during the recovery period.

Table 10. Body weight and body weight gain in dogs receiving fluensulfone for 12 months

Dietary concentration (ppm)	Males			Females		
	Mean body weight (kg)		Overall body weight gain (%)	Mean body weight (kg)		Overall body weight gain (%)
	Day 1	Day 358		Day 1	Day 358	
0	8.5	13.4	57.6	7.9	12.0	51.4
5	9.1	12.3	35.5	7.7	12.6	63.8
50	8.2	12.1	47.6	7.6	11.5	50.5
100	8.4	12.1	43.1	7.4	10.9	46.5
500	8.6	11.4	32.9*	7.7	10.1	32.0

*: $P < 0.05$
Source: Braun (2011b)

The NOAEL was 100 ppm (equal to 3 mg/kg bw per day), based on altered red cell parameters, increased pigment deposition in the liver and increased liver weights at 500 ppm (equal to 16 mg/kg bw per day) (Braun, 2011b).

2.3 Long-term studies of toxicity and carcinogenicity

Mice

Groups of 50 male and 50 female CD-1 mice were treated for 78 weeks with 0, 30, 200 or 1200 ppm fluensulfone (purity 96.7%; batch 36372130-291-PF1) in their feed. Achieved mean intakes were 0, 4.2, 28 and 152 mg/kg bw per day for males and 0, 6.4, 39 and 188 mg/kg bw per day for females, respectively. Additionally, six males and six females were used for liver enzyme determination after 13 weeks of treatment. Viability/mortality, clinical signs, feed consumption and body weights were recorded periodically throughout the study. Blood samples from all main group mice were taken after 52 and 78 weeks for determination of haematology and at 78 weeks for determination of limited clinical biochemistry parameters. After 13 weeks, satellite group mice were killed; livers were removed and homogenized, and ex vivo activity of metabolic liver enzymes was determined. All animals sacrificed after 78 weeks of treatment with fluensulfone as well as all animals

Table 11. Selected haematology parameters in male dogs receiving fluensulfone for 12 months

Dietary concentration (ppm)	Hb (mmol/L)	MCH (fmol)	MCHC (mmol/L)	HDW (mmol/L)	Reti abs ($\times 10^9$/L)	Reti rel	Plat ($\times 10^9$/L)
Pretest							
0	8.2	1.37	20.77	1.01	57	0.010	419
5	8.1	1.36	21.03	1.05	59	0.009	324
50	8.0	1.37	21.21	1.08	52	0.009	391
100	7.8	1.33	20.80	1.05	63	0.011	443
500	8.3	1.34	20.94	1.04	65	0.011	384
Week 6							
0	8.7	1.37	21.00	1.08	57	0.009	373
5	8.8	1.38	21.29	1.13	61	0.009	299
50	8.5	1.35	21.03	1.11	51	0.008	339
100	8.9	1.35	21.08	1.13	61	0.010	403
500	8.4	1.33	20.18**	1.44**	108**	0.020*	396
Week 52							
0	10.8	1.45	21.76	1.08	60	0.008	342
5	10.4	1.45	21.82	1.14	65	0.009	278
50	10.5	1.43	21.67	1.13	79	0.012	398
100	10.0	1.40	21.55	1.13	62	0.010	451
500	9.3**	1.34**	20.47**	1.53**	108*	0.015*	457
Week 8 of recovery							
0	12.4	1.41	21.00	1.01	58	0.007	257
500	10.7	1.39	21.42	1.13	72	0.010	318

Hb: haemoglobin; HDW: haemoglobin distribution width; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; Plat: platelets; Reti abs: absolute reticulocyte count; Reti rel: relative reticulocyte count (ratio of reticulocytes to total erythrocytes); *: $P < 0.05$; **: $P < 0.01$
Source: Braun (2011b)

Table 12. Selected haematology parameters in female dogs receiving fluensulfone for 12 months

Dietary concentration (ppm)	Hb (mmol/L)	MCH (fmol)	MCHC (mmol/L)	HDW (mmol/L)	Reti abs ($\times 10^9$/L)	Reti rel	Plat ($\times 10^9$/L)
Pretest							
0	8.5	1.36	20.81	0.98	64	0.009	382
5	8.5	1.35	20.97	1.03	69	0.011	418
50	8.2	1.39	21.22	1.11*	76	0.011	377
100	9.1	1.39	21.20	1.14**	98	0.016	391
500	8.8	1.38	20.81	1.04	90	0.014	439
Week 6							
0	9.5	1.37	21.15	1.10	74	0.011	347
5	9.5	1.36	21.12	1.11	72	0.011	352
50	8.6	1.39	21.11	1.11	49	0.008	361
100	9.9	1.40	21.37	1.12	92	0.014	343
500	8.4**	1.36	20.10**	1.40**	108	0.017	454

Table 12 (continued)

Dietary concentration (ppm)	Hb (mmol/L)	MCH (fmol)	MCHC (mmol/L)	HDW (mmol/L)	Reti abs ($\times 10^9$/L)	Reti rel	Plat ($\times 10^9$/L)
Week 52							
0	10.6	1.44	21.72	1.07	57	0.007	387
5	10.5	1.43	21.85	1.14	72	0.009	417
50	9.9	1.46	21.80	1.13	63	0.009	406
100	10.5	1.45	21.73	1.13	78	0.011	398
500	9.8	1.40	20.66**	1.41**	110**	0.015*	557*
Week 8 of recovery							
0	10.9	1.41	21.86	1.09	74	0.008	374
500	10.5	1.41	21.51	1.10	70	0.009	375

Hb: haemoglobin; HDW: haemoglobin distribution width; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; Plat: platelets; Reti abs: absolute reticulocyte count; Reti rel: relative reticulocyte count (ratio of reticulocytes to total erythrocytes); *: $P < 0.05$; **: $P < 0.01$
Source: Braun (2011b)

Table 13. Kidney and liver weights in dogs (n = 4) exposed to fluensulfone for 1 year

	Control[a]	5 ppm	50 ppm	100 ppm	500 ppm[a]
Males					
Kidney weight					
- Absolute (g)	60 *(56)*	53	53	63	64 *(56)*
- Relative to body weight (%)	0.45 *(0.42)*	0.44	0.45	0.53	0.68* *(0.47)*
Liver weight					
- Absolute (g)	380 *(326)*	322	315	372	351 *(330)*
- Relative to body weight (%)	2.8 *(2.5)*	2.7	2.7	3.1	3.2 *(2.8)*
Females					
Kidney weight					
- Absolute (g)	49 *(46)*	47	46	50	50 *(46)*
- Relative to body weight (%)	0.40 *(0.43)*	0.40	0.41	0.48	0.52 *(0.45)*
Liver weight					
- Absolute (g)	317 *(326)*	314	278	316	366 *(296)*
- Relative to body weight (%)	2.5 *(3.0)*	2.5	2.5	3.1	3.7* *(2.9)*

*: $P < 0.05$
[a] Recovery animals shown in parentheses.
Source: Braun (2011b)

found dead or killed in extremis during the study were necropsied, and tissues and organs were collected. Organ weights were recorded. A defined part of the liver was used for enzyme activity determinations, and the remaining portion of the liver as well as all other organs were fixed for histopathological evaluation. A wide range of tissues from control and top-dose animals was examined histopathologically, with lungs and gross lesions examined from the mid- and low-dose groups.

Survival to termination in week 78 was greater than 70% in all groups. There were no effects on survival, clinical signs, organ weights, palpable nodules and masses or clinical chemistry parameters. Absolute feed consumption was reduced throughout the study in both sexes at 1200 ppm: by 17% in males and 24% in females. Absolute mean body weight and body weight gain were statistically significantly decreased by the treatment in a dose-dependent manner in males at 200 and 1200 ppm and in females at 1200 ppm. The mean body weights at the end of the treatment period were reduced by 6% and 17% in the males of the mid- and high-dose groups and by 12% in the females of the high-dose group.

After 52 weeks, decreases were seen in absolute red blood cell count (< 10%) in mice of both sexes at 1200 ppm and decreased absolute white blood cell count in females only (no specific cell type affected). None of these changes was still present at terminal sacrifice after 78 weeks.

After 13 weeks of treatment, fluensulfone caused a slight decrease of hepatic ALAT activity in females, but no change in ALAT activity was recorded after 78 weeks of treatment. In addition, after 13 weeks, a weak increase in microsomal cytochrome P450 content, accompanied by a slight increase in the activities of Cyp4a1 and Cyp1a1 in females only, and a slight to moderate induction of phase II enzymes (UDPGT, GST and EH), mainly in females, were recorded (Supplementary Table 5).

Absolute weights of a number of organs were reduced in a manner consistent with the lower body weights. Increases in mean organ weights were associated with a small number of animals with very high organ weights. The only pathological changes of any note were in the lungs. An increased incidence of nodules in the lung of females, but not males, was seen at 200 and 1200 ppm (Table 14). The incidence and severity of bronchiolization of the lungs (described as a change from flattened epithelium to cuboidal epithelium) was increased at 200 and 1200 ppm (Table 14). An increased number of alveolar/bronchiolar adenomas was observed in females treated at 200 and 1200 ppm. The number of alveolar/bronchiolar carcinomas in females treated at 1200 ppm did not attain statistical significance with a Fisher’s exact test, whereas it was positive with the Peto trend test (Table 14). In control and 30 ppm females, the first lung lesion was seen at terminal sacrifice; at 200 ppm, three mice with lung tumours died during the study (first seen at week 67); at 1200 ppm, the first lung tumour was seen in an animal that died at week 50, with five animals dying during the study having lung tumours. The incidence of adenomas was clearly outside the historical control range (Table 14).

The NOAEL for carcinogenicity was 30 ppm (equal to 6.4 mg/kg bw per day), based on increased incidences of alveolar/bronchiolar adenomas and carcinomas in females at 200 ppm (equal to 39 mg/kg bw per day). The NOAEL for non-neoplastic effects was 30 ppm (equal to 4.2 mg/kg bw per day), based on decreased body weights and lung lesions (bronchiolization) at 200 ppm (equal to 28 mg/kg bw per day) (Kaiser, 2011a).

Mechanism of action of lung tumours in mice and supporting data

The notifier presented a relevance assessment of the lung tumours in mice based on the International Programme on Chemical Safety (IPCS) framework (Strupp et al., 2012). This was based on a number of elements:

- Published literature indicates that species-specific lung tumours can be produced in the mouse by a number of chemicals. The underlying cause is a high metabolic activity of the mouse lung, due to relatively high abundance of Clara cells in the mouse compared with humans and the mouse-specific cytochrome P450 isoform 2f2 in the Clara cells. The compounds are activated to reactive intermediates, leading to local cytotoxicity that promotes sustained cell

Table 14. Pathology findings in lungs of mice receiving fluensulfone for 78 weeks

	Males				Females				Historical control range for females
	0 ppm	30 ppm	200 ppm	1 200 ppm	0 ppm	30 ppm	200 ppm	1 200 ppm	
Survival week 78	36	42	46	41	43	37	36	36	–
No. examined	50	50	50	50	50	50	50	50	265
Nodules	11	8	8	12	1	6	11	13	–
Bronchiolar/alveolar hyperplasia	3	3	1	2	0	3	3	2	–
Lymphoid hyperplasia	17	26	22	16	26	22	26	33	–
Bronchiolization	1	0	24**	31**	5	7	43**	48**	–
- Mean grade	1.0	0	1.3	1.6	1.0	1.0	1.8	2.6	–
Alveolar/bronchiolar adenoma	7	9	5	12	2	4	14**	9*	0–6% Mean 3.5%
Alveolar/bronchiolar carcinoma	8	3	3	4	2	1	1	4#	0–10% Mean 3.5%
Adenoma and carcinoma combined	15	12	8	16	4	5	15**	13*	–

#: $P < 0.05$ (Peto trend test); */**: $P < 0.05$ and $P < 0.01$, respectively (Fisher's exact test, one-sided and pairwise)
Source: Kaiser (2011a)

proliferation, leading finally to tumour formation. Rats have lower metabolic activity in the lung compared with mice (below the threshold needed to cause lung tumours upon lifetime exposure), and activity in humans is orders of magnitude lower than in rats. The carcinogenic risk in the human lung is very low for this mode of action.

- Fluensulfone has shown an increased incidence of lung tumours in female mice at doses above a threshold of 30 ppm. Lung tumours are not seen in rats.
- Fluensulfone is not genotoxic.
- Fluensulfone was found to be extensively metabolized by mouse lung microsomes, whereas no metabolic activity is seen in human lung microsomes.
- Mouse-specific Cyp2f2 is a major contributor in fluensulfone's metabolism, and Cyp2e1 is not involved.
- Administration of fluensulfone to mice led to an early increase in Clara cell proliferation.

The Meeting considered that the proposed carcinogenicity relevance assessment was not extensive enough to conclusively identify the mode of action, having a number of deficiencies:

- It is based substantially on generic data on compounds such as isoniazid, which produced lung tumours in both sexes.
- It does not address the issue of why the non-neoplastic lesions are produced equally in male and female mice, but there is an increase in tumours only in female mice.
- The metabolic data show no differences between male and female mice. In males, there are no tumours.
- Alternative modes of action have not been addressed. There are no specific genotoxicity data on lung tissue/cells.
- The temporal association is unclear. There is an initial Clara cell proliferation at 2 weeks, but no histopathological findings at or before 13 weeks, and then bronchiolization and tumours at 50 weeks and beyond.

The Meeting concluded that the relevance to humans of the mouse lung tumours produced by fluensulfone cannot be entirely excluded, but the weight of evidence is that the mode of action is non-genotoxic and threshold based.

The full IPCS framework assessment is available as a supplementary document on the JMPR website.

Rats

In a combined chronic toxicity and carcinogenicity study in rats, four groups of HanRcc Wistar rats (50 males and 50 females in each group) were treated for 104 weeks with 0, 30, 200 or 1200 ppm fluensulfone (purity 96.7%; batch 36372130-291-PF1) in their diets. Achieved intakes were 0, 1.4, 9.6 and 58 mg/kg bw per day for males and 0, 1.7, 12 and 69 mg/kg bw per day for females, respectively. Satellite groups of 20 males and 20 females were similarly treated for 52 weeks and used for clinical chemistry, haematology and urine analysis investigations. Viability/mortality, clinical signs, feed consumption and body weights were recorded periodically throughout the study. Water consumption was recorded once during week 78. Ophthalmoscopic examinations were performed during acclimatization, in week 52 (satellite animals) and week 104. A functional observational battery was performed in week 52 (satellite groups). Blood and urine samples from all satellite animals were drawn in weeks 13 and 26 and after 52 weeks for determination of haematology, coagulation, clinical biochemistry and urine parameters. Blood samples from all main group animals were drawn after 78 and 104 weeks for determination of haematology parameters. All animals were necropsied, and tissues and organs were collected. Organ weights were recorded. In five satellite animals per group, a part of the liver was used for enzyme activity determinations; the remaining portion of the liver as well as all other organs fixed in neutral phosphate-buffered 4% formaldehyde solution underwent histopathological evaluation. Histological examinations were performed on organs and tissues from all control and high-dose animals and the lungs and oesophagus of animals from the mid- and low-dose groups. After 52 and 104 weeks, the right femur and the incisors of the maxilla of 10 animals of each sex per group were taken for fluoride determination.

There were two deaths (control females) in the satellite group, and survival to termination at 104 weeks was greater than 65% in all groups. Treatment with fluensulfone at dietary concentrations up to 1200 ppm for up to 104 weeks had no effects on survival, clinical signs, functional observational battery/motor activity results or ophthalmoscopic findings. There were no increases in palpable nodules or masses or in any type of neoplastic finding. There were no treatment-related histopathological changes in the liver, kidney, femur, joints or teeth.

Feed consumption in males treated with 1200 ppm was slightly lower during the first 4 weeks of treatment, but no further effects on feed consumption were recorded thereafter. Absolute mean body weight was decreased (< 10%) by the treatment in a dose-dependent manner in males treated at 200 and 1200 ppm.

A number of changes in haematology and biochemistry parameters were noted, mainly in animals treated with 1200 ppm. Two-fold increases in liver marker enzyme activities were seen in top-dose animals, but are not considered adverse in isolation. Dose-related, statistically significant increases in sodium levels were seen throughout the study in males and in females at week 52 (Tables 15 and 16). The sodium levels were all within the concurrent control ranges, and there were no fluctuations in sodium levels in the 90-day rat study with fluensulfone (all groups had mean values of 142 mmol/L); therefore, the fluctuations in sodium levels were considered not to be an adverse effect of treatment. Urinary ketone levels were increased in top-dose animals.

At necropsy after 52 weeks, no gross lesions that could be attributed to treatment with fluensulfone were observed. Absolute and relative weights of livers, kidneys and adrenals were increased in males treated at 1200 ppm. Kidney weight relative to body weight was also increased in females at 1200 ppm (Supplementary Table 6). Microscopically, hyperkeratosis of the oesophagus of minimal severity was observed in animals of both sexes treated with 1200 ppm, but no abnormality was noted in lungs. Phase II enzymes were significantly induced at 1200 ppm.

Table 15. Clinical chemistry parameters for male rats receiving fluensulfone in satellite group

(a) Week 13

Dietary concentration (ppm)	Chol (mmol/L)	Trigl (mmol/L)	Phos-lip (mmol/L)	ASAT (U/L)	ALAT (U/L)	LDH (U/L)	Na (mmol/L)
0	2.15	0.33	2.06	82.1	36.8	173.9	151.8
30	2.06	0.33	2.06	82.4	36.3	107.0**	148.9
200	2.29	0.36	2.18	79.8	38.1	114.8**	148.5
1 200	2.86**	0.47*	2.70**	87.0	34.8	165.6	149.9
Dietary concentration (ppm)	**K (mmol/L)**	**Cl (mmol/L)**	**Ca (mmol/L)**	**P (mmol/L)**	**Tot prot (g/L)**	**Alb (g/L)**	**Glob (g/L)**
0	4.01	108.4	2.77	2.01	74.44	45.45	28.99
30	3.91	107.2	2.76	2.01	72.43	44.27	28.17
200	3.90	106.6	2.74	2.03	71.34	43.88*	27.46
1 200	4.05	105.5*	2.83	2.18**	75.45	46.30	29.04

(b) Week 26

Dietary concentration (ppm)	Chol (mmol/L)	Trigl (mmol/L)	Phos-lip (mmol/L)	ASAT (U/L)	ALAT (U/L)	LDH (U/L)	Na (mmol/L)
0	2.47	0.39	1.83	78.8	32.8	166.2	145.5
30	2.49	0.43	1.88	80.1	33.9	162.6	145.6
200	2.60	0.46	1.93	89.5	34.8	192.6	147.0**
1 200	3.50**	0.66**	2.57**	89.9	36.0	269.4**	149.2**
Dietary concentration (ppm)	**K (mmol/L)**	**Cl (mmol/L)**	**Ca (mmol/L)**	**P (mmol/L)**	**Tot prot (g/L)**	**Alb (g/L)**	**Glob (g/L)**
0	3.81	102.7	2.75	1.76	73.87	42.84	31.03
30	3.79	102.9	2.76	1.82	74.27	42.81	31.46
200	3.88	103.5	2.74	1.74	74.57	43.37	31.20
1 200	4.11**	103.0	2.89**	1.98**	80.53**	46.02**	34.52**

(c) Week 52

Dietary concentration (ppm)	Chol (mmol/L)	Trigl (mmol/L)	Phos-lip (mmol/L)	ASAT (U/L)	ALAT (U/L)	LDH (U/L)	Na (mmol/L)
0	2.50	0.66	1.83	70.9	30.7	109.7	146.2
30	2.76	0.82	2.05	77.6	34.5	126.8	147.2*
200	2.82	0.72	2.05	79.7	31.5	123.3	149.2**
1 200	3.83**	1.05**	2.63**	91.8**	31.8	181.3**	152.0**
Dietary concentration (ppm)	**K (mmol/L)**	**Cl (mmol/L)**	**Ca (mmol/L)**	**P (mmol/L)**	**Tot prot (g/L)**	**Alb (g/L)**	**Glob (g/L)**
0	3.83	102.6	2.74	1.41	73.96	42.59	31.37
30	3.88	104.0**	2.77	1.47	75.88*	42.40	33.48**
200	3.93	106.1**	2.80**	1.55*	76.64**	43.48	33.16*
1 200	4.27**	105.3**	2.94**	1.57**	82.91**	46.00**	36.91**

ALAT: alanine aminotransferase; Alb: albumin; ASAT: aspartate aminotransferase; Chol: cholesterol; Glob: globulin; LDH: lactate dehydrogenase; Phos-lip: phospholipids; Tot prot: total protein; Trig: triglycerides; U: unit; *: $P < 0.05$; **: $P < 0.01$

Source: Kaiser (2011b)

Table 16. Clinical chemistry parameters for female rats receiving fluensulfone in satellite group

(a) Week 13

Dietary concentration (ppm)	Chol (mmol/L)	Trigl (mmol/L)	Phos-lip (mmol/L)	ASAT (U/L)	ALAT (U/L)	LDH (U/L)	Na (mmol/L)
0	1.88	0.24	2.19	90.6	34.4	125.0	148.8
30	1.64	0.24	2.09	79.0*	29.2	115.9	144.9**
200	2.02	0.27	2.32	84.3	27.4*	121.0	146.7**
1 200	2.16	0.26	2.51*	79.6	28.1*	157.8*	148.9
Dietary concentration (ppm)	**K (mmol/L)**	**Cl (mmol/L)**	**Ca (mmol/L)**	**P (mmol/L)**	**Tot prot (g/L)**	**Alb (g/L)**	**Glob (g/L)**
0	3.48	108.0	2.77	1.41	77.51	51.50	25.95
30	3.26	105.5**	2.66**	1.46	77.28	52.18	24.98
200	3.34	106.2**	2.68**	1.61*	76.55	50.05	26.50
1 200	3.52	107.1	2.70	1.57	77.51	49.95	27.56*

(b) Week 26

Dietary concentration (ppm)	Chol (mmol/L)	Trigl (mmol/L)	Phos-lip (mmol/L)	ASAT (U/L)	ALAT (U/L)	LDH (U/L)	Na (mmol/L)
0	2.40	0.32	2.29	94.0	30.7	193.6	148.1
30	2.02	0.33	2.00	90.9	27.6	189.9	143.1**
200	2.58	0.35	2.41	85.6	28.1	143.4	144.9**
1 200	2.53	0.32	2.35	75.7*	25.3*	179.6	146.4**
Dietary concentration (ppm)	**K (mmol/L)**	**Cl (mmol/L)**	**Ca (mmol/L)**	**P (mmol/L)**	**Tot prot (g/L)**	**Alb (g/L)**	**Glob (g/L)**
0	3.19	104.8	2.82	1.47	81.37	52.31	29.06
30	3.29	102.3**	2.76	1.45	78.29*	51.52	26.77**
200	3.29	103.2**	2.78	1.44	78.39*	50.64	27.75
1 200	3.43*	103.9	2.79	1.36	78.46*	49.87*	28.59

(c) Week 52

Dietary concentration (ppm)	Chol (mmol/L)	Trigl (mmol/L)	Phos-lip (mmol/L)	ASAT (U/L)	ALAT (U/L)	LDH (U/L)	Na (mmol/L)
0	2.25	0.30	1.96	116.2	40.4	131.3	144.7
30	2.11	0.33	1.97	101.5	38.5	126.6	145.0
200	2.63	0.35	2.21	105.3	32.9	160.6	147.2**
1 200	2.67	0.40**	2.47**	89.3*	28.6**	149.9	151.5**
Dietary concentration (ppm)	**K (mmol/L)**	**Cl (mmol/L)**	**Ca (mmol/L)**	**P (mmol/L)**	**Tot prot (g/L)**	**Alb (g/L)**	**Glob (g/L)**
0	3.44	102.6	2.73	1.32	78.12	48.89	29.23
30	3.48	102.8	2.80*	1.35	79.17	50.49	28.68
200	3.53	104.8**	2.80*	1.40	79.38	49.51	30.26
1 200	3.51	106.5**	2.87**	1.40	80.39	50.85	29.54

ALAT: alanine aminotransferase; Alb: albumin; ASAT: aspartate aminotransferase; Chol: cholesterol; Glob: globulin; LDH: lactate dehydrogenase; Phos-lip: phospholipids; Tot prot: total protein; Trig: triglycerides; U: unit; *: $P < 0.05$; **: $P < 0.01$

Source: Kaiser (2011b)

At necropsy after 104 weeks, an increased number of macroscopic foci in the lungs was recorded in the treated animals, compared with controls, attaining statistical significance only in males at 1200 ppm (Table 17). Enlarged livers were recorded in four females (compared with one in the control group) treated at 1200 ppm. Microscopically, an increased incidence of hyperkeratosis in the oesophagus was recorded in animals of both sexes treated at 200 and 1200 ppm. The incidence of chronic interstitial inflammation in the lungs was increased in males (Table 17) and females treated at 1200 ppm and in females treated at 200 ppm. This lesion was characterized by focal/multifocal changes consisting of interstitial or intra-alveolar inflammatory cells associated with hypertrophied reactive type II pneumocytes, associated with the presence of foamy intra-alveolar macrophages, which was also increased in mean severity grade. Although the incidences were not all statistically significant, the pattern of effects was considered outside the normal range by the study report authors.

Table 17. Incidence of lungs and oesophagus lesions in male rats receiving fluensulfone

Finding	0 ppm		30 ppm		200 ppm		1 200 ppm	
Lung foci ($n = 50$)	3	4	5	8	5	9	15**	11
Lung interstitial inflammation ($n = 50$)	2	3	4	1	2	7	13*	9
Oesophageal hyperkeratosis (week 52; $n = 20$)	0	0	0	0	0	0	12**	6*
Oesophageal hyperkeratosis (week 104; $n = 50$)	3	2	2	3	7	8*	21**	20**

*: $P < 0.05$; **: $P < 0.01$
Source: Kaiser (2011b)

Fluoride measurements revealed a marked increase in the fluoride content in the ashes from bones and teeth at 200 and 1200 ppm and in bones from 30 ppm rats at week 104 (Supplementary Table 7). Changes in bone and tooth fluoride levels were not associated with signs of dental fluorosis (discoloration of the teeth) or skeletal fluorosis (mobility problems, changes in external appearance of bones, changes in bone histopathology).

The NOAEL for chronic treatment was 30 ppm (equal to 1.4 mg/kg bw per day), based on hyperkeratitis of the oesophagus, chronic interstitial inflammation in the lungs and reduced body weight gain at 200 ppm (equal to 9.6 mg/kg bw per day). Fluensulfone showed no carcinogenic potential in rats at dose levels up to 1200 ppm (equal to 58 mg/kg bw per day), the highest dose tested (Kaiser, 2011b).

2.4 Genotoxicity

Fluensulfone has been tested for reverse mutation in bacterial and mammalian cells, for clastogenicity in vitro and for the induction of micronuclei in mouse bone marrow erythrocytes (Table 18). A weakly positive result was seen with *Salmonella typhimurium* TA100 in an early study, but not in two subsequent studies. Positive results were seen in the chromosomal aberration test at markedly cytotoxic concentrations. The micronucleus test was negative. It is concluded that fluensulfone is not genotoxic in vivo.

2.5 Reproductive and developmental toxicity

The reproductive toxicity of fluensulfone was evaluated in a two-generation reproduction study in rats. Developmental toxicity was assessed in rats and rabbits.

(a) Two-generation reproductive toxicity study in the rat

In a two-generation study, groups of 24 parent male and female rats (HanRcc Wistar) received fluensulfone (purity > 96%; batch 36372130-291-PF1) in their diets at a concentration of 0, 30, 250 or 1800 ppm during a 10-week pre-pairing period, throughout the mating period, during

Table 18. Results of studies of genotoxicity with fluensulfone

Test substance (vehicle)	End-point	Test object	Concentration	Purity; batch	Result	Reference
In vitro						
Fluensulfone (DMSO)	Reverse mutation	*Salmonella typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537	16–5 000 µg/plate, ±S9 mix Repeat 1 000–6 000 µg/plate with TA100	98.8%; NLL6692-5	Weak positive TA100 ±S9 Negative in other strains	Herbold (2002a,b)
Fluensulfone (DMSO)	Reverse mutation	*S. typhimurium* strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* strain WP2 uvrA	3.16–5 000 µg/plate (±S9 mix)	96.8%; 411-33-1	Negative	Donath (2011)
Fluensulfone (DMSO)	Reverse mutation	*S. typhimurium* strains TA98, TA100, TA1535 and TA1537 and *E. coli* strain WP2 uvrA	3–5 000 µg/plate (±S9 mix)	96.2%; 36372130-291-PF1	Negative	Sokolowski (2008)
Fluensulfone (DMSO)	Reverse mutation	Chinese hamster lung V79 cells – *HPRT* locus	24–72 µg/mL (−S9 mix) 20–80 µg/mL (+S9 mix)	98.4%; NLL6692-13.1	Negative	Herbold (2003a)
Fluensulfone (DMSO)	Chromosomal aberration	Human lymphocytes (4 and 24 h exposures)	62.5–1 000 µg/mL (−S9 mix) 125–1 000 µg/mL (+S9 mix)	96.5%; 36372130-291-PF1	Positive at cytotoxic concentrations ±S9 mix	Flügge (2010)
In vivo						
Fluensulfone (DMSO)	Bone marrow micronuclei	NMRI mice (M; n = 5)	75, 150 and 300[a] mg/kg bw, twice by intraperitoneal route	98.4%; NLL6692-13.1	Negative	Herbold (2003b)

DMSO: dimethyl sulfoxide; HPRT: hypoxanthine–guanine phosphoribosyltransferase; M: male; S9: 9000 × *g* supernatant fraction from rat liver homogenate

[a] Increased normochromatic erythrocye:polychromatic erythrocyte ratio.

pregnancy and up to weaning of the F_1 generation (on day 21 postpartum). Treatment of the F_1 generation (24 males and 24 females per group) continued during development to adulthood (at least 91 days), mating, pregnancy and lactation of the F_2 generation until weaning of the F_2 pups. Mean

achieved intakes were 0, 2.0, 16 and 122 mg/kg bw per day for males and 0, 2.8, 23 and 169 mg/kg bw per day for females at 0, 30, 250 and 1800 ppm, respectively.

Animals of both generations were observed regularly for clinical signs and viability/mortality. Body weight and feed consumption were recorded routinely, except during the pairing period. F_1 and F_2 pups were culled on day 4 postpartum to yield as nearly as possible four males and four females per litter. Pups were weighed on days 0 or 1, 4, 7, 14 and 21 postpartum; sex ratio was assessed on days 0 or 1, 4 and 21. The onset of ear and eye opening, lower incisor eruption and hair growth, and, for the F_1 animals selected for breeding of the F_2 generation, the age and weight at which vaginal opening or preputial separation occurred were recorded. At termination, all animals were necropsied, organ weights were determined, and reproductive organs as well as target organs were fixed and examined in control and high-dose animals. Liver, kidney and thyroid gland were also examined in the 30 and 250 ppm groups. Incisors and femur bones were collected from all F_1 adult animals; fluoride content was determined in right femur and half of the incisors from 10 F_1 parent animals of each sex per group and from 10 F_2 pups (aged 21 days) of each sex per group. Sperm analyses were performed in males of the control and high-dose groups of both generations. Quantitative evaluation of primordial follicles and antral follicles from 10 sections per ovary in the first 10 females of the P and F_1 generations of the control and high-dose groups was also performed.

The clinical signs noted during the study were not remarkable. At 1800 ppm, treatment with the test item resulted in a statistically significant reduction of feed consumption in both P and F_1 generations during the pre-pairing period. In the F_1 generation, this reduction was prolonged into the after pairing period in males and through the gestation period and on days 7–14 of lactation in females. Body weight was statistically significantly reduced in both generations, whereas body weight gain was decreased in the P generation only during the pre-pairing period. Kidney and liver weights were statistically significantly increased in both generations.

At 250 ppm, a reduction in feed consumption in F_1 parent males was recorded during the first half of the pre-pairing period. As feed consumption recovered and there was no corresponding decrease in body weight at this dose level, this was not considered to be adverse.

No effects on any reproductive parameters were recorded; slight increases in postnatal loss to day 4 at 1800 ppm did not have an adverse effect on litter size (Table 19). Analysis of sperm motility, sperm morphology and sperm head count did not reveal any effects. Developmental indices (onset of ear and eye opening, lower incisor eruption and hair growth) and sexual maturation (vaginal opening and preputial separation) did not indicate any test item–related effect, in terms of date or body weight.

In both F_1 and F_2 pups, pup weight was statistically significantly reduced (10%) during the last week of lactation at 1800 ppm. Secondarily, spleen weight was also reduced. During the histopathological examinations, glycogen deposits were reduced in the liver of F_2 male and female pups, possibly associated with the lower body weight.

At 1800 ppm, centrilobular hypertrophy of the liver was noted at minimal to slight severity in both P and F_1 parental animals. This finding correlated with the increase in liver weight. The incidence and severity of renal tubular hyaline droplets were increased in males of P and F_1 generation parental animals. The incidence and/or severity of tubular basophilia, mononuclear cell infiltration and tubular casts were also increased in males of P and F_1 generation parental males. These lesions were considered to be secondary lesions resulting from the hyaline droplets described above. Follicular hypertrophy of the thyroid gland was recorded at minimal to slight severity in four males of the P generation and six F_1 generation parental males.

In the F_1 generation parental animals and the F_2 generation pups, there were significant increases in the fluoride content of bones and teeth (Supplementary Table 8). There were no effects on incisor length or the coloration of the teeth reported in either the F_1 adults or the F_2 pups.

The NOAEL for systemic toxicity in parental animals of both generations was 250 ppm (equal to 16 mg/kg bw per day), based on the effects on body weight development throughout the study in males plus liver and kidney weight increases at 1800 ppm (equal to 122 mg/kg bw per day).

Table 19. Breeding data for the P generation of rats receiving fluensulfone

Parameter	Mean ± SD[a]			
	0 ppm	30 ppm	250 ppm	1 800 ppm
No. of litters	23	24	22	24
Duration of gestation (days)	21.6 ± 0.59	21.4 ± 0.58	21.6 ± 0.49	21.5 ± 0.51
No. of implantations	13.6 ± 2.13	14.1 ± 1.72	13.5 ± 2.30	14.2 ± 1.58
Postimplantation loss (% of implantations)	2.0 ± 2.40	1.2 ± 1.53	0.9 ± 0.97	1.6 ± 1.41
Living pups at first litter check	11.7 ± 3.41	12.9 ± 2.07	12.6 ± 2.48	12.6 ± 1.79
Postnatal loss days 0–4 postpartum				
- No. of litters affected	1	2	3	6
- Total no.	1	2	4	8*
- Mean ± SD	0.0 ± 0.21	0.1 ± 0.28	0.2 ± 0.50	0.3 ± 0.64
Living pups day 4 postpartum	7.6 ± 1.70	8.0 ± 0.00	8.0 ± 0.00	8.0 ± 0.00
Living pups day 21 postpartum	7.6 ± 1.70	8.0 ± 0.20	8.0 ± 0.0	8.0 ± 0.20

SD: standard deviation; *: $P < 0.05$
[a] Except where noted otherwise.
Source: Ceccatelli (2011)

The NOAEL for offspring toxicity was 250 ppm (equal to 16 mg/kg bw per day in males), based on reduced pup weight noted at 1800 ppm (equal to 122 mg/kg bw per day).

The NOAEL for reproductive effects was 1800 ppm (equal to 122 mg/kg bw per day), the highest dose tested (Ceccatelli, 2011).

(b) Developmental toxicity

Rats

In a developmental toxicity study, groups of 30 mated Wistar rats received fluensulfone (purity 96–97%; batch NLL6692-7-5) in 2% Cremophor, 0.5% carboxymethyl cellulose and deionized water at 0, 7.7, 49 or 292 mg/kg bw per day on days 6–19 of gestation. All dams were sacrificed on gestation day 20, at which time gross external and internal necropsies were performed. All fetuses were evaluated for external anomalies. Approximately half of all fetuses from each litter were examined for visceral effects at necropsy and also evaluated for cranial effects using Wilson's technique. The remaining fetuses underwent an evaluation for general skeletal (including cartilage) development.

Three dams from the mid-dose group and four from the top-dose group were found dead. Maternal compound-related findings observed in dams treated at 292 mg/kg bw per day included red vaginal discharges ($n = 3$) and body weight loss coupled with decreased feed consumption (Table 20) at the start of dosing that persisted to the end of the study. The initial body weight deficits (20 g/rat on days 6–10) were consistent with the reduction in feed consumption (6 g/rat per day). Increased absolute and relative (10%) liver and kidney weights were seen at the top dose level.

There was a decrease in the number of fetuses per litter at the low and top doses but not the middle dose (Table 21). This was due in part to reduced implantation rates, which would be unrelated to dosing. Four non-viable fetuses were seen in one top-dose litter. No compound-related effects on fetal external, visceral or skeletal malformations were observed. There were no increases in external or visceral variations. There was evidence of slightly increased ossification, particularly of the skull, with significantly lower incidences of enlarged fontanelles/sutures in the mid- and high-dose groups (Table 21). These latter findings are not considered adverse.

Table 20. Body weight and feed consumption in rats administered fluensulfone during gestation

Day of pregnancy	Mean ± standard error			
	Control	7.7 mg/kg bw per day	49 mg/kg bw per day	292 mg/kg bw per day
Body weight (g)				
0	208.2 ± 2.33	207.7 ± 2.88	211.0 ± 2.35	206.1 ± 2.43
6	228.8 ± 2.59	224.8 ± 3.42	228.7 ± 2.38	228.3 ± 2.90
7	230.4 ± 2.80	227.1 ± 3.68	228.7 ± 2.49	223.5 ± 3.28
10	241.0 ± 2.85	235.9 ± 4.30	240.4 ± 2.66	220.2 ± 3.38**
15	258.5 ± 4.09	255.8 ± 3.53	262.8 ± 2.71	237.1 ± 4.39**
20	310.0 ± 5.06	299.9 ± 5.20	314.1 ± 4.24	284.2 ± 5.17**
Feed consumption (g/kg bw per day)				
4–6	84.9 ± 1.70	79.2 ± 1.65	83.2 ± 2.68	86.1 ± 2.23
6–8	83.8 ± 3.03	82.4 ± 2.36	78.4 ± 2.63	52.6 ± 3.83**
8–10	85.8 ± 3.01	78.0 ± 2.97	82.0 ± 2.64	52.5 ± 3.10**
10–12	86.6 ± 2.82	78.7 ± 2.73	82.3 ± 2.14	60.8 ± 3.87**
12–14	87.3 ± 2.54	83.6 ± 2.91	82.9 ± 2.21	97.4 ± 9.80
18–20	71.9 ± 2.32	76.0 ± 2.39	74.7 ± 2.46	75.1 ± 8.71

**: $P < 0.01$
Source: Young (2003)

Table 21. Reproductive data in rats administered fluensulfone during gestation

Parameter	0 mg/kg bw per day	7.7 mg/kg bw per day	49 mg/kg bw per day	292 mg/kg bw per day
No. of pregnant rats	24	27	26	24
No. found dead	0	3	0	4
No. of litters	24	24	26	20
Litter size				
- Mean ± SE	10.7 ± 0.63	9.2 ± 0.76	11.4 ± 0.44	9.9 ± 0.50
- Range	1–15	0–14	5–15	5–14
No. of viable fetuses	257	220	296	193
- Mean ± SE per dam	10.7 ± 0.63	9.2 ± 0.76	11.4 ± 0.44	9.6 ± 0.56**
No. of corpora lutea (mean)	13.3	12.9	13.4	13.4
No. of implantations	11.3	10.8	12.2	10.4
Postimplantation loss (% of implantations)	0.6	1.6	0.8	0.7
No. of non-viable fetuses	0	0	0	4
Median % male fetuses	57	50	59	50
Mean weight of fetuses (g) (mean ± SE)	3.6 ± 0.05	3.6 ± 0.05	3.6 ± 0.04	3.4 ± 0.07
Malformations				
Fetal incidence	3	0	1	1
Litter incidence	3	0	1	1
Variations				
Fetal incidence (%)	150	127	167	121

Parameter	0 mg/kg bw per day	7.7 mg/kg bw per day	49 mg/kg bw per day	292 mg/kg bw per day
	(58.4%)	(57.7%)	(56.4%)	(62.7%)
Litter incidence	23	22	26	20
Enlarged anterior fontanelle	41	23	19**	11**
Enlarged sagital suture	39	22	18**	6**
Enlarged posterior fontanelle	42	32	18**	7**
Enlarged squamosal suture	13	8	4*	1*
Left-sided umbilical artery	15	12	11	20

SE: standard error; *: $P < 0.05$; **: $P < 0.01$
Source: Young (2003)

The NOAEL for maternal toxicity was 49 mg/kg bw per day, based on reduced body weight and clinical signs at 292 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 49 mg/kg bw per day, based on the four non-viable fetuses in a litter at 292 mg/kg bw per day. Fluensulfone was not teratogenic to rats (Young, 2003).

Rabbits

In a developmental toxicity study, groups of 20 mated Himalayan rabbits were administered fluensulfone (purity 96.7%; batch 36372130-291-PF1) in 0.5% aqueous carboxymethyl cellulose at 0, 2.5, 10 or 40 mg/kg bw per day at a dosing volume of 5 mL/kg bw on days 6–27 of gestation. All does were sacrificed on gestation day 28, at which time gross external and internal necropsies were performed. Fetuses were removed from the uterus, sexed, weighed individually and examined for gross external and visceral abnormalities. The heads of 50% of the fetuses were serially sectioned and examined. The remaining fetuses were used for skeletal examinations (alizarin red S and alcian blue staining).

All does survived until the scheduled necropsy, and no clinical signs related to treatment were observed. Mean body weight gain was slightly reduced in does treated at 40 mg/kg bw per day between days 6 and 9, attaining statistical significance on days 7 and 8. This effect was accompanied by a reduction in feed intake noted since the beginning of treatment, more evident between days 18 and 21 and attaining statistical significance between days 21 and 24 (−22.2% compared with the control group). Over the initial dosing period, rabbits in the top-dose group had a slightly lower feed consumption than controls, but the deficit in feed consumption (3 g/rabbit per day) does not match the body weight deficit of 42 g/rabbit over 3 days. Over the period from days 9 to 15, the top-dose rabbits had a body weight gain higher than that of any other groups; although the feed consumption was higher than that of the controls, it was below that of the low- and mid-dose groups. Overall body weight gain (days 6–28) in the top-dose group was approximately 10% less than that of the controls, but was in line with those of the low- and mid-dose groups (Table 22).

There were no adverse effects on measured litter or fetal parameters (Supplementary Table 9). A lower fetal weight in the top-dose group was secondary to the higher number of fetuses per litter and is not considered adverse.

The NOAEL for embryo and fetal toxicity was 40 mg/kg bw per day, the highest dose tested. The maternal NOAEL was 10 mg/kg bw per day, based on body weight loss. Fluensulfone was not teratogenic to rabbits (Ceccatelli, 2009).

2.6 Special studies

(a) Neurotoxicity

Acute neurotoxicity in the rat

In a preliminary acute neurotoxicity study, groups of three male and three female RccHan Wistar rats were administered single oral doses of fluensulfone at 0, 100, 500, 1000 or 2000 mg/kg

Table 22. Body weight, body weight gain and feed consumption in pregnant rabbits dosed with fluensulfone from days 6 to 27

	0 mg/kg bw per day	2.5 mg/kg bw per day	10 mg/kg bw per day	40 mg/kg bw per day
Group size[a]	20	19	19	17
Body weight (g)				
- Day 6	2 551	2 546	2 576	2 561
- Day 7	2 552	2 550	2 581	2 535
- Day 8	2 555	2 556	2 583	2 530
- Day 15	2 641	2 640	2 663	2 642
- Day 28	2 773	2 753	2 782	2 761
Body weight gain (g)				
- Days 6–9	13	24	10	−29
- Days 9–12	19	10	23	34
- Days 12–15	58	60	54	76
- Days 21–24	33	37	34	28
- Days 6–28	222	207	206	200
Mean feed consumption (g/rabbit per day)				
- Days 6–9	95	113	103	92
- Days 9–12	100	108	106	99
- Days 12–15	94	103	100	99
- Days 21–24	108	103	105	84*
- Days 6–28	98	101	105	93

*: $P < 0.05$

[a] Animals with viable litters only.

Source: Ceccatelli (2009)

bw (purity 98.7%; batch 36372130-291-PF1) in PEG 300 and were observed for the following 8 days. At the end of the observation period, all surviving animals were sacrificed and assessed by gross pathology. All animals of the high-dose group (2000 mg/kg bw) died spontaneously within 24 hours after dose administration after they had developed signs of overt toxicity from 0.5 hour. All animals of the remaining dose groups survived to terminal sacrifice without showing any signs of toxicity. No macroscopic findings were recorded in any animals of any dose groups at necropsy. A peak systemic effect was observed on the day of dosing in animals of both sexes treated at 2000 mg/kg bw approximately 5 hours after administration (Sommer, 2009).

Groups of 10 male and 10 female RccHan Wistar rats were administered single oral doses of fluensulfone at 0, 100, 400 or 1200 mg/kg bw (purity 98.7%; batch 36372130-291-PF1) in PEG 300 and observed for 14 days. General cage-side observations were made in all animals prior to treatment start and once daily thereafter throughout the study. In addition, a functional observational battery evaluation was performed on all animals prior to treatment start and on days 1 (approximately 1–4 hours after dose administration), 7 and 14. Motor activity was assessed over a period of 30 minutes after each functional observational battery evaluation. Feed consumption and body weights were measured regularly. At the end of the scheduled observation period, five rats of each sex per group were perfusion fixed in situ, and selected nervous system tissues were dissected, processed and examined neurohistopathologically.

One female dosed at 1200 mg/kg bw died within 24 hours after dose administration from unknown causes. On day 8, the mean body weight gain was lower in males and females treated at 1200 mg/kg bw. Feed consumption was dose dependently reduced in males and females at 400 and 1200 mg/kg bw on days 1–8, with statistical significance attained in females at 1200 mg/kg bw only.

Administration of a single oral gavage dose of fluensulfone to Wistar rats at 100, 400 or 1200 mg/kg bw produced reversible effects on motor activity and functional observational battery parameters. At 100 mg/kg bw, a rapidly reversible effect on motor activity was observed in females only, and an increased incidence of abnormal righting reflex was seen in males. Changes in functional observational battery parameters (Tables 23 and 24) were observed at all dose levels on day 1, but not subsequently. Detailed weekly clinical observations revealed no treatment-related effects on days 1, 8 and 15. No test item–related effects on absolute or relative brain weights were evident in males or females. There were no findings in the nervous system during macroscopic and microscopic pathology examinations.

Table 23. Incidences of remarkable observations noted during functional observational battery assessments at 1–4 hours post-dosing in rats (n = 10) administered fluensulfone

Day 1 finding	0 mg/kg bw		100 mg/kg bw		400 mg/kg bw		1 200 mg/kg bw	
	M	F	M	F	M	F	M	F
Soft faeces	0	0	0	0	0	1	2	4
Skin cold to touch	0	0	0	0	0	0	2	0
Reduced activity	0	0	3	1	6	6	8	7
Decreased rearing	0	0	2	1	4	8	6	8
Piloerection	0	0	3	0	2	2	8	6
Righting response reduced	0	0	4	1	7	7	7	8
Hunched posture	0	0	0	0	0	0	2	0
Salivation	0	0	0	0	1	0	0	1

F: females; M: males
Source: Sommer (2010)

Table 24. Summary of locomotor activity at 1–4 hours post-dosing (day 1) in rats administered fluensulfone

Parameter	Dose (mg/kg bw)	Males		Females	
		Activity counts	% difference from control	Activity counts	% difference from control
Total distance	0	2 487.9 ± 513	–	2 615.2 ± 812	–
	100	2 565.4 ± 853	+3	1 939.4* ± 614	−26
	400	1 418.6* ± 529	−43	1 504.8** ± 441	−42
	1 200	1 016.0** ± 679	−59	1 088.5** ± 525	−58
Centre time	0	124 ± 111	–	124 ± 79	–
	100	107 ± 51	−14	68* ± 39	−45
	400	67 ± 45	−46	51** ± 27	−59
	1 200	27** ± 29	−78	29** ± 26	−77
Rearing activity	0	48 ± 27	–	48 ± 20	–
	100	43 ± 43	−10	27** ± 21	−44
	400	20** ± 20	−58	17** ± 8	−65
	1 200	11** ± 13	−77	9** ± 7	−81

*: $P < 0.05$; **: $P < 0.01$ (Dunnett's test)
Source: Sommer (2010)

The lowest-observed-adverse-effect level (LOAEL) for general toxicity and neurotoxicity was 100 mg/kg bw, the lowest dose tested, in males and females, based on clinical signs and reduced activity in the functional observational battery and decreased motor activity. There was no neuropathological finding at any dose level (Sommer, 2010).

Using the United States Environmental Protection Agency's (USEPA) benchmark dose software (BMDS version 2.2), the following $BMDL_{SD}$ values ($BMDL_{SD}$ is the 95% lower confidence limit of the dose corresponding to a change equivalent to 1 standard deviation in the response for unexposed animals) were derived using the lowest value from the models with a good fit for dichotomous data or Hill model for continuous data (Table 25).

Table 25. BMD_{10}/BMD_{SD} and $BMDL_{10}$/$BMDL_{SD}$ values for end-points from the acute neurotoxicity study with fluensulfone

End-point	$BMDL_{10}$/$BMDL_{SD}$ (mg/kg bw)	BMD_{10}/BMD_{SD} (mg/kg bw)	Models
Reduced righting response: males	31	47	Gamma, Weibull, quantal linear (10% change)
Locomotor distance: males	54 / 147	335 / 371	Hill (extra 10%) / Hill (1 SD)
Locomotor distance: females	7 / 33	20 / 94	Hill (extra 10%) / Hill (1 SD)
Centre time: males	10 / 124	78 / 470	Hill (extra 10%) / Hill (1 SD)
Centre time: females	0.5 / 6	9 / 74	Hill (extra 10%) / Hill (1 SD)
Rearing activity: males	9 / Did not run	87 / 368	Hill (extra 10%) / Hill (1 SD)
Rearing activity: females	3 / 19	12 / 59	Hill (extra 10%) / Hill (1 SD)

BMD_{10}: benchmark dose for a 10% response; BMD_{SD}: benchmark dose for a 1 standard deviation response; $BMDL_{10}$: lower 95% confidence limit on the benchmark dose for a 10% response; BMD_{SD}: lower 95% confidence limit on the benchmark dose for a 1 standard deviation response; SD: standard deviation

The large variability in results in the control groups (standard deviation [SD] often approximating to the value of the mean) indicates that using a 10% change is unrealistic, and the default of the software to use 1 SD is considered to be more appropriate for the continuous data sets. The lowest $BMDL_{SD}$ is 6 mg/kg bw for centre time in females, but this is a value with a low confidence level, given the 12-fold gap from the $BMDL_{SD}$ to the BMD_{SD}. Other $BMDL_{SD}$ values for females, the more sensitive sex, from the low end of the distribution are 19 and 33, and it is concluded that these support a point of departure (POD) of 25 mg/kg bw.

[The notifier performed a simple linear regression, which indicated that the NOAEL would be in the region of 25 mg/kg bw.]

Subchronic neurotoxicity in the rat

In a subchronic dietary neurotoxicity study, groups of 12 male and 12 female Wistar rats received fluensulfone (purity 95.7%; batch 36372130-291-PF1) in their diet at a concentration of 0 (control), 100, 500 or 2500 ppm for 90 days. Achieved intakes were 0, 6, 31 and 153 mg/kg bw per day for males and 0, 7, 34 and 162 mg/kg bw per day for females, respectively. General cage-side observations, body weights and feed consumption values were determined routinely. Functional observational batteries were performed on all animals prior to first administration and in weeks 2, 5, 9 and 13. Locomotor activities were assessed after each functional observational battery evaluation. Ophthalmoscopic examinations were performed on all animals once prior to first administration and in animals of groups 1 and 4 in week 13. At the end of the treatment period, five rats of each sex per group were perfusion fixed in situ. Selected nervous system tissues were dissected, processed and examined microscopically.

All animals scheduled for neurohistological examination survived the scheduled study period. One mid-dose female died on day 87, unrelated to treatment. Treatment-related effects were confined to lower feed consumption, body weight and body weight gain in males at 2500 ppm. Terminal body weight in top-dose males was 15% lower than in controls. Detailed clinical observations and functional observational battery evaluations of clinical symptoms revealed no treatment-related effects. Locomotor activity (total distance and rearing counts) was significantly lower in males at 2500 ppm during week 5, but not at week 9 or 13, and this is considered to be a chance finding. There were no notable effects on neuropathological examination.

The NOAEL for neurotoxicity was 2500 ppm (equal to 153 mg/kg bw per day), the highest dose tested. The NOAEL for systemic toxicity was 500 ppm (equal to 31 mg/kg bw per day), based on lower body weight in males at 2500 ppm (equal to 153 mg/kg bw per day) (Sommer, 2011).

(b) Immunotoxicity in mice

Although immunotoxicity end-points were already included in the 28-day dietary study in rats and mice, fluensulfone was also tested in mice, according to the applicable USEPA guideline, to evaluate its immunotoxic potential.

In a study evaluating potential immunotoxicity, groups of 10 female CD-1 mice received fluensulfone (purity 96.7%; batch 36372130-291-PF1) in the diet at a concentration of 0, 100, 500 or 2500 ppm. After 1 week of treatment, one mouse in the high-dose group died, and signs of overt toxicity (decreases in body weight, feed and water consumption, clinical signs in a few animals) were noted in the other animals of this group. Following 3 days on untreated diet, the dietary concentration was therefore reduced to 1500 ppm for this group, and treatment was continued for an additional 21 days. The treatment period for all groups was extended to 32 days in order to complete the planned 28-day treatment period for all treated and negative control animals on study. Achieved mean intakes were 0, 17, 86 and 204 mg/kg bw per day (note that the study report erroneously presents intakes on a daily basis when they are weekly). Cyclophosphamide was used as a positive control and was administered intraperitoneally at 25 mg/kg bw per day during days 27–31 to a group of 10 female mice. On day 27, a single intravenous dose of 0.2 mL of 1×10^8 sheep red blood cells (sRBC) in phosphate-buffered saline was administered to all surviving animals. Five days after immunization (day 32), all mice were weighed and euthanized, serum samples were collected from each mouse for anti-sRBC IgM titres, and the spleen and thymus were weighed.

Apart from the effects observed when the highest-dose animals were receiving 2500 ppm in their diet, treatment did not affect body weight and feed or water consumption in mice at 100 and 500 ppm, and these parameters returned to, or were similar to, vehicle control values in the highest-dose mice when treated at 1500 ppm. No effects on absolute or relative spleen and thymus weights were noted. The mean anti-sRBC IgM values for the 100, 500 and 2500/1500 ppm groups were reduced in a dose-related manner, but with a small magnitude (Table 26). The magnitude of the changes and the extremely wide variation seen in these assays are not indicative of fluensulfone having significant immunotoxic potential.

Table 26. Anti-sRBC IgM titres in an immunotoxicity study in mice

	Mean ± standard error of duplicate samples				
	0 ppm	100 ppm	500 ppm	2 500/1 500 ppm	Positive control
Anti-sRBC IgM (U/mL)	1 526.9 ± 371.64	1 008.2 ± 214.27	1 036.9 ± 169.65	917.6 ± 154.52	167.4 ± 57.55**

IgM: immunoglobulin M; sRBC: sheep red blood cells; U: units; **: $P < 0.01$
Source: Struve (2011)

The NOAEL for systemic toxicity was 500 ppm (equal to 86 mg/kg bw per day), based on deaths and clinical signs at 2500 ppm. The NOAEL for immunotoxicity was 2500/1500 ppm (equal to a mean intake of 204 mg/kg bw per day), the highest dose tested (Struve, 2011).

(c) Investigation of possible modes of action leading to decreases in ALAT activity

A series of studies was performed to investigate possible modes of action leading to decreases in ALAT activity in serum and liver homogenates of animals exposed to fluensulfone. In a study with fluensulfone-treated dog plasma samples, the addition of excess pyridoxal-5′-phosphate had no significant effect on the measured activity of ALAT (Sagelsdorff, 2009), and measurements of pyridoxal-5′-phosphate in a 2-week dog study with fluensulfone (500 ppm) were unaltered, although ALAT activity was reduced by approximately 85% (Braun, 2011a). The addition of fluensulfone to 100 × g supernatants of dog liver homogenates did not produce any inhibition of measured ALAT activity. Although it would be expected that these homogenates would have some metabolic capability and levels of fluensulfone decreased during the assays, there were no investigations to confirm that the range of fluensulfone metabolites seen in vivo was produced (Sagelsdorff, 2010). There were no marked changes in ALAT messenger ribonucleic acid (mRNA) or protein expression in the livers of dogs exposed to fluensulfone (500 ppm) for 2 weeks (Braun, 2011a). Although these studies failed to identify the underlying cause, the evidence is indicating that direct inhibition of ALAT by fluensulfone itself, altered mRNA expression and an action involving pyridoxal-5′-phosphate do not appear to be involved. A paper by Fuentes-Almagro et al. (2012) showed that reactive molecules similar to the butene metabolites of fluensulfone could directly inhibit related enzymes.

ALAT enzyme depression subsequent to dietary administration of fluensulfone in laboratory animals was not considered to represent an adverse effect in humans (Gordon, 2011). The Meeting concluded that as the inhibition of ALAT was reversible and as alternative metabolic pathways are available, the reductions in hepatic ALAT activity seen in animals exposed to fluensulfone were not adverse.

(d) Lung tumour mechanistic studies

Comparative metabolic activity of human and mouse lung microsomes against fluensulfone

Lung microsomes were prepared from CD-1 (ICR) mice (12 of each sex) and from commercial material from 10 human non-smokers. Microsomes were analysed for CYP2E1 and Cyp2f2 enzyme activities against a standard substrate (chloroxazone) and the ability to metabolize fluensulfone (2 µmol/L). The results showed that the mouse microsomes were 300- to 500-fold more active than human microsomes against chloroxazone (Table 27). Mouse microsomes exhibited significant capability to metabolize fluensulfone, whereas human microsomes had negligible, if any, activity (Table 27). The microsomes from male mice had a specific activity similar to those from females. The use of a specific Cyp2f2 inhibitor (5-phenyl-1-pentyne) showed that the mouse-specific Cyp2f2 played a significant role in the metabolism of fluensulfone.

Table 27. Relative metabolic capability of mouse and human lung microsomes

	Male mouse microsomes	Female mouse microsomes	Human microsomes
Chloroxazone activity (pmol/mg per minute)	86.2	68.4	0.22
Fluensulfone remaining at 120 min (%); no inhibitor	2.1	4.6	102
Fluensulfone remaining at 120 min (%); + CYP2E1 inhibitor	6.2	8.7	94
Fluensulfone remaining at 120 min (%); + Cyp2f2 inhibitor	27	23	96

Source: Jaeger (2011)

Mouse lung cell proliferation assay

Groups of 10 female CD-1 mice received fluensulfone (purity 96.2%; batch 36372130-291-PF1) at 0 or 1200 ppm for 3 or 7 days or isoniazid (positive control) at 1305 ppm in the diet for 3 or 7 days. At 14 and 2 hours prior to termination, animals received an intraperitoneal injection of 5-bromo-2′-deoxyuridine (BrdU). After termination, lung (bronchiolar epithelial) cells were assessed for cell proliferation (BrdU-positive cells). With both isoniazid and fluensulfone, there was an approximate 5-fold increase in the numbers of BrdU-positive cells after 3 days of exposure, but after 7 days, the results were similar in control and test groups. This study demonstrates that exposure to fluensulfone induces an initial transient proliferation in bronchiolar epithelial cells, similar to that produced by isoniazid (Kaiser, 2011c).

(e) Toxicity studies on metabolites

Thiazole sulfonic acid (metabolite M-3625)

Thiazole sulfonic acid is of low acute oral toxicity (Table 28). It was not genotoxic in an Ames test and was negative for chromosomal aberrations in mammalian cells and in an in vivo micronucleus assay in rats (Table 29). Thiazole sulfonic acid is of lower acute oral toxicity than fluensulfone and is not genotoxic.

Table 28. Summary of acute toxicity data for fluensulfone metabolites

Species	Strain	Sex	Route	LD_{50} (mg/kg bw)	LC_{50} (mg/L)	Metabolite; purity; batch	Vehicle	Reference
Rat	HanRcc: Wistar	F	Oral	> 2 000 (OECD TG 423)	–	Thiazole sulfonic acid (M-3625) 93.6% 231PAL071	Water	Arcelin (2010a)
Rat	HanRcc: Wistar	F	Oral	300–2 000 (OECD TG 423)	–	Methyl sulfone (M-3626) 98.8% 231PAL052	Corn oil	Mallaum (2010)
Rat	HanRcc: Wistar	F	Oral	> 2 000 (OECD TG 423)	–	Butene sulfonic acid (M-3625) 99.5% 215PAL44	Water	Arcelin (2010b)

F: female; LC_{50}: median lethal concentration; LD_{50}: median lethal dose; OECD TG: Organisation for Economic Co-operation and Development Test Guideline

Methyl sulfone derivative (metabolite M-3626)

The methyl sulfone derivative is of moderate acute oral toxicity, with an LD_{50} of ≥ 300 mg/kg bw (Table 28). It was weakly mutagenic in an Ames test for test strain *Salmonella typhimurium* TA100 at the highest tested concentration in the absence of metabolic activation, was equivocal for gene mutation in mammalian cells in the presence and absence of metabolic activation and was negative in an in vivo micronucleus assay in rats and for unscheduled deoxyribonucleic acid (DNA) synthesis in rats (Table 29). The methyl sulfone derivative is of similar acute oral toxicity to fluensulfone and is not genotoxic in vivo.

Table 29. Results of studies of genotoxicity with fluensulfone metabolites

Test substance (vehicle)	End-point	Test object	Concentration	Purity; batch	Result	Reference
In vitro						
Thiazole sulfonic acid (M-3625) (water)	Reverse mutation	*Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537; *Escherichia coli* (WP2uvrA)	3–5 000 µg/plate, ±S9 mix	93.6% 231PAL071	Negative	Sokolowski (2010a)
Thiazole sulfonic acid (M-3625) (water)	Chromosomal aberration	Chinese hamster V79 cells	592, 1 185 or 2 370 µg/mL, ±S9 mix	93.6% 231PAL071	Negative	Hall (2010a)
Methyl sulfone (M-3626) (DMSO)	Reverse mutation	*S. typhimurium* strains TA98, TA100, TA1535, TA1537; *E. coli* (WP2uvrA)	3–5 000 µg/plate, ±S9 mix	98.8% 231PAL052	Weakly positive −S9 Negative +S9	Sokolowski (2010b)
Methyl sulfone (M-3626) (DMSO)	Reverse mutation (HPRT)	Chinese hamster V79 cells	3.8–175 µg/mL −S9 mix 37.5–1 000 µg/mL +S9 mix	98.8% 231PAL052	Equivocal ±S9	Hall (2011)
Butene sulfonic acid (M-3627) (water)	Reverse mutation	*S. typhimurium* strains TA98, TA100, TA1535, TA1537; *E. coli* (WP2uvrA)	3–5 000 µg/plate, ±S9 mix	99.5% 215PAL44	Negative	Sokolowski (2010c)
Butene sulfonic acid (M-3627) (water)	Chromosomal aberration	Chinese hamster V79 cells	533, 1 066 or 2 131 µg/mL, ±S9 mix	99.5% 215PAL44	Negative	Hall (2010b)
In vivo						
Thiazole sulfonic acid (M-3625) (water)	Bone marrow micronuclei	Wistar rats (M)	0, 500, 1 000 or 2 000 mg/kg bw (gavage) single	93.6% 231PAL071	Negative	Merker (2011a)
Methyl sulfone (M-3626) (corn oil)	Bone marrow micronuclei	Wistar rats (M)	0, 125, 250, 500 mg/kg bw (gavage) single	98.8% 231PAL052	Negative	Merker (2011b)
Methyl sulfone (M-3626) (corn oil)	Liver UDS	Wistar rats (M)	0, 250, 500 mg/kg bw (gavage) single	98.8% 231PAL052	Negative	Merker (2011c)
Butene sulfonic acid (M-3627) (water)	Bone marrow micronuclei	Wistar rats (M)	0, 500, 1 000 or 2 000 mg/kg bw (gavage) single	99.5% 215PAL44	Negative	Merker (2010)

DMSO: dimethyl sulfoxide; HPRT: hypoxanthine–guanine phosphoribosyltransferase; M: male; S9: 9000 × *g* supernatant fraction from rat liver homogenate; UDS: unscheduled DNA synthesis

Butene sulfonic acid (metabolite M-3627)

The butene sulfonic acid is of low acute oral toxicity (Table 28). It was not genotoxic in an Ames test and was negative for chromosomal aberrations in mammalian cells and in an in vivo micronucleus assay in rats (Table 29). The butene sulfonic acid metabolite is of lower acute oral toxicity than fluensulfone and is not genotoxic.

3. Observations in humans

Medical data on fluensulfone are limited, but no reports of adverse effects were identified during routine monitoring of production plant workers and among personnel involved in the experimental biological testing or field trials. There is no evidence or data available to support any findings in relation to poisoning with fluensulfone.

3.1 Medical data

(a) Report on medical surveillance on manufacturing plant personnel

A declaration of health record of industrial workers has been provided by Makhteshim Chemical Works, Beer Sheva, 84100 Israel. More than 20 workers were estimated to have been involved with the development of the synthesis and manufacturing of fluensulfone and formulations. Additional workers were exposed during the regulatory and field biological testing in Israel and elsewhere. Makhteshim, up to 25 December 2011, did not receive any reports of illness or adverse health effects attributed to exposures associated with the handling, testing or manufacturing of fluensulfone and formulations (Exponent, 2012).

Comments

Biochemical aspects

Absorption of fluensulfone administered by gavage at 5 mg/kg bw is rapid, with maximal plasma concentrations achieved within 4 hours. At 5 and 500 mg/kg bw, the extent of oral absorption is high (> 80%). Fluensulfone is widely distributed in the body. High concentrations of both butene- and thiazole-labelled material were found in the liver and kidney, and the butene-labelled material was also found at high concentrations in the lung. Thiazole-labelled material partitioned readily to red blood cells. Two hours after administration at both high and low doses, the thyroid had a high concentration of thiazole-labelled material relative to whole blood, although levels in thyroid were comparable to the whole blood concentrations by the subsequent time point (51 hours). The labelled material was rapidly excreted via urine (> 70%), with faecal excretion accounting for no more than 5–13%. A small proportion (< 5% of the administered dose at 5 mg/kg bw) was exhaled as carbon dioxide. Absorbed fluensulfone was extensively metabolized, with almost no unmetabolized parent compound detected. Other than low amounts of thiazole sulfonic acid, no other faecal metabolites were present at levels above 5% of the administered dose. The parent compound probably reacts with glutathione and cleaves, giving rise to thiazole mercapturate and butene sulfinic acid, the major urinary metabolites. The excretion pattern, tissue distribution of radioactivity and metabolite profile were essentially unaltered when the administration of thiazole-labelled substance was preceded by 14 days of administration of the unlabelled material.

Fluensulfone has a molecular weight of 272 Da and contains three fluorine atoms. If all the available fluorine is released, this means that a fluensulfone dose of 100 mg/kg bw could provide about 20 mg of free fluoride ion per kilogram body weight. Approximately 50% of the administered dose is excreted as fluoride-containing compounds.

Toxicological data

Fluensulfone was of moderate acute toxicity in rats via the oral route (LD_{50} = 671 mg/kg bw), but caused no mortality at limit doses after dermal (LD_{50} > 2000 mg/kg bw) or inhalation (LC_{50} > 5.1 mg/L air) exposure. Fluensulfone was slightly irritating to the skin of rabbits, but not irritating to the eyes of rabbits. It was a skin sensitizer in guinea-pigs.

In all species, the liver was a target organ, with increases in weight and hepatocellular hypertrophy. Investigations on liver enzyme activities in all species revealed that fluensulfone administration does not result in significant induction of cytochrome P450 subfamilies, but that the (conjugating) phase II enzymes were induced to a limited extent. Effects on kidney, observed in studies in rats conducted at high doses, were most prominent in males, but some renal effects were

observed in female rats treated at high doses and in dogs treated for 1 year. Reduced body weight gain, often associated with reductions in feed consumption, was a consistent, sensitive end-point. Fluorosis leading to discoloration of the teeth was reported only in the 90-day rat study at 2000 ppm (equal to 139 mg/kg bw per day), but there were no other overt signs of fluorosis. Determinations of fluoride content of bones and teeth showed significantly increased levels of total fluoride, even at low doses of fluensulfone. Examinations of tooth colour were performed and bones were examined histopathologically, but no specific investigations of bone density, thickness or bending resistance were performed. The Meeting concluded that the dietary intake of fluoride associated with the use of fluensulfone as a nematicide should be included in an overall assessment of fluoride intake from all sources. Upper levels for fluoride intake have been proposed by a number of organizations.

In mice, rats and dogs, decreases in ALAT activity in both plasma and liver were recorded. Mode of action investigations were performed in dogs, the most susceptible species for this effect. It was found that direct binding of fluensulfone itself did not cause the decrease in ALAT activity and that there was no significant interaction with the cofactor pyridoxal-5′-phosphate. mRNA levels appeared slightly induced, whereas protein expression appeared stable. No effect on the general health of the dogs accompanied the reduction of hepatic ALAT activity at low doses (50 ppm, equal to 1.5 mg/kg bw per day), even when the reduction was greater than 40%. The reduced ALAT activity was reversible, and alternative metabolic pathways are available. The Meeting concluded that a reduction in hepatic ALAT activity per se is not adverse.

In a 28-day study of toxicity in mice, dietary concentrations were 0, 100, 500 and 2000 ppm (equal to 0, 30, 101 and 375 mg/kg bw per day for males and 0, 41, 155 and 353 mg/kg bw per day for females, respectively). The NOAEL was 500 ppm (equal to 101 mg/kg bw per day), based on body weight loss, changes in erythrocyte parameters (e.g. increased reticulocytes) and liver toxicity (altered cytoplasmic structure, single-cell necrosis, bile duct hyperplasia) at 2000 ppm (equal to 353 mg/kg bw per day).

In a 90-day study of toxicity in mice, dietary concentrations were 0, 60, 300 and 1500 ppm (equal to 0, 11, 51 and 229 mg/kg bw per day for males and 0, 18, 68 and 252 mg/kg bw per day for females, respectively). The NOAEL was 60 ppm (equal to 11 mg/kg bw per day), on the basis of haematological findings in males and hepatocyte hypertrophy observed at 300 ppm (equal to 51 mg/kg bw per day).

In a 28-day study of toxicity in rats, dietary concentrations were 0, 125, 500 and 2000 ppm (equal to 0, 10, 43 and 152 mg/kg bw per day for males and 0, 12, 37 and 166 mg/kg bw per day for females, respectively). The NOAEL was 125 ppm (equal to 10 mg/kg bw per day), based on reductions in body weight gain and kidney lesions in male rats at 500 ppm (equal to 43 mg/kg bw per day).

In a 90-day study of toxicity in rats, dietary concentrations were 0, 60, 120, 500 and 2000 ppm (equal to 0, 4, 8, 35 and 139 mg/kg bw per day for males and 0, 5, 12, 53 and 149 mg/kg bw per day for females, respectively). The NOAEL was 120 ppm (equal to 8 mg/kg bw per day), on the basis of forestomach hyperplasia, increased triglycerides and increased water consumption in females and decreased body weight/body weight gains in males at 500 ppm (equal to 35 mg/kg bw per day).

In a 28-day dietary study in which dogs were administered fluensulfone at 0, 50, 200 or 900 ppm (equal to 0, 1.9, 7 or 31 mg/kg bw per day for males and 0, 2, 8 or 30 mg/kg bw per day for females, respectively), the NOAEL was 200 ppm (equal to 7 mg/kg bw per day), based on reductions in feed consumption and body weight gain and increases in liver and thyroid weights at 900 ppm (equal to 30 mg/kg bw per day). In a 90-day study in which dogs received fluensulfone in the diet at 0, 5, 50 or 500 ppm (equal to 0, 0.2, 1.7 and 17 mg/kg bw per day for males and 0, 0.2, 1.8 and 18 mg/kg bw per day for females, respectively), the NOAEL was 50 ppm (equal to 1.7 mg/kg bw per day), based on increases in reticulocytes and liver weights at 500 ppm (equal to 17 mg/kg bw per day). In a 1-year study in dogs in which fluensulfone was administered in the diet at 0, 5, 50, 100 or 500 ppm (equal to 0, 0.1, 1.5, 3 and 16 mg/kg bw per day, respectively), the NOAEL was 100 ppm (equal to 3 mg/kg bw per day), on the basis of changes in erythrocyte parameters, liver weight increases and increased pigment deposition in the liver at 500 ppm (equal to 16 mg/kg bw per day).

The overall NOAEL for the 90-day and 1-year studies was 100 ppm (equal to 3 mg/kg bw per day), and the overall LOAEL was 500 ppm (equal to 16 mg/kg bw per day).

In an 18-month toxicity and carcinogenicity study in mice, dietary concentrations were 0, 30, 200 and 1200 ppm (equal to 0, 4.2, 28 and 152 mg/kg bw per day for males and 0, 6.4, 39 and 188 mg/kg bw per day for females, respectively). The NOAEL was 30 ppm (equal to 4.2 mg/kg bw per day), based on decreased body weights and bronchiolization at 200 ppm (equal to 28 mg/kg bw per day). The NOAEL for tumours was 30 ppm (equal to 6.4 mg/kg bw per day), based on increased incidences of alveolar/bronchiolar adenomas and carcinomas in females receiving 200 ppm (equal to 39 mg/kg bw per day).

In a 2-year chronic toxicity and carcinogenicity study in rats, dietary concentrations were 0, 30, 200 and 1200 ppm (equal to 0, 1.4, 9.6 and 58 mg/kg bw per day for males and 0, 1.7, 12 and 69 mg/kg bw per day for females, respectively). Fluensulfone showed no carcinogenic potential in rats. The NOAEL for non-neoplastic effects was 30 ppm (equal to 1.4 mg/kg bw per day), on the basis of chronic interstitial inflammation in the lungs of females, oesophageal hyperkeratosis and decreased body weight gain in males at 200 ppm (equal to 9.6 mg/kg bw per day). Dose-related increases in plasma sodium levels were seen between weeks 13 and 52. The sodium levels in the fluensulfone-treated groups were within the control ranges in the study, and this finding was not consistent with the results of the 90-day rat study, in which plasma sodium levels were unaltered by treatment at week 13. The Meeting concluded that the increases in plasma sodium levels were not an adverse effect of fluensulfone administration.

The Meeting concluded that fluensulfone is carcinogenic in female mice but not male mice or rats.

An adequate battery of in vitro and in vivo mutagenicity studies has been performed with fluensulfone. Weak positive results were seen with one strain of *Salmonella typhimurium* in one Ames test but not in two other Ames tests using the same strains. An equivocal finding was noted at high concentrations in an assay for chromosome damage. No evidence of genotoxicity was seen in an adequately performed bone marrow micronucleus assay in mice.

The Meeting concluded that fluensulfone is unlikely to be genotoxic in vivo.

Mechanistic studies were carried out to determine the relevance for humans of the lung tumour findings in mice. Species-specific lung tumours in the mouse have been induced by a number of chemicals. The underlying cause is attributed to a high metabolic activity of the mouse lung, due to a relatively high abundance of Clara cells in the mouse compared with humans and the mouse-specific Cyp2f2 in the Clara cells. The compounds are activated to reactive intermediates, leading to local cytotoxicity that promotes sustained cell proliferation, leading finally to tumour formation. Rats have lower metabolic activity in the lungs compared with mice (below the threshold needed to cause lung tumours upon lifetime exposure), and this metabolic activity in humans is reported to be significantly lower than in rats. A limited package of data specific for fluensulfone showed it to be extensively metabolized by male and female mouse lung microsomes, whereas essentially no metabolic activity was seen in human lung microsomes. Mouse-specific Cyp2f2 was shown to be a significant contributor to fluensulfone's metabolism. Administration of fluensulfone to mice led to an early increase in bronchiolar epithelial cell proliferation; however, no equivalent data are available for human or rat lung preparations. Bronchiolization indicative of a chronic inflammatory response was noted in male as well as female mice. These data do not address the fact that no increases in lung tumours were seen in male mice.

Considering the submitted mode of action, the Meeting concluded that the work undertaken is not extensive enough to conclusively identify the mode of action or to entirely exclude human relevance. However, the mode of action for mouse lung tumours in female mice administered fluensulfone is likely to be non-genotoxic and threshold dependent.

The Meeting concluded that fluensulfone is unlikely to be genotoxic in vivo and that there is a clear threshold for lung tumours in female mice. Therefore, fluensulfone is unlikely to pose a carcinogenic risk to humans from the diet.

In a two-generation study of reproductive toxicity in rats, dietary concentrations were 0, 30, 250 and 1800 ppm (equal to mean intakes of 0, 2.0, 16 and 122 mg/kg bw per day for males and 0, 2.8, 23 and 169 mg/kg bw per day for females, respectively). The NOAEL for reproductive effects was 1800 ppm (equal to 122 mg/kg bw per day), the highest dose tested. The parental NOAEL was 250 ppm (equal to 16 mg/kg bw per day), based on effects on body weight throughout the study in males and increased liver and kidney weights at 1800 ppm (equal to 122 mg/kg bw per day). The NOAEL for effects on offspring was 250 ppm (equal to 16 mg/kg bw per day), based on reduced pup weight at 1800 ppm (equal to 122 mg/kg bw per day).

In a study of developmental toxicity in rats dosed at 0, 7.7, 49 or 292 mg/kg bw per day, there was no evidence of teratogenicity. The Meeting noted a decrease in the incidence of some variations of the skull bones but considered that this was not toxicologically relevant. The NOAEL for maternal toxicity was 49 mg/kg bw per day, on the basis of decreased body weight gain and clinical signs at 292 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 49 mg/kg bw per day, based on four non-viable fetuses at 292 mg/kg bw per day.

In a study of developmental toxicity in rabbits dosed at 0, 2.5, 10 or 40 mg/kg bw per day, the NOAEL for maternal toxicity was 10 mg/kg bw per day, based on body weight loss at 40 mg/kg bw per day, and the NOAEL for embryo and fetal toxicity was 40 mg/kg bw per day, the highest dose tested.

The Meeting concluded that fluensulfone is not teratogenic in rats or rabbits.

The acute neurotoxicity of fluensulfone was investigated in rats at dose levels of 0, 100, 400 and 1200 mg/kg bw. Clinical signs and reduced activity in a functional observational battery on day 1, but not subsequently, were seen at all dose levels, with females being more sensitive than males. Benchmark dose modelling gave reliable $BMDL_{SD}$ values in the range 19–33 mg/kg bw for females, and the Meeting identified 25 mg/kg bw as a reference point or POD. There were no indications of neuropathy.

In a subchronic (90-day) neurotoxicity study in rats, dietary concentrations were 0, 100, 500 and 2500 ppm (equal to 0, 6, 31 and 153 mg/kg bw per day for males and 0, 7, 34 and 162 mg/kg bw per day for females, respectively). The NOAEL for neurotoxicity was 2500 ppm (equal to 153 mg/kg bw per day), the highest dose tested, and the NOAEL for general toxicity was 500 ppm (equal to 31 mg/kg bw per day), based on reduced body weight gain in males at 2500 ppm (equal to 153 mg/kg bw per day).

In a 28-day immunotoxicity study in female mice, dietary concentrations were 0, 100, 500 and 2500/1500 ppm (equal to 0, 17, 86 and 204 mg/kg bw per day). There were no significant effects on anti-sRBC IgM titres at any dose level. The NOAEL for general toxicity was 500 ppm (equal to 86 mg/kg bw per day), based on deaths and clinical signs at 2500 ppm. Fluensulfone did not affect splenic or bone marrow cell counts or IgA, IgG or IgM titres in rats exposed to concentrations up to 2000 ppm (equal to 152 mg/kg bw per day) in the diet in the 28-day study of toxicity in rats.

Toxicological data on metabolites and/or degradates

Acute oral toxicity and genotoxicity studies were performed on the following metabolites: thiazole sulfonic acid (M-3625), methyl sulfone derivative (M-3626) and butene sulfonic acid (M-3627). Thiazole sulfonic acid and butene sulfonic acid were of low acute oral toxicity (LD_{50} > 2000 mg/kg bw) and were not genotoxic in vitro or in vivo. The methyl sulfone derivative had an acute oral LD_{50} of ≥ 300 mg/kg bw. It was weakly positive in the Ames test for test strain *S. typhimurium* TA100 at the highest concentration tested (5000 μg/plate) in the absence of metabolic activation and equivocal in a reverse mutation assay in Chinese hamster cells. Two in vivo genotoxicity studies, for bone marrow micronuclei and liver unscheduled DNA synthesis, were negative.

Human data

No adverse effects have been reported in a group of over 20 individuals involved in the manufacturing, handling and testing of fluensulfone.

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

Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) for fluensulfone of 0–0.01 mg/kg bw on the basis of the NOAEL of 1.4 mg/kg bw per day for chronic interstitial inflammation in the lungs, oesophageal hyperkeratosis and decreased body weight from the rat chronic toxicity and carcinogenicity study. A safety factor of 100 was applied. This provides a margin of exposure of 3900 between the upper bound of the ADI and the LOAEL for tumours in female mice.

The Meeting established an acute reference dose (ARfD) for fluensulfone of 0.3 mg/kg bw, on the basis of the POD of 25 mg/kg bw for changes in the functional observational battery in the acute neurotoxicity study in rats. A safety factor of 100 was applied.

Levels relevant to risk assessment of fluensulfone

Species	Study	Effect	NOAEL	LOAEL
Mouse	Ninety-day study of toxicity[a]	Toxicity	60 ppm, equal to 11 mg/kg bw per day	300 ppm, equal to 51 mg/kg bw per day
	Eighteen-month study of toxicity and carcinogenicity[a]	Toxicity	30 ppm, equal to 4.2 mg/kg bw per day (M)	200 ppm, equal to 28 mg/kg bw per day (M)
		Carcinogenicity	30 ppm, equal to 6.4 mg/kg bw per day (F)	200 ppm, equal to 39 mg/kg bw per day (F)
Rat	Acute neurotoxicity study[b]	Neurotoxicity	25 mg/kg bw[c]	100 mg/kg bw[d]
	Ninety-day study of toxicity[a]	Toxicity	120 ppm, equal to 8 mg/kg bw per day	500 ppm, equal to 35 mg/kg bw per day
	Two-year study of toxicity and carcinogenicity[a]	Toxicity	30 ppm, equal to 1.4 mg/kg bw per day	200 ppm, equal to 9.6 mg/kg bw per day
		Carcinogenicity	1 200 ppm, equal to 69 mg/kg bw per day[e]	–
	Multigeneration reproductive toxicity study[a]	Reproductive toxicity	1 800 ppm, equal to 122 mg/kg bw per day[e]	–
		Parental toxicity	250 ppm, equal to 16 mg/kg bw per day	1 800 ppm, equal to 122 mg/kg bw per day
		Offspring toxicity	250 ppm, equal to 16 mg/kg bw per day	1 800 ppm, equal to 122 mg/kg bw per day
	Developmental toxicity study[b]	Maternal toxicity	49 mg/kg bw per day	292 mg/kg bw per day
		Embryo and fetal toxicity	49 mg/kg bw per day	292 mg/kg bw per day
Rabbit	Developmental toxicity study[b]	Maternal toxicity	10 mg/kg bw per day	40 mg/kg bw per day
		Embryo and fetal toxicity	40 mg/kg bw per day[e]	–
Dog	Ninety-day and 1-year studies of toxicity[a,f]	Toxicity	100 ppm, equal to 3 mg/kg bw per day	500 ppm, equal to 16 mg/kg bw per day

$BMDL_{SD}$: 95% lower confidence limit on the benchmark dose for a 1 standard deviation response; F: female; LOAEL: lowest-observed-adverse-effect level; M: male; NOAEL: no-observed-adverse-effect level; POD: point of departure

[a] Dietary administration.
[b] Gavage administration.
[c] POD based on $BMDL_{SD}$ values of 19–33 mg/kg bw in females.
[d] Lowest dose tested.
[e] Highest dose tested.
[f] Two or more studies combined.

Estimate of acceptable daily intake

0–0.01 mg/kg bw

Estimate of acute reference dose

0.3 mg/kg bw

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

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

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

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid, plasma T_{max} is 4 h; > 80%
Dermal absorption	No data
Distribution	Widely distributed; highest concentrations in liver, kidney, red blood cells and thyroid
Potential for accumulation	Potential accumulation in erythrocytes
Rate and extent of excretion	Largely cleared within 48 h at low dose; primarily via urine (> 70%) and, to a lesser extent, faeces (5–13%)
Metabolism in animals	Extensive; mainly by cleavage to yield the thiazole glutathione conjugate and butene sulfinic acid, which are further metabolized
Toxicologically significant compounds in animals, plants and the environment	Fluensulfone; fluoride ion
Acute toxicity	
Rat, LD_{50}, oral	671 mg/kg bw
Rat, LD_{50}, dermal	> 2 000 mg/kg bw
Rat, LC_{50}, inhalation	> 5.1 mg/L
Rabbit, dermal irritation	Slightly irritating
Rabbit, ocular irritation	Not irritating
Dermal sensitization	Sensitizing (guinea-pig maximization test)
Short-term studies of toxicity	
Target/critical effect	Body weight gain, haematology; liver and kidney weights
Lowest relevant oral NOAEL	3 mg/kg bw per day (dog)
Lowest relevant dermal NOAEL	No data
Lowest relevant inhalation NOAEC	No data

Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Lung, oesophagus, body weight
Lowest relevant NOAEL	1.4 mg/kg bw per day (rat)
Carcinogenicity	Unlikely to be carcinogenic to humans from the diet
Genotoxicity	
	Not genotoxic in vivo
Reproductive toxicity	
Target/critical effect	Lower pup weight at parentally toxic dose
Lowest relevant parental NOAEL	16 mg/kg bw per day
Lowest relevant offspring NOAEL	16 mg/kg bw per day
Lowest relevant reproductive NOAEL	122 mg/kg bw per day, the highest dose tested
Developmental toxicity	
Target/critical effect	Pup viability at maternally toxic dose (rat)
Lowest relevant maternal NOAEL	10 mg/kg bw per day (rabbit)
Lowest relevant embryo/fetal NOAEL	40 mg/kg bw per day, the highest dose tested (rabbit); 49 mg/kg bw per day (rat)
Neurotoxicity	
Acute neurotoxicity NOAEL	POD 25 mg/kg bw (based on $BMDL_{SD}$) (rat)
Subchronic neurotoxicity NOAEL	153 mg/kg bw per day, the highest dose tested (rat)
Other toxicological studies	
Immunotoxicity	Not immunotoxic (mouse and rat)
Studies on metabolites	Thiazole sulfonic acid (M-3625): Oral LD_{50} > 2 000 mg/kg bw; not genotoxic Methyl sulfone derivative (M-3626): Oral LD_{50} ≥ 300 mg/kg bw; not genotoxic in vivo Butene sulfonic acid (M-3627): Oral LD_{50} > 2 000 mg/kg bw; not genotoxic
Medical data	
	No adverse effects reported

$BMDL_{SD}$: lower 95% confidence limit on the benchmark dose for a 1 standard deviation response; LC_{50}: median lethal concentration; LD_{50}: median lethal dose; NOAEC: no-observed-adverse-effect concentration; NOAEL: no-observed-adverse-effect level; POD: point of departure: T_{max}: time to reach the peak concentration in plasma

Summary

	Value	Study	Safety factor
ADI	0–0.01 mg/kg bw	Two-year toxicity/carcinogenicity study in rats	100
ARfD	0.3 mg/kg bw	Acute neurotoxicity study in rats	100

ADI: acceptable daily intake; ARfD: acute reference dose

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IMAZAPIC

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Explanation

Imazapic is the International Organization for Standardization–approved name for (*RS*)-2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)-5-methylnicotinic acid (International Union of Pure and Applied Chemistry), with Chemical Abstracts Service No. 104098-48-8. It is a new herbicide that belongs to the imidazolinone family. The proposed mode of action is specific to plants and involves the disruption of protein synthesis via the inhibition of acetohydroxyacid synthase, an enzyme not found in mammalian tissues.

Imazapic 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 daily intake

1. Biochemical aspects

The absorption, distribution, metabolism and excretion, as well as the toxicokinetics, of imazapic have been investigated in Crl: CD®BR rats. Summaries of the relevant data are presented below.

1.1 Absorption, distribution and excretion

The absorption, distribution, metabolism and excretion of imazapic in Sprague-Dawley rats were investigated using the active substance radiolabelled in the pyridine ring. The test item was a mixture of ^{14}C-labelled imazapic (pyridine label; Fig. 1) and unlabelled imazapic. The data generated with imazapic (including intravenous dosing) indicated that it is rapidly and extensively absorbed, with almost complete bioavailability (Cheng, 1993). The study design is summarized in Table 1.

Fig. 1. Radiolabelled form of impazapic used in absorption, distribution, metabolism and excretion studies: structure and position of label

* Position of radiolabel

Source: Cheng (1993)

Table 1. Dosing groups for balance/excretion experiments with [^{14}C]imazapic

Test group	Dose of labelled material (mg/kg bw)	Number of rats of each sex	Remarks
Preliminary	10	2	Expired $^{14}CO_2$, organic volatiles, urine and faeces were collected at 0–12 and 12–24 h post-dosing and daily thereafter for 7 days post-dosing. Expired $^{14}CO_2$ was trapped in a 1 : 1 mixture of ethanolamine and ethoxyethanol. Expired organic volatiles were collected in activated charcoal.
Single oral low dose	10	5	Urinary and faecal samples were collected at 0–6, 6–12 and 12–24 h post-dosing and every 24 h thereafter through 7 days post-exposure. Urine and faeces were collected from the carrier control group at 0–24 h post-dosing only. Cage washings and wipes were also collected after the final excreta collection. Tissues and carcass were collected on day 7 post-dosing after the animals were terminated. The tissues that were removed included bone (femur), brain, fat (reproductive area), testes, ovaries, heart, kidneys, liver, lungs, muscle (thigh), spleen, uterus and residual carcass. Blood was collected by cardiac puncture.
Single oral high dose	1 000	5	
Repeat oral low dose	10 (14 × ^{12}C, 1 × ^{14}C)	5	
Single intravenous low dose	10	5	
Control carrier	–	1	

bw: body weight
Source: Cheng (1993)

Radiolabelled imazapic was administered via oral gavage in a water/ammonium hydroxide vehicle.

Total radioactive recovery at 168 hours ranged from 97.5% to 106% of the administered dose, with the main route of excretion being the urine for all exposures (Table 2). Urinary excretion was primarily completed in the first 6 hours post-exposure, with recovery of 76.8–97.2% of the administered radioactivity (combined urine and cage wash). At the end of 168 hours, between 94% and 102% of the administered radioactivity was recovered in the urine. Faecal excretion comprised 1.95–3.5% of the administered radioactivity in oral groups and 0.59–0.79% in the intravenous group, indicating little in the way of biliary excretion. Faecal excretion peaked between 6 and 24 hours. Excretion was nearly complete, with trace amounts to 0.43% of the administered dose recovered in the carcass.

Table 2. Excretion balance in rats at 168 hours post-dosing

Route; time (h)	% of administered radioactivity							
	Single oral low dose (10 mg/kg bw)		Single i.v. low dose (10 mg/kg bw)		Repeat oral low dose (10 mg/kg bw per day)		Single oral high dose (1 000 mg/kg bw)	
	Males	Females	Males	Females	Males	Females	Males	Females
Urine 0–6	82.3	62.6	85.4	85.7	80.6	61.7	71.0	68.2
Cage wash	12.5	20.1	9.31	8.53	16.6	24.2	5.83	12.2
Urine 6–12	3.86	14.9	1.65	1.47	2.06	2.84	14.1	14.2
Cage wash	0.73	1.90	0.27	0.66	0.93	1.62	0.89	1.35
Urine 12–24	0.36	0.72	0.31	0.67	0.68	1.73	1.29	1.35
Urine 24–48	0.10	0.29	0.08	0.22	0.24	0.54	0.21	0.28
Urine 48–72	0.04	0.19	0.05	0.23	0.09	0.27	0.13	0.19
Urine 72–96	0.03	0.15	0.04	0.12	0.03	0.18	0.07	0.13
Urine 96–120	0.03	0.10	0.03	0.07	0.03	0.16	0.05	0.06
Urine 120–144	0.02	0.09	0.02	0.06	0.02	0.12	0.04	0.06
Urine 144–168	0.03	0.06	0.02	0.11	0.01	0.09	0.04	0.06
Cage wash	0.28	0.40	0.10	0.57	0.33	0.68	0.26	0.27
Cage wipe	0.09	0.28	0.03	0.17	0.15	0.42	0.07	0.13
Subtotal urine	**100.00**	**102.00**	**97.30**	**98.60**	**102.00**	**94.50**	**94.00**	**98.50**
Faeces 0–6	< 0.01	< 0.01	0.01	< 0.01	0.16	0.03	0.01	< 0.01
Faeces 6–12	1.10	1.01	0.29	0.13	1.43	0.93	0.78	1.27
Faeces 12–24	1.09	0.89	0.45	0.41	1.35	2.03	2.40	2.11
Faeces 24–48	0.12	0.06	0.03	0.04	0.13	0.37	0.23	0.12
Faeces 48–72	n.d.	n.d.	< 0.01	< 0.01	< 0.01	0.02	0.01	< 0.01

Table 2 (continued)

Route; time (h)	% of administered radioactivity							
	Single oral low dose (10 mg/kg bw)		Single i.v. low dose (10 mg/kg bw)		Repeat oral low dose (10 mg/kg bw per day)		Single oral high dose (1 000 mg/kg bw)	
	Males	Females	Males	Females	Males	Females	Males	Females
Faeces 72–96	n.d.	n.d.	n.d.	< 0.01	n.d.	< 0.01	< 0.01	< 0.01
Faeces 96–120	n.d.	n.d.	< 0.01	n.d.	n.d.	n.d.	< 0.01	< 0.01
Faeces 120–144	n.d.	n.d.	n.d.	n.d	n.d.	n.d.	< 0.01	< 0.01
Faeces 144–168	n.d.	n.d.	n.d	< 0.01	< 0.01	n.d.	< 0.01	< 0.01
Subtotal faeces	**2.31**	**1.95**	**0.79**	**0.59**	**3.07**	**3.39**	**3.44**	**3.50**
Tissues	n.d	n.d	n.d.	n.d.	n.d.	n.d.	< 0.01	< 0.01
Carcass	< 0.01	0.08	0.02	0.07	< 0.01	0.43	0.12	0.13
Total	**103.0**	**104.0**	**98.10**	**99.20**	**106.0**	**98.30**	**97.50**	**102.10**

bw: body weight; i.v.: intravenous; n.d.: not detected
Source: Cheng (1993)

Following termination at 168 hours, the carcass and select tissues of the animals in the above study were examined. In animals given the low or repeated doses, there was no residual radioactivity other than small to trace amounts in the carcass (Table 2). In the animals given imazapic at 1000 mg/kg body weight (bw), small amounts of residual radioactivity were found in the residual carcass, blood, liver and bone of males and females and in the kidneys and fat of females.

1.2 Biotransformation

The urinary and faecal samples from the above study were subjected to high-performance liquid chromatographic analysis to determine the metabolic fate of imazapic. The vast majority of imazapic was excreted as unchanged parent via either the urine or faeces. None of the metabolites produced from oxidation, reduction and hydrolysis (including the metabolites CL 263,284, CL 280,442 and several other unidentified metabolites) accounted for more than about 1.3% of the administered dose in the urine and faeces (Tables 3 and 4).

The proposed metabolic pathway in the rat is shown in Fig. 2.

2. Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

The results of acute toxicity studies with imazapic administered orally, dermally or by inhalation are summarized in Table 5. Imazapic is of low acute toxicity in rats via the oral and dermal routes. The acute inhalation toxicity study was difficult to interpret, because the mass median aerodynamic diameter values were too high (6.47 ± 0.21 µm at 3.67 mg/L and 8.28 ± 0.85 µm at 4.83 mg/L). There were no clinical signs or gross necropsy changes in the acute oral or dermal studies. In the inhalation study, eye clenching and genital wetting were observed at 3.67 mg/L, and nasal discharge and genital wetting were observed at 4.83 mg/L. Lung haemorrhages were noted in males at both concentrations at necropsy (Lowe & Fischer, 1992, 1993a; Hershman, 1993).

Table 3. Metabolites identified in rat excreta as percentage of radioactivity in sample

Compound	% of recovered dose											
	Single oral low dose 10 mg/kg bw Urine: 0–6 h		Single i.v. low dose 10 mg/kg bw Urine: 0–6 h		Repeat oral low dose 10 mg/kg bw Urine: 0–6 h		Single oral high dose 1 000 mg/kg bw					
							Urine: 0–6 h		Urine: 6–12 h		Faeces: 12–24 h	
	M	F	M	F	M	F	M	F	M	F	M	F
Parent	93.5	105	96.8	98.9	97.8	108	94.5	102	101	95.6	66.0	65.9
CL 263,284	n.d.	n.d.	n.d.	n.d.	0.225	n.d.	n.d.	n.d.	n.d.	0.249	1.96	0.854
CL 312,622	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.373	0.199
CL 290,610	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.686	0.704
CL 303,459	n.d.	n.d.	n.d.	n.d.	n.d.	0.107	n.d.	n.d.	n.d.	0.576	4.29	5.38
CL 280,422	n.d.	0.098	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Unknown 1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.210	n.d.
Unknown 2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.266	n.d.
Unknown 3	n.d.	n.d.	n.d.	n.d.	0.111	n.d.	n.d.	n.d.	0.257	n.d.	n.d.	n.d.
Unknown 4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.249	0.373	0.451
Unknown 5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.22	1.26
Unknown 6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.215	n.d.	n.d.	0.398
Unknown 7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.266	n.d.
Unknown 8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.317	n.d.
Unknown 9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.115	n.d.	0.222	1.86	1.58
Unknown 10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.75	0.752
Unknown 11	n.d.	n.d.	n.d.	n.d.	n.d.	0.160	n.d.	n.d.	0.129	0.144	1.59	1.16
Unknown 12	0.480	0.759	0.548	0.555	0.168	0.539	n.d.	n.d.	0.408	1.31	0.424	1.56
Unknown 13	n.d.	0.098	n.d.	n.d.	n.d.	0.144	n.d.	n.d.	n.d.	0.51	1.15	0.757
Unknown 14	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.553
Unknown 15	n.d.	n.d.	0.196	0.226	n.d.	n.d.	n.d.	n.d.	0.108	0.117	n.d.	n.d.
Unknown 16	0.180	n.d.	n.d.	0.226	n.d.	n.d.	0.283	0.408	0.408	0.393	1.75	1.26
Unknown 17	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.210	0.199
Total identified	**94.3**	**106**	**97.5**	**99.9**	**98.3**	**109**	**94.8**	**103**	**103**	**99.4**	**84.7**	**83.0**

bw: body weight; F: females; i.v.: intravenous; M: males; n.d.: not detected, set at < 0.1% by the sponsor
Source: Cheng (1993)

(b) Ocular irritation

In an eye irritation study, 0.1 g imazapic (purity 96.9%; batch AC 7591-51A) was applied into the conjunctival sac of the left eye of six male New Zealand White rabbits for 4 hours. The eyes were washed with tap water at that time point. Animals were then observed for 3 or 4 days. Irritation was scored by the method of Draize. Corneal opacities were observed in 2/6 animals, but cleared by 72 hours. Maximal conjunctival redness, chemosis and discharge were observed in rabbits from 1 to 24 hours, with conjunctival redness persisting in 3/6 rabbits until 72 hours. In this study, imazapic was mildly irritating to the eye based on the maximum average score of 3.7/110 and persistence at the 72-hour reading (Lowe & Hess, 1995).

Table 4. Chemical structures for identified metabolites

Metabolite designation (code)	Retention time (min)	Structure	Urine	Faeces
Imazapic = CL 263,222	21.8	CL 263,222	+	+
CL 301,414	13.5		–	–
CL 312,622	18.0		–	+
CL 290,610	19.8		–	+
CL 263,284	21.8		+	+
CL 303,459	25.1		+	+
CL 280,442	26.7		+	–

Source: Cheng (1993)

Fig. 2. Proposed metabolic pathway of imazapic in rat

CL 263,222

Oxidation

Ring closure reduction

CL 263,284

CL 280,442

Hydrolysis

CL 303,459

* Proposed intermediate.

Hydrolysis

CL 290,610

Source: Cheng (1993)

Six adult female New Zealand White rabbits were exposed to 0.1 g imazapic (purity 96.9%; batch AC 7591-51A) applied into the conjunctival sac of the left eye. The eye was rinsed with tap water 24 hours after application, and ocular lesions were scored at 1, 24, 48 and 72 hours. At 1 hour post-application, there were no signs of corneal irritation or iritis; however, slight to moderate redness and/or chemosis and moderate to copious discharge were noted in all animals. At 24 hours, 2/6 animals exhibited mild corneal opacity and mild to marked conjunctivitis (Lowe & Fischer, 1993c).

Table 5. Acute toxicity of impazapic

Species	Strain	Sex	Route	Purity (%)	Result	Reference
Rat	CrlCD(SD)BR (Sprague-Dawley derived)	Male and female	Oral	96.9	LD_{50} > 5 000 mg/kg bw	Lowe & Fischer (1992)
Rabbit	New Zealand White	Male and female	Dermal	96.9	LD_{50} > 2 000 mg/kg bw	Lowe & Fischer (1993a)
Rat	Outbred Sprague-Dawley	Male and female	Inhalation	93.7	LC_{50} > 4.83 mg/L (26–33% of particles < 4.7 µm)	Hershman (1993)

LC_{50}: median lethal concentration; LD_{50}: median lethal dose

(c) Dermal irritation

In a dermal irritation study, six adult male New Zealand White rabbits were exposed to 0.5 g imazapic (purity 96.9%; batch AC 7591-51A) for 4 hours to 6.5 cm^2 of body surface area with occlusion. Following exposure, the test site was washed with tap water, and the animals were observed for 3 days. At 1 hour, 2/6 animals exhibited very slight erythema, which cleared by 24 hours. No oedema was observed (Lowe & Fischer, 1993b).

(d) Dermal sensitization

The skin sensitization potential of imazapic (purity 93.7%; lot no. AC 5270-11) was investigated using the method of Buehler. Ten male Hartley albino guinea-pigs (test group) were subjected to an induction phase of nine applications of 0.43 g of imazapic in 0.43 g saline to a 6.5 cm^2 clipped test site for 6 hours, with occlusion, over 3 weeks. Animals were observed for 2 weeks, and then a challenge application was applied to a naive test site for a contact period of 6 hours. A concurrent positive control group of 10 males was subjected to the same procedure using 0.1% (weight per volume [w/v]) 1-chloro-2,4-dinitrobenzene suspension in 50% ethanol : 0.9% saline solution. A naive control group of 10 males was retained under conditions similar to those of the test and positive control groups, but was not subjected to the induction phase. Irritation during the induction phase and following the challenge application was graded according to Draize.

No irritation was noted in the test animals during the induction phase, and no irritation was noted in the test or naive control group following the challenge application. The response in the positive control animals validated the test. It was concluded that imazapic does not have a sensitizing effect on the skin in the Buehler test (Costello, 1992).

The above ocular and dermal irritation and dermal sensitization studies are summarized in Table 6.

2.2 Short-term studies of toxicity

(a) Oral administration

Rats

In a 13-week study, groups of 20 Sprague-Dawley rats of each sex per dose received imazapic (purity 93.7%) in the diet at a dose level of 0, 5000, 10 000 or 20 000 parts per million (ppm) (equal to 0, 386, 760 and 1522 mg/kg bw per day for males and 0, 429, 848 and 1728 mg/kg bw per day for females, 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. Clinical chemistry, haematology and urine analysis parameters were

Table 6. Irritation and skin sensitization potential of impazapic

Species	Strain	Sex	End-point (method)	Purity (%)	Result	Reference
Rabbit	New Zealand White	Male	Skin irritation	96.9	Non-irritating	Lowe & Fischer (1993b)
Rabbit	New Zealand White	Male	Eye irritation	96.9	Mildly irritating	Lowe & Hess (1995)
Rabbit	New Zealand White	Female	Eye irritation	96.9	Minimally irritating	Lowe & Fischer (1993a)
Guinea-pig	Hartley	Male	Skin sensitization (Buehler)	93.7	Not sensitizing	Costello (1992)

measured at the termination of the study. At necropsy, the weights of selected organs were recorded, assessed by gross examination and examined histopathologically.

There were no treatment-related effects observed on mortality, clinical signs of toxicity, body weight, feed consumption, haematological parameters tested, clinical chemistry parameters tested, organ weights and/or gross pathological and histopathological parameters. In this study, ophthalmoscopy, neurotoxicity battery, blood clotting potential, cholesterol and urea measurements were not conducted, and the epididymides and thymus were not weighed.

The no-observed-adverse-effect level (NOAEL) was 20 000 ppm (equal to 1522 mg/kg bw per day), the highest dose tested (Fischer, 1992).

Dogs

In a range-finding oral toxicity study, three Beagle dogs of each sex per dose were administered imazapic (purity 98.0%) in the diet for 14 days at a concentration of 0, 5000, 10 000, 20 000 or 40 000 ppm (equivalent to approximately 0, 375, 750, 1500 and 3000 mg/kg bw per day). The animals were observed for clinical signs of toxicity, mortality and moribundity daily, feed consumption was measured daily and body weights were assessed weekly. Clinical chemistry and haematological parameters were measured prior to the initiation of dosing and after 14 days of treatment. Select organ weights were recorded at necropsy, along with gross observations. No histopathological examination was performed.

There was no effect on mortality, clinical signs of toxicity, body weight, clinical chemistry, organ weights or changes at gross necropsy. Feed consumption was decreased compared with controls at 40 000 ppm in females; however, as there was no adverse effect on body weight, the change was not considered adverse.

The NOAEL in this range-finding study was 40 000 ppm (equivalent to 3000 mg/kg bw per day), the highest dose tested (Balmer, 1987).

In a short-term oral toxicity study, six Beagle dogs of each sex per dose were administered imazapic (purity 96.9%) in the diet for 1 year at a concentration of 0, 5000, 20 000 or 40 000 ppm (equal to 0, 137, 501 and 1141 mg/kg bw per day for males and 0, 180, 534 and 1092 mg/kg bw per day for females, respectively). The animals were subjected to examinations 3 times per day for gross clinical signs, morbidity and moribundity, as well as weekly detailed clinical observations, weekly recording of body weight and daily recording of feed consumption. Ophthalmoscopic evaluations were performed at 3, 6 and 12 months. Blood samples for haematology and plasma clinical chemistry were withdrawn and urine samples were collected from all animals predosing and at approximately months 1, 3, 6 and 12. Water intake was measured on days 94–96, 176–178 and 351–353. Urine volume was measured on days 36–38, 94–96, 176–178 and 351–353. The animals were terminated on

days 362–366 and subjected to necropsy and postmortem examination of major organs and tissues. Organs were weighed, and a full range of tissues was preserved, processed and examined by light microscopy. At the request of the sponsor, skeletal muscle slides from all animals were sent to a consultant pathologist, and comments were integrated into the study report. No separate report was available. The haematological data were reviewed by an independent consultant. A measure of blood clotting potential was not taken, and the spleen, epididymides, uterus and thyroid were not weighed.

Clinical signs of toxicity were limited to increased salivation at doses of 20 000 ppm and above and increased emesis at 40 000 ppm (Table 7). The first incidences of salivation occurred on days 51 and 65 in males, at 20 000 and 40 000 ppm, respectively, and on days 156 and 134 in females, at 20 000 and 40 000 ppm, respectively. Feed emesis started as early as day 6 in high-dose females and as late as day 358 in control males. Frothy emesis was noted starting at day 13 in females at 5000 ppm to as late as day 243 in control females (Table 7).

Table 7. Clinical observations in dogs administered imazapic for approximately 12 months

Observation	Males				Females			
	0 ppm	5 000 ppm	20 000 ppm	40 000 ppm	0 ppm	5 000 ppm	20 000 ppm	40 000 ppm
Salivation								
- Animals affected	1/6	1/6	3/6	5/6	1/6	0/6	3/6	6/6
- Total observations	1	15	37	203	1	0	3	177
- Range of first incidence	D149	D22	D51–156	D65–148	D259	–	D156–334	D134–156
Emesis: Feed								
- Animals affected	4/6	4/6	5/6	6/6	4/6	5/6	4/6	6/6
- Total observations	12	18	9	134	16	34	19	214
- Range of first incidence	D18–358	D19–282	D15–338	D35–266	D17–320	D45–353	D37–94	D6–198
Emesis: Other (white, yellow, foamy)								
- Animals affected	4/6	3/6	3/6	5/6	6/6	5/6	5/6	6/6
- Total observations	7	6	9	87	15	25	7	80
- Range of first incidence	D36–238	D21–307	D40–311	D36–202	D34–243	D13–160	D37–279	D28–64

D: days
Source: Wolford (1993)

Body weight and body weight gain were decreased in males and females at 40 000 ppm, and feed consumption was decreased at doses of 20 000 ppm and higher in females and at 40 000 ppm in males (Tables 8 and 9). Males and females exhibited discoloration of the lungs at 40 000 ppm (Table 10). There was a non-adverse increase in urinary pH in both sexes at 40 000 ppm.

Changes to the haematopoietic system consisted of decreased haematocrit and haemoglobin and increased anisokaryocytes, hypochromic cells and polychromic cells in males and females; decreased mean cell volume (MCV) and mean cell haemoglobin (MCH), increased target cells and macrocytes and increased erythropoiesis in the spleen and bone marrow in males; and decreased red blood cells in females at doses of 20 000 ppm and above. At 40 000 ppm, males and females exhibited decreased mean cell haemoglobin concentrations (MCHC), increased poikilocytes, increased dark, red, gelatinous bone marrow in the femur and bone marrow congestion; males exhibited decreased red blood cells; and females exhibited decreased MCV, MCH and target cells and increased erythropoiesis in the spleen and bone marrow (Tables 10 and 11). These changes were confirmed by the haematological consultant, who concluded that they were consistent with inhibition of one or more enzymes involved in haemoglobin synthesis with adaptive compensation.

Table 8. Mean body weight and body weight gain of dogs administered imazapic for approximately 12 months

Parameter	Males (n = 6)				Females (n = 6)			
	0 ppm	5 000 ppm	20 000 ppm	40 000 ppm	0 ppm	5 000 ppm	20 000 ppm	40 000 ppm
Body weight (g)[a]								
- Day 0	9 634.2 ± 705.7	9 845.8 ± 511.9	9 849.3 ± 1061.1	9 739.3 ± 282.7	7 840.3 ± 648.6	7 805.7 ± 460.3	7 879.7 ± 624.7	7 907.5 ± 627.5
- Day 355	13 476 ± 2 130.9	14 553 ± 958.47	16 684 ± 2 981.1*	12 576 ± 1 253.5	11 807 ± 1 166.7	11 045 ± 2 380.7	12 632 ± 1 386.3	10 844 ± 1 090.9
Body weight relative to controls (%)	–	↑8	↑24	↓7	–	↓6	↑6	↓8
Overall body weight gain (g)	3 841.8	4 707.2	6 834.7	2 836.7	3 966.7	3 239.3	4 752.3	2 936.5
Overall body weight gain relative to controls (%)	–	↑22	↑78	↓26	–	↓18	↑20	↓25

*: $P \leq 0.05$ (two-tailed *t*-test)
[a] Mean ± standard deviation.
Source: Wolford (1993)

Table 9. Feed consumption in dogs administered imazapic for 12 months

Dietary concentration (ppm)	Mean feed consumption (g/day) (± standard deviation)							
	Males (n = 6)				Females (n = 6)			
	Day 5	Day 13	Day 29	Day 51	Day 5	Day 13	Day 29	Day 51
Control	321.1 ± 80.3	344.4 ± 65.2	386.0 ± 30.5	391.4 ± 19.9	280.9 ± 61.5	302.3 ± 71.6	337.5 ± 44.3	324.5 ± 96.7
5 000	321.3 ± 46.9	312.8 ± 59.9	364.3 ± 31.6	386.2 ± 21.3	302.3 ± 71.6	335.8 ± 40.6	347.4 ± 48.6	248.9 ± 61.2
20 000	310.7 ± 49.4	328.1 ± 60.6	342.6 ± 42.3	390.0 ± 17.1	337.5 ± 44.3	258.7 ± 27.3	276.5 ± 52.9*	329.3 ± 41.3
40 000	258.9 ± 26.9	301.8 ± 50.7	308.8 ± 57.1**	359.1 ± 46.5	324.5 ± 96.7	248.0 ± 35.0	243.0 ± 38.3**	262.8 ± 14.6

*: $P \leq 0.05$; **: $P \leq 0.01$ (two-tailed *t*-test)
Source: Wolford (1993)

Table 10. Incidence of selected macropathological and histopathological lesions in dogs administered imazapic for 1 year

Lesion	Males (n = 6)				Females (n = 6)			
	0 ppm	5 000 ppm	20 000 ppm	40 000 ppm	0 ppm	5 000 ppm	20 000 ppm	40 000 ppm
Macropathology								
Bone marrow								
- Dark	0	0	0	3	0	0	0	5
- Red	0	0	0	4	0	0	0	6
- Gelatinous	0	0	0	4	0	0	0	5
Lungs								
- Discoloration	2	2	0	4	1	1	1	5

Table 10 (continued)

	Males (*n* = 6)				Females (*n* = 6)			
	0 ppm	5 000 ppm	20 000 ppm	40 000 ppm	0 ppm	5 000 ppm	20 000 ppm	40 000 ppm
Histopathology[a]								
Diaphragm								
- Infiltrates, lymphocytes, macrophages	0	1 [1]	4 [1]	3 [1.7]	0	1 [1]	4 [1]	5 [1.6]
- Degeneration/necrosis	0	0	1 [1]	4 [1.5]	0	0	1 [1]	4 [1.3]
Skeletal muscle: Vastus group								
- Infiltrates, lymphocytes, macrophages	0	3 [1]	3 [1]	5 [2.2]	0	2 [1]	6 [1]	6 [2.5]
- Degeneration/necrosis	0	3 [1]	3 [1]	5 [2.4]	0	1 [1]	2 [1]	5 [2.2]
Skeletal muscle: Abdominal muscle								
- Infiltrates, lymphocytes, macrophages	0	3 [1]	4 [1]	4 [1.8]	0	1 [1]	5 [1]	5 [1.6]
- Degeneration/necrosis	0	3 [1]	4 [1]	5 [2.2]	0	1 [1]	2 [1]	4 [1.5]
Oesophagus								
- Infiltrates, lymphocytes, macrophages	0	0	3 [1]	3 [3]	0	0	3 [1]	3 [2]
- Degeneration/necrosis	0	0	1 [1]	3 [2.3]	0	0	0	3 [1.7]
Spleen								
- Increased erythropoiesis	0	0	1 [2]	4 [2]	0	0	0	2 [2]
Bone marrow								
- Congestion	0	0	1 [2]	5 [2.3]	0	0	0	5 [1]
- Increased erythropoiesis	0	0	1 [2]	5 [2.6]	0	0	1 [2]	6 [3.2]
Bone: Sternum								
- Increased erythropoiesis	0	0	1 [2]	5 [2]	0	0	0	6 [2.2]

[a] [ ] mean severity grading; histopathological findings were graded minimal (Grade 1), slight (Grade 2), moderate (Grade 3), marked (Grade 4) and massive/severe (Grade 5). The mean severity is the sum of the gradings divided by the incidence.

Source: Wolford (1993)

Table 11. Selected haematological findings of dogs administered imazapic for 1 year[a]

Parameter	Day	Males (*n* = 5)				Females (*n* = 5)			
		0 ppm	5 000 ppm	20 000 ppm	40 000 ppm	0 ppm	5 000 ppm	20 000 ppm	40 000 ppm
Hct (%)	35	42.2 ± 2.5	40.9 ± 1.9	39.1 ± 2.0* (↓7)	36.3 ± 2.7*** (↓14)	44.8 ± 2.9	41.6 ± 1.9	42.6 ± 2.8 (↓5)	36.6 ± 3.0*** (↓18)
	42	41.8 ± 2.2	–	–	34.7 ± 3.2** (↓17)	43.9 ± 1.3	–	–	35.9 ± 2.0*** (↓18)
	91	43.7 ± 2.5	43.9 ± 2.6	39.8 ± 3.3* (↓9)	33.5 ± 2.0*** (↓23)	47.5 ± 1.7	42.6 ± 2.5**	43.5 ± 3.3* (↓8)	32.5 ± 2.1*** (↓32)

Parameter	Day	Males (n = 5)				Females (n = 5)			
		0 ppm	5 000 ppm	20 000 ppm	40 000 ppm	0 ppm	5 000 ppm	20 000 ppm	40 000 ppm
	173	45.5 ± 3.7	45.7 ± 2.6	42.9 ± 1.6 (↓6)	36.8 ± 3.1*** (↓19)	47.4 ± 3.5	43.0 ± 3.2*	45.0 ± 3.5 (↓5)	36.0 ± 3.5*** (↓24)
	357	46.4 ± 3.0	45.9 ± 1.8	47.7 ± 3.6	39.1 ± 2.7*** (↓16)	45.5 ± 3.8	44.0 ± 3.2	48.9 ± 4.6 (↑7)	38.7 ± 3.6** (↓15)
Hb (g/dL)	35	14.6 ± 0.9	14.1 ± 0.6	13.4 ± 0.7* (↓8)	12.5 ± 1.0*** (↓14)	15.7 ± 1.1	14.5 ± 0.7	14.8 ± 1.1 (↓6)	12.6 ± 1.1*** (↓20)
	42	14.4 ± 0.7	–	–	11.8 ± 1.2** (↓18)	15.3 ± 0.5	–	–	12.3 ± 0.8*** (↓20)
	91	15.3 ± 1.0	15.4 ± 0.9	13.8 ± 1.1* (↓10)	11.3 ± 0.08*** (↓26)	16.7 ± 0.8	15.0 ± 0.8**	15.1 ± 1.2** (↓10)	10.7 ± 0.7*** (↓36)
	173	15.9 ± 1.4	16.2 ± 0.8	15.0 ± 0.6 (↓6)	12.6 ± 1.3*** (↓21)	16.8 ± 1.3	15.2 ± 1.2*	16.0 ± 1.2 (↓5)	12.1 ± 1.2*** (↓28)
	357	16.6 ± 1.2	16.5 ± 0.8	17.0 ± 1.2 (↑2)	13.5 ± 1.0*** (↓19)	16.4 ± 1.3	15.9 ± 1.1	17.6 ± 1.9 (↑7)	13.2 ± 1.0*** (↓20)
RBC (× 10^{-6}/mm^3)	35	6.4 ± 0.4	6.4 ± 0.5	6.1 ± 0.4 (↓5)	5.7 ± 0.5* (↓11)	7.0 ± 0.4	6.3 ± 0.2**	6.5 ± 0.5 (↓7)	5.8 ± 0.5*** (↓17)
	42	6.3 ± 0.3	–	–	5.5 ± 0.6* (↓13)	6.8 ± 0.2	–	–	5.7 ± 0.3*** (↓16)
	91	6.6 ± 0.3	6.9 ± 0.6	6.3 ± 0.5 (↓5)	5.4 ± 0.4*** (↓18)	7.3 ± 0.3	6.3 ± 0.3***	6.7 ± 0.6** (↓8)	5.3 ± 0.3*** (↓27)
	173	7.0 ± 0.5	7.2 ± 0.7	6.9 ± 0.3	6.2 ± 0.6* (↓11)	7.3 ± 0.5	6.5 ± 0.5	7.0 ± 0.7 (↓8)	5.9 ± 0.3*** (↓29)
	357	7.1 ± 0.4	7.3 ± 0.6	7.5 ± 0.6	6.5 ± 0.4* (↓8)	7.1 ± 0.6	6.7 ± 0.4	7.5 ± 0.9 (↑6)	6.4 ± 0.5 (↓10)
Reticulocytes (%)	35	0.9 ± 0.4	0.8 ± 0.2	0.9 ± 0.3	0 .9 ± 0.5	0.8 ± 0.2	0.5 ± 0.2	0.7 ± 0.2	0.9 ± 0.3 (↑13)
	42	0.5 ± 0.3	–	–	0.4 ± 0.2 (↓20)	0.4 ± 0.2	–	–	0.5 ± 0.2 (↑25)
	91	0.2 ± 0.1	0.2 ± 0.2	0.6 ± 0.4*	0.4 ± 0.2 (↑100)	0.1 ± 0.1	0.2 ± 0.2	0.2 ± 0.2	0.4 ± 0.2** (↑300)
	173	0.3 ± 0.1	0.2 ± 0.2	0.4 ± 0.3	0.7 ± 0.2** (↑133)	0.3 ± 0.2	0.2 ± 0.2	0.3 ± 0.2	0.6 ± 0.4 (↑100)
	357	1.4 ± 0.5	1.3 ± 0.5	1.8 ± 0.6	1.5 ± 0.8 (↑7)	1.1 ± 0.3	1.1 ± 0.5	0.9 ± 0.1	1.7 ± 0.6* (↑55)
Normoblasts (per 100 WBC)	35	0 ± 0	0 ± 0	0 ± 0	3 ± 7	0 ± 0	0 ± 0	0 ± 0	2 ± 2*
	42	0 ± 0	–	–	2 ± 3	0 ± 0	–	–	2 ± 1*
	91	0 ± 0	0 ± 0	0 ± 1	5 ± 3***	0 ± 0	0 ± 0	0 ± 1	5 ± 7*

Table 11 (continued)

	Day	Males (n = 5)				Females (n = 5)			
		0 ppm	5 000 ppm	20 000 ppm	40 000 ppm	0 ppm	5 000 ppm	20 000 ppm	40 000 ppm
	173	0 ± 0	0 ± 0	1 ± 2	14 ± 12***	0 ± 0	0 ± 0	0 ± 0	4 ± 4**
	357	0 ± 0	0 ± 0	1 ± 2	6 ± 5***	0 ± 0	0 ± 0	0 ± 0	4 ± 3***
MCV (µm^3)	35	66.2 ± 1.8	63.7 ± 2.3*	64.5 ± 1.1 (↓3)	63.6 ± 1.5* (↓4)	64.3 ± 1.8	66.4 ± 1.6	65.6 ± 2.2	63.3 ± 2.69 (↓2)
	42	66.2 ± 2.0	–	–	63.2 ± 1.5* (↓5)	64.4 ± 1.6	–	–	62.9 ± 2.9 (↓2)
	91	65.9 ± 2.4	64.0 ± 2.1	63.7 ± 0.9 (↓3)	61.6 ± 1.2*** (↓7)	65.0 ± 1.2	67.4 ± 1.1	65.2 ± 2.0	61.9 ± 4.0* (↓5)
	173	65.5 ± 2.2	63.4 ± 2.3	62.7 ± 0.7* (↓4)	59.7 ± 1.5*** (↓9)	65.2 ± 1.2	66.6 ± 1.3	64.7 ± 2.0	60.9 ± 4.8* (↓7)
	357	65.0 ± 2.7	62.8 ± 2.7	64.1 ± 1.1 (↓1)	60.2 ± 0.5*** (↓7)	64.5 ± 1.5	65.6 ± 1.4	65.2 ± 2.6	60.3 ± 4.6* (↓7)
MCH (pg)	35	22.8 ± 0.7	21.9 ± 0.8*	22.1 ± 0.4 (↓3)	21.9 ± 0.6* (↓4)	22.6 ± 0.8	23.2 ± 0.6	22.9 ± 1.0	21.8 ± 1.0 (↓4)
	42	22.7 ± 0.6	–	–	21.5 ± 0.5** (↓5)	22.5 ± 0.5	–	–	21.5 ± 0.9 (↓4)
	91	23.0 ± 0.9	22.5 ± 0.9	22.1 ± 0.5* (↓4)	20.8 ± 0.5*** (↓10)	22.9 ± 0.5	23.7 ± 0.3	22.7 ± 0.9	20.4 ± 1.4*** (↓11)
	173	22.9 ± 1.0	22.5 ± 1.0	21.9 ± 0.4* (↓4)	20.4 ± 0.3*** (↓11)	23.0 ± 0.5	23.5 ± 0.4	23.0 ± 0.7	20.4 ± 1.7*** (↓11)
	357	23.2 ± 1.1	22.6 ± 0.9	22.8 ± 0.2 (↓2)	20.8 ± 0.3*** (↓10)	23.2 ± 0.8	23.7 ± 0.5	23.5 ± 0.8	20.6 ± 1.6*** (↓11)
MCHC (%)	35	34.5 ± 0.9	34.4 ± 0.4	34.2 ± 0.2	34.4 ± 0.6	35.1 ± 0.4	34.9 ± 0.4	34.8 ± 0.4	34.3 ± 0.5** (↓2)
	42	34.3 ± 0.6	–	–	34.0 ± 0.3 (↓1)	34.9 ± 0.3	–	–	34.2 ± 0.5* (↓2)
	91	34.9 ± 0.5	35.1 ± 0.3	34.7 ± 0.7	33.7 ± 0.4*** (↓3)	35.2 ± 0.4	35.2 ± 0.3	34.7 ± 0.4	33.0 ± 0.6*** (↓6)
	173	35.0 ± 0.6	35.5 ± 0.5	35.0 ± 0.5	34.2 ± 0.8 (↓2)	35.3 ± 0.3	35.2 ± 0.3	35.5 ± 0.2	33.5 ± 0.4*** (↓5)
	357	35.8 ± 0.5	36.0 ± 0.6	35.5 ± 0.4	34.5 ± 0.5*** (↓4)	36.0 ± 0.3	36.2 ± 0.4	36.0 ± 0.5	34.3 ± 0.7*** (↓5)
Platelets (× 10^{-3}/mm^3)	35	300 ± 30	310 ± 73	342 ± 34	342 ± 61 (↑14)	298 ± 46	348 ± 54	297 ± 26	335 ± 84 (↑12)
	42	317 ± 31	–	–	398 ± 48** (↑26)	290 ± 42	–	–	347 ± 109 (↑20)
	91	335 ± 39	317 ± 73	362 ± 46	418 ± 78* (↑25)	307 ± 48	377 ± 29	332 ± 51	382 ± 118 (↑24)

	Day	Males (n = 5)				Females (n = 5)			
		0 ppm	5 000 ppm	20 000 ppm	40 000 ppm	0 ppm	5 000 ppm	20 000 ppm	40 000 ppm
	173	292 ± 40	300 ± 46	348 ± 58	460 ± 62*** (↑58)	308 ± 31	373 ± 32	350 ± 75	503 ± 148*** (↑63)
	357	285 ± 49	258 ± 34	300 ± 42	391 ± 101** (↑37)	283 ± 34	372 ± 47	330 ± 57	437 ± 153** (↑54)
WBC (× 10^{-3}/mm^3)	35	11.12 ± 2.92	10.48 ± 1.56	11.87 ± 1.75	11.37 ± 1.88	10.12 ± 2.32	10.28 ± 1.63	9.83 ± 2.02	11.80 ± 1.30
	42	12.90 ± 4.06	–	–	10.07 ± 2.26	9.33 ± 1.63	–	–	11.37 ± 11.33*
	91	11.18 ± 2.49	13.20 ± 8.82	12.00 ± 1.77	11.20 ± 1.75	10.33 ± 3.88	11.20 ± 3.04	10.97 ± 1.43	12.03 ± 1.24
	173	11.77 ± 3.45	11.02 ± 1.56	12.67 ± 1.70	14.13 ± 4.66	10.33 ± 2.39	9.10 ± 2.21	12.33 ± 2.33	16.27 ± 4.80**
	357	11.60 ± 2.97	10.92 ±1.38	12.47 ± 1.68	13.54 ± 4.46	10.13 ± 2.55	10.13 ± 1.73	11.88 ± 1.17	11.45 ± 3.08
Anisocytosis	35	0/6	2/6 (1)	4/6 (1)	6/6 (1)	0/6	1/6 (1)	3/6 (1)	6/6 (1.2)
	42	3/6 (1)	–	–	4/6 (1)	2/6 (1)	–	–	6/6 (1)
	91	1/6 (1)	1/6 (1)	4/6 (1)	6/6 (1.3)	0/6	2/6(1)	4/6 (1)	6/6 (1.2)
	173	2/6 (1)	5/6 (1)	4/6 (1)	6/6 (1.8)	5/6 (1)	4/6 (1)	6/6 (1)	6/6 (2)
	357	0/6	2/6 (1)	3/6 (1)	5/5 (1)	1/6 (1)	1/6 (1)	2/6 (1)	6/6 (1)
Macrocytes	35	0/6	0/6	1/6 (1)	1/6 (1)	0/6	0/6	0/6	1/6 (1)
	42	0/6	–	–	0/6	0/6	–	–	1/6 (1)
	91	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
	173	1/6 (1)	1/6 (1)	2/6 (1)	3/6 (1.7)	0/6	1/6 (1)	0/6	5/6 (1)
	357	0/6	1/6 (1)	0/6	0/6	0/6	0/6	0/6	0/6
Poikilocytosis	35	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
	42	1/6 (1)	–	–	1/6 (1)	0/6	–	–	0/6
	91	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
	173	0/6	0/6	0/6	0/6	0/6	0/6	0/6	1/6 (1)
	357	0/6	0/6	0/6	2/5 (1)	0/6	0/6	0/6	3/6 (1)
Hypochromasia	35	0/6	0/6	2/6 (1)	1/6 (1)	0/6	0/6	0/6	4/6 (1.3)
	42	0/6	–	–	2/6 (1)	0/6	–	–	5/6 (1)
	91	0/6	0/6	2/6 (1)	3/3 (1)	0/6	0/6	1/6 (1)	3/6 (1)
	173	0/6	0/6	0/6	1/6 (1)	0/6	0/6	0/6	2/6 (1)
	357	0/6	0/6	0/6	2/5 (1)	0/6	0/6	0/6	3/6 (1)
Polychromasia	35	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
	42	0/6	–	–	0/6	0/6	–	–	0/6
	91	0/6	0/6	1/6 (1)	2/6 (1)	0/6	0/6	0/6	2/6 (1)
	173	0/6	0/6	0/6	3/6 (1)	1/6 (1)	0/6	2/6 (1)	4/6 (1.3)
	357	0/6	0/6	0/6	2/5 (1)	0/6	0/6	0/6	2/6 (1)
Target cells	35	0/6	0/6	0/6	0/6	0/6	0/6	0/6	5/6 (1.2)
	42	0/6	–	–	0/6	0/6	–	–	0/6
	91	0/6	0/6	1/6 (1)	4/6 (1)	0/6	0/6	0/6	3/6 (1)

Table 11 (continued)

Day	Males (*n* = 5)				Females (*n* = 5)			
	0 ppm	5 000 ppm	20 000 ppm	40 000 ppm	0 ppm	5 000 ppm	20 000 ppm	40 000 ppm
173	0/6	0/6	2/6 (1)	5/6 (1.2)	0/6	0/6	0/6	6/6 (1.5)
357	0/6	0/6	0/6	3/5 (1.3)	0/6	0/6	0/6	4/6 (1)

Hb: haemoglobin; Hct: haematocrit; MCH: mean cell haemoglobin; MCHC: mean cell haemoglobin concentration; MCV: mean cell volume; RBC: red blood cells; WBC: white blood cells; *: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$ (two-tailed *t*-test)

[a] Group means ± standard deviation; (↑/↓x) per cent change from control; (x) average severity in RBC morphology parameters.

Source: Wolford (1993)

Changes to the skeletal muscle consisted of increased lymphocyte and macrophage infiltrations and increased degeneration and necrosis in the muscle at and above 5000 ppm in males and females. At the lowest dose tested, Grade 1 lesions were seen in five males and three females, although primarily at one site per animal in the diaphragm, vastus group or abdominal muscles. At 20 000 ppm, all males and females exhibited Grade 1 lesions, primarily at multiple sites, and infiltration was also seen in the oesophagus of males and females and necrosis in the oesophagus of males. At 40 000 ppm, infiltrations, degeneration and necrosis were seen in all animals at multiple sites, including the oesophagus, and the gradation of the lesions had increased.

At 20 000 ppm, cholesterol (Table 12) and liver weights (Table 13) were increased in males and females and phosphorus values were increased in males. At 40 000 ppm, cholesterol, potassium, globulin values, lactate dehydrogenase, creatinine kinase, aspartate aminotransferase and alanine aminotransferase values were increased and albumin, creatinine and albumin : globulin ratios were decreased, at least for some interval during the treatment period.

Table 12. Selected clinical chemistry findings of dogs administered impazapic for 1 year[a]

Parameter	Day	Males (*n* = 5)				Females (*n* = 5)			
		0 ppm	5 000 ppm	20 000 ppm	40 000 ppm	0 ppm	5 000 ppm	20 000 ppm	40 000 ppm
Potassium (meq/L)	35	4.97 ± 0.14	4.92 ± 0.16	4.80 ± 0.17	4.88 ± 0.44 (↓2)	4.67 ± 0.14	4.65 ± 0.27	4.62 ± 0.17	4.57 ± 0.38 (↓8)
	42	4.85 ± 0.15	–	–	4.92 ± 0.26 (↑1)	4.72 ± 0.17	–	–	4.75 ± 0.28 (↑1)
	91	5.00 ± 0.24	4.88 ± 0.23	4.93 ± 0.19	5.53 ± 0.52* (↑11)	4.67 ± 0.18	4.65 ± 0.15	4.83 ± 0.44	4.98 ± 0.31 (↑7)
	173	5.00 ± 0.4	4.92 ± 0.17	4.75 ± 0.15	5.52 ± 0.72* (↑10)	4.76 ± 0.22	4.83 ± 0.44	4.82 ± 0.27	5.32 ± 0.29* (↑12)
	357	4.95 ± 0.21	4.62 ± 0.20**	4.58 ± 0.16**	5.05 ± 0.19 (↑2)	4.52 ± 0.19	4.53 ± 0.16	4.83 ± 0.37	5.15 ± 0.47** (↑14)
Phosphorus (mg/dL)	35	5.65 ± 0.26	6.56 ± 0.19	6.28 ± 0.42** (↑11)	6.38 ± 0.48*** (↑13)	4.98 ± 0.42	5.62 ± 1.01	5.48 ± 0.65 (↑10)	5.98 ± 0.58* (↑20)
	42	5.63 ± 0.39	–	–	5.82 ± 0.34 (↑3)	5.08 ± 0.20	–	–	5.80 ± 0.50**

Parameter	Day	Males (n = 5)				Females (n = 5)			
		0 ppm	5 000 ppm	20 000 ppm	40 000 ppm	0 ppm	5 000 ppm	20 000 ppm	40 000 ppm
									(↑14)
	91	5.18 ± 0.25	5.05 ± 0.43	5.37 ± 0.28 (↑4)	5.55 ± 0.53 (↑7)	4.42 ± 0.27	4.77 ± 0.73	5.02 ± 0.62 (↑14)	5.37 ± 0.67* (↑21)
	173	4.62 ± 0.35	4.40 ± 0.37	4.80 ± 0.40 (↑4)	5.10 ± 0.14* (↑10)	3.94 ± 0.32	4.23 ± 0.60	4.52 ± 0.58 (↑15)	4.66 ± 0.40* (↑18)
	357	3.68 ± 0.29	3.48 ± 0.13	3.80 ± 0.55 (↑3)	3.88 ± 0.33 (↑5)	3.37 ± 0.40	3.37 ± 0.42	4.05 ± 0.70 (↑20)	4.27 ± 0.85* (↑27)
Creatinine (mg/dL)	35	0.92 ± 0.08	0.87 ± 0.08	0.88 ± 0.04	0.82 ± 0.13 (↓11)	097 ± 0.05	0.93 ± 0.05	0.90 ± 0.06*	0.87 ± 0.05** (↓10)
	42	0.87 ± 0.10	–	–	0.68 ± 0.15* (↓11)	0.95 ± 0.05	–	–	0.73 ± 0.05*** (↓23)
	91	0.87 ± 0.05	0.90 ± 0.11	0.83 ± 0.08	0.73 ± 0.08* (↓16)	0.93 ± 0.08	0.90 ± 0.06	0.85 ± 0.10	0.80 ± 0.06** (↓14)
	173	0.88 ± 0.10	0.93 ± 0.08	0.80 ± 0.06	0.67 ± 0.08*** (↓24)	0.94 ± 0.05	0.87 ± 0.05*	0.85 ± 0.05*	0.72 ± 0.04*** (↓23)
	357	0.88 ± 0.08	0.92 ± 0.10	0.84 ± 0.05	0.68 ± 0.10** (↓23)	0.90 ± 0.11	0.87 ± 0.05	0.82 ± 0.08	0.78 ± 0.16 (↓13)
Cholesterol (mg/dL)	35	157.2 ± 28.9	183.2 ±21.2	194.7 ± 22.0* (↑24)	187.3 ± 30.7 (↑19)	156.2 ± 14.8	151.8 ± 18.1	178.3 ± 25.6* (↑14)	184.7 ± 18.9* (↑18)
	42	160.8 ± 22.4	–	–	194.5 ± 38.1 (↑21)	152.2 ± 14.9	–	–	191.3 ± 15.5** (↑26)
	91	160.7 ± 34.0	191.5 ± 21.3	217.7 ± 23.3** (↑35)	199.7 ± 33.2* (↑24)	160.5 ± 18.4	159.2 ± 22.6	197.2 ± 24.5** (↑23)	196.3 ± 22.4* (↑22)
	173	156.3 ± 40.4	177.0 ± 28.7	206.3 ± 27.5* (↑32)	199.2 ± 32.0* (↑27)	190.6 ± 61.8	173.8 ± 57.2	207.3 ± 35.8 (↑9)	215.4 ± 20.5 (↑13)
	357	155.2 ± 34.0	171.0 ± 22.7	205.2 ± 30.8* (↑32)	184.8 ± 33.1 (↑19)	184.0 ± 41.0	160.8 ± 31.3	207.7 ± 36.7 (↑13)	225.7 ± 61.2 (↑23)
Lactate dehydrogenase (IU/L)	35	144.3 ± 54.4	148.2 ± 46.2	120.3 ± 54.2	238.8 ± 127.8* (↑65)	125.7 ± 55.2	103.5 ± 51.8	119.0 ± 103.0	556.8 ± 648.6* (↑343)
	42	82.7 ± 38.6	–	–	126.3 ± 95.3 (↑53)	74.0 ± 55.4	–	–	143.7 ± 81.8 (↑94)
	91	119.2 ± 50.9	118.5 ± 38.3	138.7 ± 61.8	282.3 ± 132.6** (↑137)	81.3 ± 20.2	79.7 ± 10.9	75.7 ± 35.3	166.7 ± 68.2** (↑105)

Table 12 (continued)

	Day	Males (*n* = 5)				Females (*n* = 5)			
		0 ppm	5 000 ppm	20 000 ppm	40 000 ppm	0 ppm	5 000 ppm	20 000 ppm	40 000 ppm
	173	178.8 ± 70.9	171.2 ± 54.5	131.8 ± 38.1	277.2 ± 131.3 (↑55)	135.6 ± 62.8	112.8 ± 45.7	118.0 ± 48.5	239.4 ± 117.6* (↑77)
	357	102.0 ±42.3	100.3 ± 29.1	97.4 ± 29.1	139.3 ± 101.8 (↑37)	96.7 ± 55.0	96.2 ± 34.7	129.3 ± 29.5	140.2 ± 78.2 (↑45)
Creatinine kinase (IU/L)	35	343.3 ± 162.4	307.7 ±79.6	451.5 ± 464.5	10 682 ± 13 121* (↑3 012)	306.7 ± 102.6	285.0 ± 87.6	529.5 ± 539.2	23 208 ± 34 291* (↑7 467)
	42	209.0 ± 54.9	–	–	6 629.5 ± 6 350.0* (↑3 071)	229.3 ± 73.2	–	–	8 178 ± 4 300** (↑3 467)
	91	223.0 ± 37.5	208.3 ± 22.8	275.3 ± 141.4	324.3 ± 113.3 (↑45)	205.5 ± 58.9	159.3 ± 21.3	146.7 ± 30.3	446.8 ± 454.3 (↑117)
	173	467.3 ± 491.7	213.3 ± 39.2	173.2 ± 48.9	350.3 ± 102.3 (↓75)	201.0 ± 35.8	199.3 ± 99.9	190.0 ± 115.3	324.4 ± 153.3 (↑61)
	357	162.0 ± 34.4	171.5 ± 26.1	122.0 ± 13.7	215.5 ± 53.3* (↑33)	242.0 ± 205.2	162.0 ± 39.5	176.7 ± 37.8	239.0 ± 141.0 (↓1)
Aspartate aminotransferase (IU/L)	35	33.3 ± 2.8	33.3 ± 4.5	34.3 ± 8.3	632.2 ± 760.1* (↑1 798)	36.2 ± 6.1	32.8 ± 4.0	41.3 ± 10.8	1 093 ± 1 497* (↑2 991)
	42	32.5 ± 5.2	–	–	384.7 ± 269.2** (↑1 084)	38.0 ± 8.3	–	–	552 ± 243*** (↑1 352)
	91	32.3 ± 4.6	33.3 ± 3.0	32.8 ± 6.3	44.3 ± 16.0* (↑37)	35.7 ± 8.4	32.8 ± 1.8	30.5 ± 7.0	79.5 ± 86.1 (↑123)
	173	36.8 ± 11.7	35.5 ± 3.1	29.8 ± 1.9	55.8 ± 29.0* (↑52)	38.4 ± 7.7	34.7 ± 4.2	32.2 ± 9.6	48.8 ± 19.9 (↑27)
	357	34.0 ± 3.8	32.5 ± 3.1	29.0 ± 2.8*	32.8 ± 4.6 (↓4)	35.5 ± 9.9	75.0 ± 94.6	32.2 ± 6.5	36.7 ± 14.8 (↑3)
Alanine aminotransferase (IU/L)	35	28.2 ± 8.1	25.8 ± 2.9	27.5 ± 6.9	260.2 ± 329.7* (↑823)	30.3 ± 12.5	26.8 ± 5.6	24.8 ± 3.6	400.5 ± 530.9* (↑1 222)
	42	25.8 ± 7.8	–	–	412.3 ± 320.9* (↑1 498)	27.5 ± 7.1	–	–	493.5 ± 358.6** (↑1 695)
	91	31.2 ± 9.6	31.3 ± 2.7	38.5 ± 11.5	43.0 ± 9.8* (↑38)	31.7 ± 6.6	30.0 ± 6.7	34.5 ± 11.5	70.5 ± 44.2* (↑122)
	173	27.5 ± 7.0	27.0 ± 2.8	37.3 ± 8.5	53.2 ± 24.5** (↑93)	24.2 ± 6.3	25.2 ± 5.8	26.2 ± 5.3	46.2 ± 18.0** (↑91)
	357	30.5 ±	30.7 ±	39.2 ±	45.3 ±	25.5 ±	106.8 ±	29.5 ±	40.5 ±

	Day	Males (n = 5)				Females (n = 5)			
		0 ppm	5 000 ppm	20 000 ppm	40 000 ppm	0 ppm	5 000 ppm	20 000 ppm	40 000 ppm
		6.7	4.8	11.6	14.6* (↑49)	7.0	208.0	7.2	12.1 (↑59)
Albumin (g/dL)	35	3.47 ± 0.16	3.42 ± 0.17	3.37 ± 0.10	3.28 ± 0.12* (↓5)	3.60 ± 0.14	3.55 ± 023	3.55 ± 0.20	3.38 ± 0.12* (↓6)
	42	3.30 ± 0.18	–	–	3.02 ± 0.12** (↓8)	3.40 ± 0.09	–	–	3.22 ± 0.13* (↓5)
	91	3.43 ± 0.15	3.48 ± 0.20	3.32 ± 0.15	3.32 ± 0.15 (↓3)	3.58 ± 0.10	3.50 ± 0.22	3.57 ± 0.25	3.35 ± 0.10* (↓6)
	173	3.40 ± 0.26	3.43 ± 0.23	3.32 ± 0.12	3.27 ± 0.21 (↓4)	3.34 ± 0.22	3.38 ± 0.26	3.43 ± 0.22	3.24 ± 0.17 (↓3)
	357	3.48 ± 0.20	3.50 ± 0.13	3.64 ± 0.11	3.55 ± 0.26 (↑2)	3.40 ± 0.14	3.52 ± 0.24	3.82 ± 0.26**	3.47 ± 0.15 (↑2)
Globulin (g/dL)	35	2.32 ± 0.17	2.43 ± 0.34	2.50 ± 0.15	2.57 ± 0.30 (↑11)	2.40 ± 0.31	2.22 ± 0.31	2.40 ± 0.45	2.35 ± 0.12 (↓2)
	42	2.25 ± 0.25	–	–	2.50 ± 0.24 (↑11)	2.25 ± 0.18	–	–	2.37 ± 0.14 (↑5)
	91	2.38 ± 0.16	2.55 ± 0.21	2.62 ± 0.26	2.67 ± 0.23* (↑12)	2.33 ± 0.31	2.12 ± 0.22	2.20 ± 0.60	2.30 ± 0.28 (↓1)
	173	2.35 ± 0.21	2.62 ± 0.41	2.67 ± 0.19	2.72 ± 0.33* (↑16)	2.74 ± 0.05	2.15 ± 0.19***	2.50 ± 0.38	2.40 ± 0.20* (↓12)
	357	2.42 ± 0.10	2.68 ± 0.32	2.46 ± 0.15	2.55 ± 0.31 (↑4)	2.57 ± 0.29	2.18 ± 0.12	2.43 ± 0.61	2.63 ± 0.36 (↑2)
A : G ratio	35	1.50 ± 0.14	1.43 ± 0.24	1.35 ± 0.12	1.29 ± 0.17* (↓14)	1.52 ± 0.19	1.63 ± 0.28	1.52 ± 0.28	1.44 ± 0.08 (↓5)
	42	1.49 ± 0.21	–	–	1.22 ± 0.14* (↓18)	1.52 ± 0.12	–	–	1.36 ± 0.07* (↓11)
	91	1.44 ± 0.09	1.38 ± 0.17	1.28 ± 0.15*	1.25 ± 0.11* (↓13)	1.56 ± 0.21	1.67 ± 0.22	1.71 ± 0.42	1.48 ± 0.20 (↓5)
	173	1.46 ± 0.23	1.35 ± 0.33	1.25 ± 0.12	1.22 ± 0.15 (↓18)	1.22 ± 0.07	1.59 ± 0.20**	1.40 ± 0.25	1.36 ± 0.14 (↑11)
	357	1.43 ± 0.13	1.32 ± 0.19	1.48 ± 0.07	1.41 ± 0.23 (↓1)	1.34 ± 0.20	1.62 ± 0.17	1.65 ± 0.42	1.34 ± 0.19 (0)

A : G: albumin to globulin; eq: equivalents; IU: International Units; *: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$ (two-tailed *t*-test)

[a] Group means ± standard deviation.; (↑/↓x) per cent change from control.

Source: Wolford (1993)

Table 13. Liver weight findings (mean ± standard deviation) in dogs administered imazapic for 12 months[a]

Parameter	Males (n = 6)				Females (n = 6)			
	0 ppm	5 000 ppm	20 000 ppm	40 000 ppm	0 ppm	5 000 ppm	20 000 ppm	40 000 ppm
Absolute liver weight (g)	338.2 ± 18.4	344.0 ± 24.5 (↑2)	407.4 ± 44.2* (↑20)	385.8 ± 40.5* (↑14)	271.0 ± 40.1	263.6 ± 35.8 (↓7)	299.0 ± 21.3 (↑10)	326.4 ± 28.0* (↑20)
Relative liver weight (% of body weight)	2.7 ± 0.4	2.5 ± 0.2 (↓7)	2.6 ± 0.3 (↓4)	3.2 ± 0.5* (↑19)	2.4 ± 0.4	2.6 ± 0.4 (↑8)	2.5 ± 0.2 (↑4)	3.2 ± 0.4* (↑33)
Relative liver weight (% of brain weight)	397.3 ± 45.8	408.5 ± 47.0 (↑3)	477.9 ± 54.5* (↑20)	484.7 ± 68.5* (↑22)	337.3 ± 47.2	326.3 ± 51.5 (↓3)	400.8 ± 52.7* (↑19)	412.6 ± 49.3* (↑22)

*: $P < 0.05$
[a] (↑/↓x) per cent change from control.
Source: Wolford (1993)

The lowest-observed-adverse-effect level (LOAEL) was 5000 ppm (equal to 137 mg/kg bw per day), the lowest dose tested, based on increased lymphocyte and macrophage infiltration of the diaphragm, abdominal and thigh skeletal muscles and increased degeneration and necrosis in the abdominal and thigh skeletal muscles, all of minimal severity. A NOAEL could not be identified.

The study authors determined the LOAEL to be 20 000 ppm (501 mg/kg bw per day) and the NOAEL to be 5000 ppm (137 mg/kg bw per day) (Wolford, 1993).

(b) Dermal application

Rabbits

In a 21-day dermal toxicity study, groups of six New Zealand White rabbits of each sex per dose were treated dermally with imazapic (purity 93.7%) applied to approximately 10% of the body surface at a dose level of 0, 250, 500 or 1000 mg/kg bw per day for 3 weeks. The volume of application was approximately 3 mL/kg bw, and the duration of treatment was 6 hours daily, with occlusion, 5 days/week. The animals were observed daily for clinical signs, and, prior to initiation and immediately before each subsequent exposure period, animals were assessed for skin irritation. Animals were weighed twice pretest, weekly thereafter and at necropsy. Feed consumption was monitored daily. Blood samples for haematology and plasma chemistry were collected 3 days prior to study initiation and just prior to necropsy from fasted animals. The animals were terminated after 3 weeks of treatment and subjected to necropsy and postmortem examination of major organs and tissues. Organs were weighed, and a full range of tissues, including gross lesions, was preserved. Tissue samples from all control and high-dose animals and any gross lesions from all animals were processed and examined by light microscopy.

There were no deaths and no treatment-related general clinical signs or reactions at the dermal application sites at any dose level. There was no effect of treatment on body weight, body weight gain or feed consumption in either sex at any dose level. The haematological and clinical chemistry investigations performed in week 3 did not indicate any treatment-related effects at any dose level. There were no treatment-related gross lesions at necropsy or changes to organ weights at any dose level. The microscopic examination performed after 3 weeks of treatment did not show treatment-related changes in any of the organs examined in the group treated at 1000 mg/kg bw per day.

The NOAEL in males and females was 1000 mg/kg bw per day, the highest dose tested (Moore, 1992).

2.3 *Long-term studies of toxicity and carcinogenicity*

Mice

In a carcinogenicity study in mice, imazapic (purity 96.9%) was administered in the diet to 65 CD-1 mice of each sex per dose at 0, 1750, 3500 or 7000 ppm (equal to 0, 271, 551 and 1134 mg/kg bw per day for males and 0, 369, 733 and 1442 mg/kg bw per day for females, respectively) for 78 weeks. Ten animals of each sex per dose were terminated after 12 months of treatment. Detailed clinical signs were recorded weekly, and animals were observed daily for overt signs of toxicity and twice daily for morbidity and mortality. Body weights were recorded prior to treatment, weekly for the first 14 weeks of the study, biweekly for weeks 14–26 and monthly thereafter. Feed consumption was recorded weekly for 14 weeks and at 4-week intervals thereafter. Haematology parameters were determined in 10 animals of each sex per dose at 12 and 18 months. All animals were subjected to necropsy, postmortem examination and tissue preservation. Organ weights were recorded for 10 animals of each sex per dose. Histopathological evaluation of haematoxylin and eosin–stained tissues was performed on all animals from the control and high-dose groups terminated at 12 and 18 months, decedents from all groups and lungs, liver and kidneys from all groups. A histopathological examination of the coagulating gland, Harderian gland, lacrimal gland, pharynx, larynx and nose was not conducted.

There was no evidence of oncogenicity, and there were no effects of treatment on body weight, body weight gain or feed consumption in either sex at any dose level. The haematological investigations did not indicate any treatment-related effects at any dose level. There were no treatment-related gross lesions at necropsy or changes to organ weights at any dose level. The microscopic examination performed after 12 and 18 months of treatment did not show treatment-related changes in any of the organs examined in the group treated at 7000 ppm.

The NOAEL for chronic toxicity was 7000 ppm (equal to 1134 mg/kg bw per day), the highest dose tested (Fischer, 1994b).

Rats

In a carcinogenicity study in rats, imazapic (purity 96.9%) was administered in the diet to 65 Sprague-Dawley rats of each sex per dose at 0, 5000, 10 000 or 20 000 ppm (equal to 0, 253, 505 and 1029 mg/kg bw per day for males and 0, 308, 609 and 1237 mg/kg bw per day for females, respectively) for 104 weeks. A satellite group of 10 animals of each sex per dose was terminated at 1 year to assess chronic toxicity. The animals were observed twice daily for viability, clinical signs were recorded daily and a detailed physical examination was performed weekly. Body weights and feed consumption were recorded weekly for 15 weeks (17 weeks for feed consumption), biweekly from weeks 17 to 27 and every 4 weeks thereafter. Blood samples from 10 animals of each sex per dose taken at 6-month intervals were subject to haematological and clinical chemistry analyses. Urine analysis was also performed at these times. All animals, including decedents, were subjected to necropsy, postmortem examination and tissue preservation. The organs of all interim kill animals and 10 animals of each sex per dose were weighed. Major organs and tissues from the control and high-dose groups, premature decedents from all groups, gross lesions from all animals and liver, lungs and kidneys from all animals were processed and examined by light microscopy. Blood clotting potential was not examined. The adrenals, epididymides, spleen and heart were not weighed. The uterus and ovaries were weighed together, and only in animals killed at 24 months. The pharynx, larynx and nose were not examined histopathologically.

There was no evidence of oncogenicity, and there were no effects of treatment on body weight, body weight gain or feed consumption in either sex at any dose level. The haematological investigations did not indicate any treatment-related effects at any dose level. There were no treatment-related gross lesions at necropsy or changes to organ weights at any dose level. The microscopic examination performed after 12 and 24 months of treatment did not show treatment-related changes in any of the organs examined in the group treated at 20 000 ppm.

The NOAEL was 20 000 ppm (equal to 1029 mg/kg bw per day), the highest dose tested (Fischer, 1994a).

2.4 Genotoxicity

A range of GLP-compliant studies of genotoxicity with imazapic was conducted to assess its potential for inducing gene mutation, chromosomal aberration and unscheduled deoxyribonucleic acid (DNA) synthesis. There was no evidence for genotoxicity or mutagenicity in the available studies (summarized in Table 14), but the status of the unscheduled DNA synthesis test is unclear. In the only treated cultures evaluated for unscheduled DNA synthesis (1000, 1500, 2000 and 2500 µg/mL), precipitation of unidentified material was observed. At the two highest of these dose levels, survival appeared to be higher than observed over the "lower" range of 500–1500 µg/mL, where precipitation was also observed at concentrations of 750 µg/mL and above. It cannot be assumed that target cell exposure had increased with the concentration of test material in the culture medium; therefore, this experiment, although adequately conducted, was incapable of providing acceptable results. In a published comparative genotoxicity evaluation of imidazoline herbicides, a commercially available imazapic end-use product was tested in *Drosophila melanogaster* for an effect on mutagenicity and recombinogenic activity. There was no evidence that imazapic was genotoxic in this test. Overall, imazapic did not demonstrate any genotoxic potential.

Table 14. Genotoxicity studies with imazapic

End-point	Test object	Concentration	Purity (%)	Results	Reference
In vitro					
Reverse mutation	*Salmonella typhimurium* and *Escherichia coli*	0, 100, 500, 1 000, 2 500 or 5 000 µg/plate (±S9)	93.7	Negative	Traul (1992)
Mammalian cell gene mutation	Chinese hamster ovary cells (*HPRT* locus assay)	0, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0 or 5.0/4.0 µg/mL (±S9)	93.7	Negative	Young (1992)
Unscheduled DNA synthesis	Sprague-Dawley rat hepatocytes, male	0, 1 000, 1 500, 2 000 or 2 500 µg/mL	93.7	Unacceptable	Thilager (1992)
Chromosomal aberration	Chinese hamster ovary cells	0, 0.1, 0.3, 1.0, 3.0 µg/mL, 0.01, 0.03, 0.1, 0.3, 1.0 or 3.0 mg/mL (±S9)	93.7	Negative	Sharma (1992)
In vivo					
Chromosomal aberration	Sprague-Dawley rat bone marrow, male	0, 500, 1 667 or 5 000 mg/kg bw Harvest time: 6, 18 and 30 h	93.7	Negative	Ivett (1992)
Mutagenic and recombinogenic activity	*Drosophila melanogaster*	0, 2.5, 5.0, 10.0 or 20.0 mmol/L	700 g/kg[a]	Negative	Fragiorge et al. (2008)

HPRT: hypoxanthine-guanine phosphoribosyltransferase; S9: 9000 × *g* supernatant fraction of liver homogenate from Aroclor-treated rats

[a] Tested as commercially available Plateau 70 DR®, active ingredient 700 g/kg, inert ingredients 300 g/kg.

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

Rats

In a two-generation reproductive toxicity study in rats, imazapic (purity 93.7%) was administered in the diet to 30 Sprague-Dawley rats of each sex per dose at a concentration of 0, 5000, 10 000 or 20 000 ppm (equal to 0, 301, 605 and 1205 mg/kg bw per day for males and 0, 378, 737

and 1484 mg/kg bw per day for females, respectively; Table 15) for 14 weeks prior to mating and through lactation and weaning of the F_1 offspring. Groups of 25 male and 25 female F_1 generation offspring were then similarly treated through 21 days of lactation of the F_2 offspring. Overall achieved dose levels were within the ranges 301–365, 605–737 and 1205–1447 mg/kg bw per day for males and 368–425, 714–884 and 1446–1703 mg/kg bw per day for females at 5000, 10 000 and 20 000 ppm, respectively (Table 15). In F_0 and F_1 parental animals, toxicity and mortality inspections were performed twice daily, body weight measurement and feed consumption recording and detailed clinical observations were performed approximately weekly, and the duration of gestation was recorded. F_1 and F_2 offspring were examined daily during the lactation period for clinical signs and mortality, and full external examinations and body weight recording were performed on days 0, 4, 7, 14 and 21 of lactation. Litters were culled to four pups of each sex on day 4 of lactation. All surviving and decedent F_0 and F_1 parental animals were subjected to necropsy and gross pathological examination. High-dose and control animals were subjected to histopathological examination of the pituitary, reproductive organs and any gross lesions. Pups culled on day 4 of lactation, F_1 weanlings not selected to form the parental generation and offspring dying during lactation were subjected to gross necropsy. One male and one female weanling per litter per generation were subjected to a detailed macroscopic external and internal examination, and all gross lesions were preserved in 10% neutral buffered formalin. Sperm parameters and ovarian follicle counts as well as brain, liver, kidneys, spleen, thyroid and adrenal weights were not measured in parents. Physical development of the young, including timing of sexual maturation, was not assessed.

Table 15. Mean test substance intake of imazapic in the two-generation study in rats

	Dose (mg/kg bw per day)					
	F_0 generation			F_1 generation		
	5 000 ppm	10 000 ppm	20 000 ppm	5 000 ppm	10 000 ppm	20 000 ppm
Males	301	605	1 205	365	737	1 447
Females premating	378	737	1 484	425	884	1 703
Females gestation period	368	714	1 446	403	804	1 620
Females lactation period	–	–	–	–	–	–

Source: Schroeder (1994)

There was no evidence of effects on body weight, feed consumption, clinical signs of toxicity, survival or gross pathological or histopathological change in parents or offspring of either generation. There was no evidence of reproductive toxicity.

The NOAEL for parental toxicity, reproductive toxicity and offspring toxicity in Wistar rats was 20 000 ppm (equal to 1205 mg/kg bw per day), the highest dose tested (Schroeder, 1994).

(b) Developmental toxicity

Rats

In a range-finding developmental toxicity study, imazapic (purity 93.7%) was administered to mated female Sprague-Dawley rats (eight per dose) via gavage from days 6 to 15 of gestation at a dose level of 0, 375, 750, 1500 or 2500 mg/kg bw per day and a dosing volume of 10 mL/kg bw in corn oil. The animals were terminated on day 20 after mating for reproductive assessment. Clinical signs and body weight were recorded. Adult females were examined macroscopically at necropsy on day 20 after mating. As this was a range-finding study, no fetal assessment was performed.

One day following initial test substance administration, one 1500 mg/kg bw per day and four 2500 mg/kg bw per day dams exhibited excessive salivation (Table 16). There were no changes to body weight and no changes in the caesarean section parameters.

Table 16. Clinical signs of toxicity from the range-finding developmental toxicity study in rats

	Incidence of salivation (n = 8)				
	0 mg/kg bw per day	375 mg/kg bw per day	750 mg/kg bw per day	1 500 mg/kg bw per day	2 500 mg/kg bw per day
Salivation	0	0	0	1	4

Source: Schardein (1992)

No LOAEL or NOAEL was identified, as this was a range-finding study (Schardein, 1992).

In the main developmental toxicity study, imazapic (purity 93.7%) was administered to mated female Sprague-Dawley rats (25 per dose) via gavage from day 6 to day 15 of gestation at a dose level of 0, 250, 500 or 1000 mg/kg bw per day and a dosing volume of 10 mL/kg bw in corn oil. The animals were terminated 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 examination or skeletal examination. Maternal body weight and feed consumption were not analysed statistically, and the homogeneity and stability of the test substance in corn oil were not determined.

There were no premature deaths, and there were no clinical signs or changes in body weight or feed consumption. There was a slight increase in hydronephrosis in the kidneys of two 500 mg/kg bw per day and two 1000 mg/kg bw per day dams; however, there was no dose–response relationship, and the changes were considered incidental. There were no effects on caesarean section parameters, and there was no evidence of teratogenicity.

The NOAEL for maternal and embryo/fetal toxicity was 1000 mg/kg bw per day, the highest dose tested (Schardein, 1992).

Rabbits

In a range-finding developmental toxicity study in rabbits, imazapic (purity 93.7%) was administered to artificially inseminated female New Zealand White rabbits (five per dose) via gavage, from day 7 to day 19 of gestation, at a dose level of 0, 62.5, 125, 250, 500 or 1000 mg/kg bw per day and a dosing volume of 10 mL/kg bw in aqueous carboxymethyl cellulose (0.4% w/v). The animals were killed on day 29 after mating for reproductive assessment and external fetal examination. Clinical signs and body weight were recorded. Adult females were examined macroscopically at necropsy on day 29 after mating, and all fetuses were examined macroscopically at maternal necropsy.

There was one unscheduled death at 62.5 mg/kg bw per day, attributed to regurgitation and aspiration of the dose, one premature delivery at 125 mg/kg bw per day and one abortion at 500 mg/kg bw per day. Three animals in the 1000 mg/kg bw per day dose group were euthanized moribund, and two others were found dead. Clinical signs of toxicity (Table 17) in the dams consisted of anorexia, few or no faeces, languid presentation and prostration in the 1000 mg/kg bw per day animals. There was an increase in red, ulcerated, thickened and/or peeling stomach at 1000 mg/kg bw per day, along with enlargement and/or reddening of the inner wall of the gallbladder (Table 18). There were no effects on the caesarean section parameters of surviving animals.

No LOAEL or NOAEL was identified, as this was a range-finding study (Schardein, 1992).

In the main developmental toxicity study in rabbits, imazapic (purity 93.7%) was administered to artificially inseminated female New Zealand White rabbits (20 per dose) via gavage, from day 7 to day 19 of gestation, at a dose level of 0, 175, 350, 500 or 700 mg/kg bw per day and a dosing volume of 10 mL/kg bw in aqueous carboxymethyl cellulose (0.4% w/v). The animals were

Table 17. Clinical signs of toxicity from the range-finding developmental toxicity study in rabbits

Finding	Incidence of finding (*n* = 5)					
	0 mg/kg bw per day	6.25 mg/kg bw per day	125 mg/kg bw per day	250 mg/kg bw per day	500 mg/kg bw per day	1 000 mg/kg bw per day
Anorexia	2	2	2	3	3	5
Few or no faeces	3	3	2	4	3	5
Languid	0	0	0	0	0	4
Prostrate	0	0	0	0	0	3
Premature delivery	0	0	0	0	1	0
Moribund sacrifice	0	0	0	0	0	3
Died on test	0	1	0	0	0	2

Source: MacKenzie (1992)

Table 18. Gross necropsy findings from the range-finding developmental toxicity study in rabbits

Finding	Incidence of finding (*n* = 5)					
	0 mg/kg bw per day	6.25 mg/kg bw per day	125 mg/kg bw per day	250 mg/kg bw per day	500 mg/kg bw per day	1 000 mg/kg bw per day
Gallbladder, enlarged and/or inner wall reddened	0	1	0	0	0	4
Stomach, ulcerated, red, thickened or peeling	0	3	0	0	1	5

Source: MacKenzie (1992)

killed on day 29 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 29 after mating, and all fetuses were examined macroscopically at maternal necropsy and subsequently by detailed internal visceral examination and skeletal examination. High mortality in does at 700 mg/kg bw per day precluded results from this dose level being included in the determination of the NOAELs and LOAELs for maternal and embryo/fetal toxicity; however, treatment-related findings are included below for completeness of reporting.

Maternal mortalities were noted at all doses, including control, and were considered treatment related at 700 mg/kg bw per day, with 11/20 animals dying on test or euthanized moribund. Clinical signs of toxicity consisted of few or no faeces, languid presentation and anuria at 700 mg/kg bw per day (Table 19). Body weights, body weight gain (Table 20; Figs 3 and 4) and feed consumption (Table 21; Fig. 5) were decreased in affected does treated with 700 mg/kg bw per day, although there was no change in 700 mg/kg bw per day does surviving to day 29. At 500 mg/kg bw per day, body weight gain was decreased 350% and 217% from gestation day (GD) 7 to GD 14 and from GD 14 to GD 20, respectively (not statistically significant), and feed consumption was decreased from GD 12 (reaching statistical significance at GD 15 and GD 16) until the end of treatment. At doses less than or equal to 500 mg/kg bw per day, there were no effects on clinical signs, gross pathology (Table 22) or caesarean section parameters. There was no treatment-related effect on embryo/fetal toxicity, including malformations, up to 500 mg/kg bw per day.

Table 19. Clinical signs of toxicity in rabbit dams given imazapic during gestation

	Incidence of finding (*n* = 5)				
	0 mg/kg bw per day	175 mg/kg bw per day	350 mg/kg bw per day	500 mg/kg bw per day	700 mg/kg bw per day
Few or no faeces	10	9	10	11	16
Languid	1	1	1	1	4
Anuria	0	0	0	0	2
Moribund sacrifice	0	2	1	1	1
Died on test	1	2	4	4	11

Source: MacKenzie (1992)

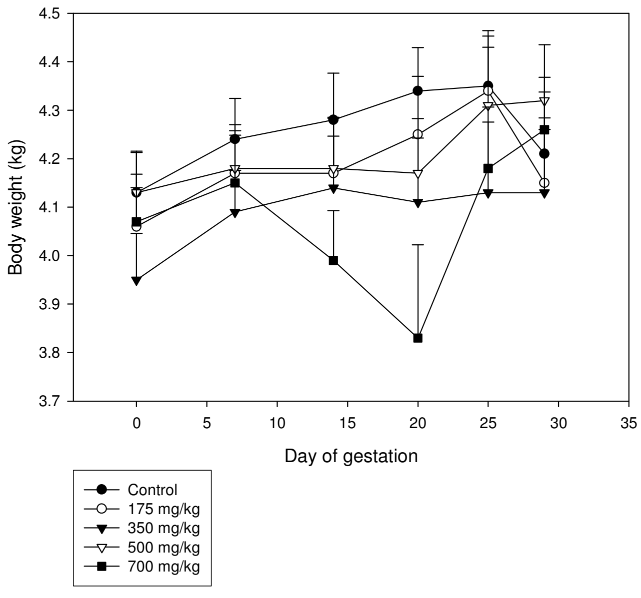
Table 20. Selected body weights of rabbits in a developmental toxicity study

	Body weight (mean ± SD; % change from control)				
	0 mg/kg bw per day	175 mg/kg bw per day	350 mg/kg bw per day	500 mg/kg bw per day	700 mg/kg bw per day
Day 0	4.13 ± 0.353 (*n* = 18)	4.06 ± 0.341 (*n* = 18)	3.95 ± 0.430 (*n* = 20)	4.13 ± 0.363 (*n* = 18)	4.07 ± 0.428 (*n* = 19)
Day 7	4.24 ± 0.358 (*n* = 18)	4.17 ± 0.372(*n* = 18)	4.09 ± 0.376 (*n* = 19)	4.18 ± 0.373 (*n* = 17)	4.15 ± 0.418 (*n* = 18)
Day 14	4.28 ± 0.398 (*n* = 17)	4.17 ± 0.453 (*n* = 16)	4.14 ± 0.440 (↓5%; *n* = 17)	4.18 ± 0.423 (*n* = 16)	3.99 ± 0.410 (↓7%; *n* = 16)
Day 20	4.34 ± 0.369 (*n* = 17)	4.25 ± 0.464 (*n* = 15)	4.11 ± 0.531 (↓5%; *n* = 16)	4.17 ± 0.437 (*n* = 15)	3.83 ± 0.577 (↓12%; *n* = 9)
Day 25	4.35 ± 0.424 (*n* = 17)	4.34 ± 0.465 (*n* = 14)	4.13 ± 0.582 (*n* = 16)	4.31 ± 0.433 (*n* = 13)	4.18 ± 0.335 (*n* = 7)
Day 29	4.21 ± 0.526 (*n* = 17)	4.15 ± 0.503 (*n* = 14)	4.13 ± 0.505 (*n* = 15)	4.32 ± 0.416 (*n* = 13)	4.26 ± 0.285 (*n* = 7)
BWG days 0–7	0.11 ± 0.091	0.10 ± 0.076	0.11 ± 0.124 (*n* = 19)	0.08 ± 0.107 (*n* = 17)	0.12 ± 0.128 (*n* = 18)
BWG days 7–14	0.04 ± 0.096 (*n* = 17)	0.04 ± 181 (*n* = 16)	0.05 ± 0.125 (*n* = 17)	−0.01 ± 0.180 (↓350%; *n* = 16)	−0.17 ± 0.237* (↓575%; *n* = 16)
BWG days 14–20	0.06 ± 0.134 (*n* = 17)	0.04 ± 0.116 (*n* = 15)	0.0 ± 0.174 (*n* = 16)	−0.07 ± 0.229 (↓217%; *n* = 15)	−0.18 ± 0.207* (↓400%; *n* = 9)
BWG days 20–25	0.01 ± 0.129 (*n* = 17)	0.10 ± 0.089 (*n* = 14)	0.02 ± 0.133 (*n* = 16)	0.10 ± 0.149 (*n* = 13)	0.14 ± 0.107 (*n* = 7)
BWG days 25–29	−0.14 ± 0.240 (*n* = 17)	−0.19 ± 0.332 (*n* = 14)	−0.07 ± 0.164 (*n* = 15)	0.00 ± 0.159 (*n* = 13)	0.09 ± 0.086* (*n* = 7)
Cumulative gain (0–29)	0.09 ± 0.420 (*n* = 17)	0.08 ± 0.404 (*n* = 14)	0.13 ± 0.440 (*n* = 15)	0.23 ± 0.206 (*n* = 13)	−0.17 ± 0.126 (*n* = 7)

BWG: body weight gain; SD: standard deviation; *: $P < 0.05$
Source: MacKenzie (1992)

The NOAEL for maternal toxicity was 350 mg/kg bw per day, based on decreased body weight gain (GDs 7–20) and feed consumption at 500 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 500 mg/kg bw per day, the highest tolerated dose (MacKenzie, 1992).

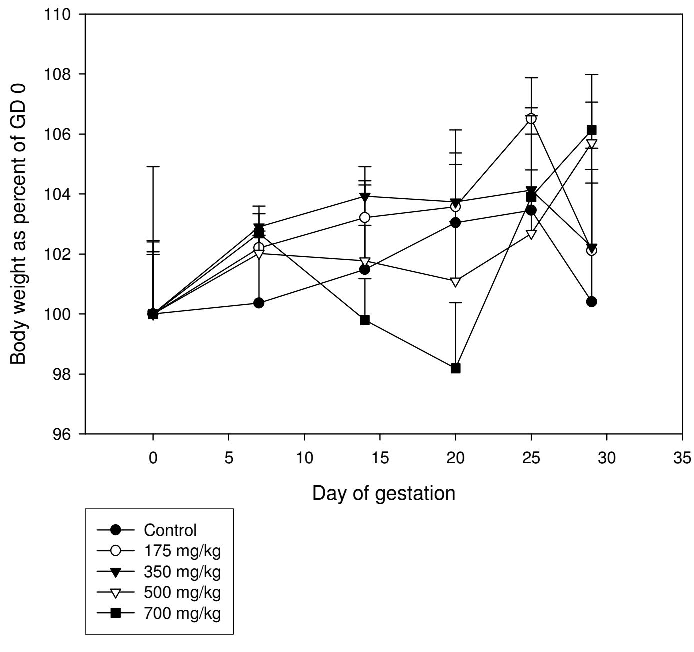
Fig. 3. Effects of imazapic on maternal body weight in a rabbit developmental toxicity study



Values are means ± SEM (SDs provided/sqrt N)

Source: MacKenzie (1992)

Fig. 4. Effects of imazapic on maternal body weight (percentage of GD 0) in a rabbit developmental toxicity study



Absolute body weights expressed as percentage of individual body weight on GD 0

Values are means ± SEM (SDs provided/sqrt N) of respective percentage body weights

Source: MacKenzie (1992)

Table 21. Feed consumption in dams in a rabbit developmental toxicity study

	Feed consumption (mean ± SD; % of control)				
	0 mg/kg bw per day	175 mg/kg bw per day	350 mg/kg bw per day	500 mg/kg bw per day	700 mg/kg bw per day
Day 8	176.9 ± 46.72 (*n* = 16)	152.4 ± 45.76 (*n* = 17)	146.8 ± 115.95 (*n* = 19)	176.2 ± 62.81 (*n* = 16)	131.8 ± 46.67 (*n* = 17)
Day 9	240.6 ± 216.32 (*n* = 17)	164.1 ± 45.70	161.6 ± 81.46	153.1 ± 47.01	112.9 ± 47.40*
Day 10	168.0 ± 76.83 (*n* = 15)	175.0 ± 42.58 (*n* = 16)	161.1 ± 53.43	153.1 ± 62.26	107.6 ± 66.19* (↓53%)
Day 11	189.4 ± 71.54 (*n* = 17)	158.8 ± 39.98	153.7 ± 54.59	153.8 ± 67.61	98.2 ± 71.00* (↓36%)
Day 12	177.6 ± 66.48	153.8 ± 67.91	153.9 ± 55.96 (*n* = 18)	126.9 ± 53.88	77.6 ± 63.50* (↓48%)
Day 13	156.5 ± 54.42	138.8 ± 60.10	154.1 ± 56.69 (*n* = 17)	120.0 ± 65.32	70.0 ± 58.42* (↓55%; *n* = 16)
Day 14	149.4 ± 76.55 (n=16)	132.5 ± 64.55	136.5 ± 63.24	106.9 ± 8.54	56.2 ± 62.60* (↓62%)
Day 15	180.6 ± 67.67	138.8 ± 71.26	132.9 ± 83.05	105.3 ± 84.42* (↓42%; *n* = 15)	43.1 ± 52.63* (↓76%)
Day 16	183.5 ± 51.95 (*n* = 17)	150.6 ± 87.21	137.6 ± 88.64	92.0 ± 64.53* (↓50%)	41.2 ± 59.65* (↓78%)
Day 17	182.5 ± 71.32 (*n* = 16)	153.1 ± 95.41	141.8 ± 100.39	110.7 ± 74.50	52.7 ± 71.66* (↓71%; *n* = 15)
Day 18	176.2 ± 70.04	155.6 ± 87.48	141.8 ± 84.65	108.7 ±8 6.84	70.9 ± 68.91* (↓60%; *n* = 11)
Day 19	163.8 ± 62.17	170.0 ± 92.88	139.4 ± 87.93	112.0 ± 84.28	63.3 ± 72.28* (↓61%; *n* = 9)
Day 20	138.1 ± 75.12	176.0 ± 76.98 (*n* = 15)	127.5 ± 72.24 (*n* = 16)	112.0 ± 76.66	72.2 ± 89.13 (↓48%)
Day 21	159.4 ± 73.16	181.4 ± 78.53 (*n* = 14)	133.1 ± 79.98	109.3 ± 66.28	98.8 ± 99.20 (↓38%; *n* = 8)
Day 22	200.0 ± 157.40	180.7 ± 68.10	130.6 ± 74.79	132.0 ± 74.37	124.3 ± 67.79 (↓38%; *n* = 7)

SD: standard deviation; * $P < 0.05$
Source: MacKenzie (1992)

2.6 Special studies

(a) Neurotoxicity

In an acute neurotoxicity study, imazapic (purity 97.8%) was administered to young adult Wistar (Crl:WI(Han)) rats (10 of each sex per dose) via gavage at a dosing volume of 10 mL/kg bw in an aqueous suspension (drinking-water containing 0.01% Cremophor EL) at a dose level of 0, 200, 600 or 2000 mg/kg bw and then maintained for a 14-day observation period. Feed consumption and body weights were recorded, and a functional observational battery of tests, including a quantitative assessment of motor activity, was performed on all animals predosing, on the day of treatment and on days 7 and 14. Five animals of each sex per dose were subjected to necropsy, postmortem examination, brain weight and dimensions recording, perfusion fixation and preservation of brain,

dorsal root fibres and ganglia, ventral root fibres, spinal cord, eyes, optic, tibial and sciatic nerves, skeletal muscle and gross lesions.

No premature deaths occurred. Clinical signs of toxicity were limited to excess salivation immediately following dosing in males and females at 2000 mg/kg bw (Table 23). The finding was treatment related, but of unknown toxicological significance, and occurred only above the limit dose and resolved within 2 hours of dosing. There were no effects on feed consumption, the functional observational battery, motor activity or postmortem examination.

The NOAEL was 600 mg/kg bw, based on increased salivation in both males and females at 2000 mg/kg bw. The study authors determined the NOAEL to be 2000 mg/kg bw (Buesen et al., 2011a).

In a 13-week neurotoxicity study, imazapic (purity 97.8%) was administered in the diet to groups of 10 male and 10 female Wistar Crl:WI(Han) rats at a target dietary dose level of 0, 100, 300 or 1000 mg/kg bw per day (equal to 0, 97, 287 and 927 mg/kg bw per day for males and 0, 98, 292 and 1038 mg/kg bw per day for females, respectively) for 13 weeks. Feed consumption and body weights were recorded weekly, and a functional observational battery of tests, including a quantitative assessment of motor activity, was performed on all animals predosing and on study days 1, 22, 50 and 85. Five animals of each sex per dose were subjected to necropsy, postmortem examination, brain weight and dimensions recording, perfusion fixation and preservation of brain, dorsal root fibres and ganglia, ventral root fibres, spinal cord, eyes, optic, tibial and sciatic nerves, skeletal muscle and gross lesions. The remaining five animals of each sex per dose were subjected to gross pathology examination.

Fig. 5. Effects of imazapic on maternal feed intake in a rabbit developmental toxicity study

Values are means ± SEM (SDs provided/sqrt N)

Source: MacKenzie (1992)

Table 22. Select gross necropsy findings in a rabbit developmental toxicity study

Finding	Incidence of finding (*n* = 20)				
	0 mg/kg bw per day	175 mg/kg bw per day	350 mg/kg bw per day	500 mg/kg bw per day	700 mg/kg bw per day
Trachea, contains frothy/foamy fluid	0	2	4	2	5
Trachea, dark, reddish-black areas on mucosa	0	0	2	1	2
Trachea, contains blood/red fluid	1	0	0	0	2
Lungs, contain frothy/foamy fluid	0	1	1	0	2
Lungs, contain red fluid	0	0	0	0	1
Lungs, dark red	1	2	1	1	4
Lungs, mottled	0	0	0	0	1
Lungs, consolidated	0	0	0	0	1
Stomach, ulcerated/reddened/ depressed areas	0	0	1	1	5
Thoracic cavity, contains dark, black grainy, thick material	0	0	0	0	2

Source: MacKenzie (1992)

Table 23. Select clinical signs of toxicity in rats given a single oral dose of imazapic in an acute neurotoxicity study

	Males (*n* = 10)				Females (*n* = 10)			
	0 mg/kg bw	200 mg/kg bw	600 mg/kg bw	2 000 mg/kg bw	0 mg/kg bw	200 mg/kg bw	600 mg/kg bw	2 000 mg/kg bw
Salivation	0	0	0	6	0	0	0	7

Source: Buesen et al. (2011a)

There were no effects on clinical signs, the functional observational battery, motor activity counts or postmortem examinations. There were no effects on body weight, body weight gain or feed consumption in males. In females, terminal body weights were decreased at 300 ppm, but there was no dose–response relationship. There was an apparent increase in feed consumption at 1000 ppm in females; however, according to the study authors, this was due entirely to one female and was likely a result of food wastage. The Meeting considered the change incidental (Table 24).

The NOAEL was 927 mg/kg bw per day, the highest dose tested (Buesen et al., 2011b).

Table 24. Mean body weight, body weight gain and cumulative feed consumption data for rats administered imazapic for at least 91 days in a neurotoxicity study

	Mean values ± standard deviation							
	Males (n = 10)				Females (n = 10)			
	0 ppm	100 ppm	300 ppm	1 000 ppm	0 ppm	100 ppm	300 ppm	1 000 ppm
Body weight (g)								
- Day 0	214.79 ± 6.26	216.63 ± 9.94	217.53 ± 4.93	219.81 ± 9.03	160.92 ± 8.02	163.08 ± 3.55	164.41 ± 5.55	158.35 ± 6.62
- Day 91	433.36 ± 29.75	431.15 ± 34.12	442.86 ± 34.72	449.11 ± 30.63	265.95 ± 14.28	255.40 ± 13.93	251.66 ± 16.04	256.16 ± 19.79
% change relative to control[a]	–	*−0.51*	*2.19*	*3.63*	–	*−3.97*	*−5.37*	*−3.68*
Overall body weight gain (g)	218.57 ± 26.98	214.52 ± 26.40	225.33 ± 32.54	229.30 ± 28.87	105.03 ± 9.18	92.32 ± 11.68	87.25 ± 16.30	98.10 ± 15.39
% change relative to control[a]	–	*−1.85*	*3.09*	*4.91*	–	*−12.10*	*−16.93*	*−6.60*
Feed consumption (g/animal)[b]								
- Days 6–7	23.61 ± 2.84	23.49 ± 2.81	23.16 ± 3.82	23.69 ± 1.71	16.39 ± 1.68	15.01 ± 0.78	15.54 ± 1.87	16.28 ± 2.66
% change relative to control	–	*−0.51*	*−1.91*	*0.34*	–	*−8.42*	*−5.19*	*−0.67*
- Days 41–42	22.83 ± 0.75	21.94 ± 0.20	19.38 ± 3.85	22.28 ± 1.16	18.36 ± 3.71	17.07 ± 0.41	17.16 ± 2.01	22.92 ± 6.90
% change relative to control	–	*−3.90*	*−15.11*	*−2.41*	–	*−7.03*	*−6.54*	*24.84*
- Days 62–63	23.53 ± 6.75	21.46 ± 1.27	21.46 ± 4.95	27.04 ± 11.34	20.31 ± 7.59	15.54 ± 1.44	15.44 ± 0.76	19.52 ± 5.28
% change relative to control	–	*−8.80*	*−8.80*	*14.92*	–	*−23.49*	*−23.98*	*−3.91*
- Days 83–84	21.91 ± 3.49	21.90 ± 2.46	20.87 ± 3.66	23.75 ± 2.87	15.13 ± 1.85	16.33 ± 1.94	18.14 ± 2.66	26.03 ± 8.44
% change relative to control	–	*−0.05*	*−4.75*	*8.40*	–	*7.93*	*19.89*	*72.04*
- Days 90–91	19.98 ± 0.96	20.63 ± 0.89	19.18 ± 0.45	20.68 ± 1.10	17.00 ± 0.17	18.54 ± 0.42	18.72 ± 2.09	30.58 ± 3.08
% change relative to control	–	*3.25*	*−4.00*	*3.50*	–	*9.06*	*10.12*	*79.88*

* $P \leq 0.05$

[a] Values may not calculate exactly due to rounding of mean values (values given in the table are based on unrounded means).

[b] Values calculated based on group mean daily feed consumption.

Source: Buesen et al. (2011b)

(b) Immunotoxicity

In an immunotoxicity study, imazapic (purity 99.2%) was administered in the diet to C57BL/6J Rj mice (eight females per dose) at a dose level of 0, 500, 1500 or 5000 ppm (equal to 0, 130, 450 and 1364 mg/kg bw per day, respectively) for 4 weeks. A similar group of mice, given daily oral (gavage) doses of cyclophosphamide monohydrate at 10 mg/kg bw per day for 4 weeks, acted as positive controls. All animals received a sensitizing intraperitoneal inoculation of sheep red blood cells (sRBC) (0.5 mL; 4 × 10^8 sRBC/mL) 6 days prior to termination. Body weights and feed consumption were measured weekly, water consumption was noted via a daily visual inspection of the water bottles, and blood samples for total and differential white blood cell counts were withdrawn at termination. All animals were subjected to necropsy after 4 weeks of treatment, and the weights of spleen and thymus were recorded. Lymphocyte counts and humoral immunity assessment (anti-sRBC

immunoglobulin M [IgM] enzyme-linked immunosorbent assay) were performed on blood collected at study termination.

All animals survived the scheduled treatment period, and there were no treatment-related clinical signs or effects on body weight gain or feed consumption at any dose level.

The parameters used for detection of potential test substance–related alterations in the immune system included (a) the morphology of the immune system as evidenced by lymphoid organ weights (spleen and thymus) and (b) functional parameters comprising the analysis of the primary humoral (IgM response) immune response to sRBCs. None of the parameters mentioned above was affected by treatment with imazapic up to the highest dose level.

Concurrent treatment with the positive control substance, cyclophosphamide (10 mg/kg bw per day by oral gavage), induced clear signs of immunotoxicity, demonstrating the reliability of the test system under the study conditions employed.

The NOAEL for immunotoxic effects was 5000 ppm (equal to 1364 mg/kg bw per day), based on the lack of any morphological or functional change in the elements of the immune system investigated up to the limit dose of 1364 mg/kg bw per day. The NOAEL for systemic effects was 5000 ppm (equal to 1364 mg/kg bw per day), the highest dose tested (Buesen et al., 2011c).

3. Observations in humans

No information was submitted.

Comments

Biochemical aspects

Radiolabelled imazapic administered by oral gavage is rapidly and extensively absorbed, minimally metabolized and excreted primarily in the urine after single low (10 mg/kg bw) or high doses (1000 mg/kg bw) or repeated low doses (10 mg/kg bw per day) over 14 days to rats. Biliary excretion was minimal. The majority of radioactivity was excreted as the parent compound within the first 6 hours post-dosing. Less than 2% of the administered dose was detected in the carcass, with trace amounts detected in blood, kidneys and liver of the high-dose group at 168 hours post-dosing; however, radiolabelled test substance was not detected in any other organs. There was no evidence of accumulation. There were no notable differences in absorption or excretion between the sexes. Imazapic and its metabolites were not excreted in expired air. Parent compound accounted for more than 94% of the administered dose in the urine and 2.3% of the administered dose in the faeces. The metabolites produced from oxidation, reduction and hydrolysis, including CL 263,284 (M715H001 or 5-hydroxymethyl metabolite), CL 280,442 (no common name assigned) and several other unidentified metabolites, accounted for a total of approximately 6% of the administered dose in the urine and faeces.

Toxicological data

Imazapic is of low acute oral toxicity in the rat (LD_{50} > 5000 mg/kg bw) and low acute dermal toxicity in the rabbit (LD_{50} > 2000 mg/kg bw). Imazapic appears to be of low acute inhalation toxicity, but characterization by this route was limited due to the high median particle sizes. In the rabbit, imazapic was non-irritating to the skin and mildly irritating to the eyes. Imazapic was not a dermal sensitizer in guinea-pigs.

Overall, imazapic showed low mammalian toxicity on repeated administration. Most of the rodent studies found no adverse effects, including target organ toxicity, up to the limit dose. The dog and, to a lesser extent, the rabbit were more sensitive than the rat or mouse to imazapic-induced toxicity. The target tissues in the dog were skeletal muscle and, at higher doses, bone marrow.

In a 13-week oral toxicity study in the rat, the NOAEL was 20 000 ppm (equal to 1522 mg/kg bw per day), the highest dose tested.

In a 1-year oral toxicity study in the dog, animals were exposed to 0, 5000, 20 000 or 40 000 ppm (equal to 0, 137, 501 and 1141 mg/kg bw per day for males and 0, 180, 534 and 1092 mg/kg bw per day for females, respectively). There was an increase in lymphocyte and macrophage infiltration in the diaphragm, abdominal and thigh skeletal muscles and an increase in degeneration or necrosis of the abdominal and thigh muscles at doses greater than or equal to 5000 ppm (equal to 137 mg/kg bw per day), which was therefore the LOAEL for this study. The effects observed at the LOAEL were of minimal severity. Grade 1 lesions were seen primarily in one site per animal at the lowest dose tested, in multiple sites per animal at the middle dose and in multiple sites at increased grades at the high dose. However, in the absence of any information on the mode of toxicological action for the effects of imazapic on the muscles of dogs, the effects were considered adverse and potentially relevant to humans.

The chronic toxicity and carcinogenicity of imazapic have been investigated in mice and rats. In the mouse study, there was no evidence of toxicity up to 7000 ppm (equal to 1134 mg/kg bw per day), the highest dose tested. In the rat study, there was no evidence of toxicity up to 20 000 ppm (equal to 1029 mg/kg bw per day), the highest dose tested.

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

Imazapic has been tested in an adequate range of genotoxicity studies in vitro and in vivo. There was no evidence of genotoxicity.

The Meeting concluded that imazapic is unlikely to be genotoxic.

Based on the lack of genotoxicity and the absence of carcinogenicity in mice and rats, the Meeting concluded that imazapic is unlikely to pose a carcinogenic risk to humans.

A multigeneration reproductive toxicity study was performed with imazapic in rats. The NOAEL for reproductive, parental and offspring toxicity was 20 000 ppm (equal to 1205 mg/kg bw per day), the highest dose tested.

In a developmental toxicity study in rats, the NOAEL for maternal and embryo and fetal toxicity was 1000 mg/kg bw per day, the highest dose tested.

In a developmental toxicity study in rabbits, pregnant females were dosed at 0, 175, 350, 500 or 700 mg/kg bw per day; however, the highest dose was not evaluated for embryo and fetal toxicity due to excessive maternal mortality. The NOAEL for maternal toxicity was 350 mg/kg bw per day, based on a loss of body weight and a decrease in body weight gain compared with controls and a decrease in feed consumption at 500 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 500 mg/kg bw per day, the highest dose at which fetuses were evaluated.

The Meeting concluded that imazapic is not teratogenic in rats or rabbits.

In an acute neurotoxicity study in rats, animals were given imazapic as a single oral gavage dose at 0, 200, 600 or 2000 mg/kg bw. The NOAEL was 600 mg/kg bw, based on increased salivation in males and females at 2000 mg/kg bw.

In a 13-week dietary neurotoxicity study in rats, the NOAEL was 927 mg/kg bw per day, the highest dose tested.

In a 28-day dietary immunotoxicity study in mice, no effects on IgM response to sRBCs or any other signs of immunotoxicity were observed at 5000 ppm (equal to 1364 mg/kg bw per day), the highest dose tested.

Human data

No specific information on the effects of imazapic on production plant workers or others was available. There are no reports of poisoning cases with imazapic.

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

Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) of 0–0.7 mg/kg bw on the basis of the LOAEL of 5000 ppm (equal to 137 mg/kg bw per day), the lowest dose tested, for effects on skeletal muscles in a 1-year study of toxicity in dogs. A safety factor of 200 was applied, with an additional safety factor of 2 being used to account for the use of a LOAEL instead of a NOAEL; the effects observed at the LOAEL were of minimal severity. No adverse effects were observed in chronic studies in rats or mice, up to the limit dose.

The plant metabolite CL 189,215 is the glucoside conjugate of the 5-hydroxymethyl imazapic, which occurs as a minor metabolite in rats. The Meeting concluded that this plant metabolite would be covered by the ADI for imazapic.

The Meeting concluded that it was not necessary to establish an acute reference dose (ARfD) for imazapic 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. The effects observed in the acute neurotoxicity study occurred above 500 mg/kg bw.

Levels relevant to risk assessment of imazapic

Species	Study	Effect	NOAEL	LOAEL
Mouse	Two-year study of toxicity and carcinogenicity[a]	Toxicity	7 000 ppm, equal to 1 134 mg/kg bw per day[b]	–
		Carcinogenicity	7 000 ppm, equal to 1 134 mg/kg bw per day[b]	–
Rat	Thirteen-week study of toxicity[a]	Toxicity	20 000 ppm, equal to 1 522 mg/kg bw per day[b]	–
	Two-year study of toxicity and carcinogenicity[a]	Toxicity	20 000 ppm, equal to 1 029 mg/kg bw per day[b]	–
		Carcinogenicity	20 000 ppm, equal to 1 029 mg/kg bw per day[b]	–
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	20 000 ppm, equal to 1 205 mg/kg bw per day[b]	–
		Parental toxicity	20 000 ppm, equal to 1 205 mg/kg bw per day[b]	–
		Offspring toxicity	20 000 ppm, equal to 1 205 mg/kg bw per day[b]	–
	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	350 mg/kg bw per day	500 mg/kg bw per day
		Embryo and fetal toxicity	500 mg/kg bw per day[b]	–
Dog	One-year study of toxicity[a]	Toxicity	–	5 000 ppm, equal to 137 mg/kg bw per day[d]

LOAEL: lowest-observed-adverse-effect level; NOAEL: no-observed-adverse-effect level

[a] Dietary administration.
[b] Highest dose tested.
[c] Gavage administration.
[d] Lowest dose tested.

Estimate of acceptable daily intake

0–0.7 mg/kg bw

Estimate of acute reference dose

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 imazapic

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid; extensive
Dermal absorption	No data
Distribution	Rapidly eliminated; highest residues in blood, bone, carcass and liver in males and females and fat and kidneys in females
Potential for accumulation	No evidence of accumulation
Rate and extent of excretion	Largely complete within 48 h; primarily via urine (94.5–102%) with < 5% via faeces
Metabolism in animals	Mostly excreted unchanged
Toxicologically significant compounds in animals, plants and the environment	Imazapic, 5-hydroxymethyl metabolite
Acute toxicity	
Rat, LD_{50}, oral	> 5 000 mg/kg bw
Rabbit, LD_{50}, dermal	> 2 000 mg/kg bw
Rat, LC_{50}, inhalation	No reliable data
Rabbit, dermal irritation	Non-irritating
Rabbit, ocular irritation	Mildly irritating
Dermal sensitization	Not sensitizing (Buehler method in guinea-pigs)
Short-term studies of toxicity	
Target/critical effect	Skeletal muscle
Lowest relevant oral NOAEL	< 137 mg/kg bw per day, the lowest dose tested (dog)
Lowest relevant dermal NOAEL	1 000 mg/kg bw per day (rabbit)
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	No long-term effects up to limit dose
Lowest relevant oral NOAEL	1 029 mg/kg bw per day, the highest dose tested (rat)
Carcinogenicity	Not carcinogenic

Genotoxicity	
	Not genotoxic
Reproductive toxicity	
Target/critical effect	No reproductive toxicity
Lowest relevant parental NOAEL	1 205 mg/kg bw per day, the highest dose tested
Lowest relevant offspring NOAEL	1 205 mg/kg bw per day, the highest dose tested
Lowest relevant reproductive NOAEL	1 205 mg/kg bw per day, the highest dose tested
Developmental toxicity	
Target/critical effect	Body weight and feed consumption in dams
Lowest relevant maternal NOAEL	350 mg/kg bw per day (rabbit)
Lowest relevant embryo/fetal NOAEL	500 mg/kg bw per day, the highest dose tested (rabbit)
Neurotoxicity	
Acute and subchronic neurotoxicity NOAEL	Salivation: 600 mg/kg bw
Immunotoxicity	
	Not immunotoxic
Medical data	
	No studies submitted

LC_{50}: median lethal concentration; LD_{50}: median lethal dose; NOAEC: no-observed-adverse-effect concentration; NOAEL: no-observed-adverse-effect level

Summary

	Value	Study	Safety factor
ADI	0–0.7 mg/kg bw	One-year oral toxicity study (dog)	200
ARfD	Unnecessary	—	—

ADI: acceptable daily intake; ARfD: acute reference dose

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IMAZAPYR

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Explanation

Imazapyr (Fig. 1) is the International Organization for Standardization–approved name of 2-[(*RS*)-4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl]nicotinic acid (International Union of Pure and Applied Chemistry), with Chemical Abstracts Service No. 81334-34-1. Imazapyr is a herbicide used for the control of grasses and broadleaf weeds in a variety of crops, including major uses in soya bean, sunflower, rice, maize, sugar cane, rape, wheat and non-crop areas such as vegetation management and forestry and minor uses in tobacco and oil palm. Imazapyr kills weeds by inhibiting the activity of the plant-specific enzyme acetohydroxyacid synthase, which catalyses the production of three branched-chain amino acids (valine, leucine and isoleucine) required for protein synthesis and cell growth. The rate of plant death is usually slow (several weeks) and is likely related to the amount of stored amino acids available to the plant.

Fig. 1. Chemical structure of imazapyr

Imazapyr 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 daily intake

1. Biochemical aspects

1.1 Absorption, distribution, metabolism and excretion

Rats

To determine the excretion pattern in rats after oral dosing with [^{14}C]imazapyr, a pilot study was undertaken. Each of four Crl:CD (SD) BR rats (two of each sex, 6–10 weeks old, weighing 150–250 g) was given a single dose of [^{14}C]imazapyr at a nominal rate of 10 mg/kg body weight (bw) by gavage. After dosing, the rats were individually housed in glass metabolism cages. Urine, faeces and expired air were collected daily for 3 days following dosing and analysed quantitatively for radioactive residues of [^{14}C]imazapyr. [^{14}C]Imazapyr used in this study had a specific activity of 0.39 MBq/mg and a radiochemical purity of 95.25%. The results showed that over a 3-day period, approximately 70–75% of the [^{14}C]imazapyr-related radioactivity was excreted in the urine and cage rinse, about 14–25% was excreted in the faeces and 0.05–0.15% was in the expired air. The excretion pattern was similar in both male and female rats. An average of about 90% of the dosed radioactivity was excreted within the first 24 hours. It was concluded that imazapyr was rapidly excreted from the body of the rat. The amount of radiocarbon found in the expired air was essentially "nil", leading to the conclusion that further metabolism studies of [^{14}C]imazapyr in rats need not be conducted in a closed system.

The study was GLP compliant, and a quality assurance (QA) statement was attached (Wu, 1992).

The definitive rat metabolism study was conducted to examine the absorption, distribution, metabolism and excretion of imazapyr, to aid the evaluation of test results from toxicological studies and extrapolation of data from experimental animals to humans. [^{14}C]Imazapyr labelled with ^{14}C at the 6-carbon position of the pyridine ring was used in this study. The position of ^{14}C label in imazapyr was considered to be metabolically stable, which allowed determination of the metabolic profile in rats. The ^{14}C tracer had a specific activity of 1.6 MBq/mg, with a radiochemical purity of 96.8% and a chemical purity of 93.4%. This material was diluted with non-radiolabelled imazapyr that had a chemical purity of 99.5%, to give final specific activities of 0.35 MBq/mg for the low dose and 0.01 MBq/mg for the high dose used in this study. The radiolabelled imazapyr was dissolved in corn oil for all oral dosing solutions, and the intravenous solution was prepared in normal saline. The study was conducted using both male and female Sprague-Dawley Crl:CD rats (53–66 days old; weight 150–250 g). The animals were divided into five groups. The sex and group distribution, route of administration and target doses are shown in Table 1.

Urine and faeces were collected from all treatment groups at specified time intervals, and selected samples were analysed for parent material and metabolites. Animals were sacrificed 7 days after administration of the test substance, and radiocarbon distributions were determined in select tissue samples. The collection of expired air in the definitive phase studies (Groups A, B, C and D) was not necessary, as the pilot study (Wu, 1992) had shown only trace amounts of radiocarbon in the expired air over a 3-day period.

The results revealed that imazapyr is quickly absorbed and excreted following administration to rats. The percentage of [^{14}C]imazapyr dose absorbed in the single oral dose group and multiple oral low-dose groups was calculated by comparing the percentage of the dose excreted in urine by these groups with the percentage of the dose excreted in urine by the intravenous low-dose group. The

Table 1. Details of the rat metabolism study

Group	Route of administration	Target dose (mg/kg bw)	Number of males	Number of females
A	Single oral low via gavage	9.5	5	5
B	Single oral high via gavage	924	5	5
C	Repeated oral low via gavage	14 days of unlabelled material (~10 mg/kg bw) followed by a single [^{14}C]imazapyr dose at 9.26 mg/kg bw	5	5
D	Single intravenous low	9.94	5	5
E (control)	Single oral via gavage (vehicle only)	Corn oil only	2	2

Source: Wu (1994)

Table 2. Route of excretion and total recovery of imazapyr in rats after 7 days

Group	Target dose (mg/kg bw)	Route of administration	Sex of animal	% of administered dose ± standard deviation			
				Urine[a]	Faeces	Carcass	Total
A	9.5	Single oral low	M	81.1 ± 5.9	26.4 ± 2.7	0.2 ± 0.2	107.7 ± 4.3
			F	78.5 ± 13.4	25.9 ± 9.2	0.1 ± 0.1	104.5 ± 6.3
B	924	Single oral high	M	78.8 ± 5.3	21.1 ± 5.3	< 0.1	99.9 ± 1.9
			F	76.7 ± 7.1	21.9 ± 6.2	0.1 ± 0.03	98.7 ± 2.6
C	9.26	Repeated oral low	M	75.0 ± 2.9	31.1 ± 3.3	0.1 ± 0.1	106.2 ± 4.5
			F	67.8 ± 4.6	33.2 ± 6.8	0.2 ± 0.07	101.2 ± 4.5
D	9.94	Intravenous	M	94.6 ± 7.7	6.6 ± 5.6	0.1 ± 0.02	101.3 ± 2.4
			F	86.5 ± 6.4	5.5 ± 4.1	0.1 ± 0.06	92.1 ± 3.8

F: female; M: male

[a] Includes cage wash and cage wipe.

Source: Wu (1994)

calculated absorption percentages were 80.6% and 80.0% for oral low-dose (Group A) males and females, respectively, and 75.6% and 71.4% for the repeated oral dose (Group C) males and females, respectively. For the oral high-dose group (Group B), the absorption percentages were 84.4% and 81.7% for males and females, respectively.

The majority of the administered dose was excreted through the urine and, to a lesser degree, through faeces. The test substance was mostly excreted as intact imazapyr, indicating a low level of biochemical alteration of the parent compound. Trace levels of polar and non-polar metabolites were formed and excreted in the urine and faeces. Only trace amounts of residues were detected in the liver and kidneys of the high-dose group.

Urine was the major route of ^{14}C excretion (67.8–94.6%) (Table 2). Most of the elimination occurred within the first 24 hours after dosing (56.9–90.6%). The total level of ^{14}C radioactivity recovered in faeces ranged from 5.5% to 33.2% (Table 2), with a majority also eliminated within the first 24 hours (3.0–23.8%). Regardless of the treatment regimen, [^{14}C]imazapyr was rapidly eliminated by the rats, primarily through urine.

From the above, it can be concluded that regardless of the treatment regimen, imazapyr was eliminated rapidly by rats, and urine was the primary elimination route. The residue levels were below the limit of detection in tissues of rats dosed at 9.5 or 924 mg/kg bw, except for kidneys and liver from the high-dose group and ovaries from the multiple-dose group. There were no appreciable sex-related differences in absorption, elimination or distribution of radioactivity for rats dosed orally with

[^{14}C]imazapyr. The parent imazapyr was metabolized only to a limited degree and represented the major radiocarbon fraction in urine, faeces and examined tissues. Trace levels of polar and non-polar metabolites were formed and excreted in the urine and faeces.

The proposed metabolic pathway of imazapyr is shown in Fig. 2.

Fig. 2. Metabolic pathway of imazapyr

CL 243,997

CL 252,974

CL 60,032

The study was GLP compliant, and a QA statement was attached (Wu, 1994).

The absorption, distribution, elimination and biotransformation of imazapyr were studied in male rats dosed orally with [^{14}C]imazapyr. Fifteen male Sprague-Dawley rats (age not specified), each weighing approximately 225 g, were housed in individual metabolism cages (Acme Metal Products, Inc., Chicago, Illinois) constructed to facilitate the separate collection of urine and faeces. Twelve rats were treated with [^{14}C]imazapyr at a dose of 4.4 mg/kg bw per day, and the remaining three rats were used as control animals. Three treated rats were sacrificed at each time interval (1, 2, 5 and 8 days) after dosing. One control rat was sacrificed on the 5th day, and two were sacrificed on the 8th day.

Approximately 87.2%, 93.3% and 94.9% of the total administered dose was rapidly excreted in the urine and faeces in 1, 2 and 4 days after treatment, respectively. Elimination of the administered dose was essentially completed by day 6 and accounted for 95.1% of the total dose. Overall recovery from the urine, faeces and cage rinse was 98.0% of the total administered dose by day 8 after treatment: 58.8% in urine, 36.3% in faeces and 2.9% in cage rinse. After 1 day, about 55.3% of the dose was excreted in the urine, and about 31.9% was excreted in the faeces. The half-life of imazapyr

in the rat was less than 1 day. The tissue residue level was less than 0.01 parts per million (ppm) on day 8.

The study was not GLP compliant, nor was any QA statement attached (Mallipudi, 1983).

2. Toxicological studies

2.1 Acute toxicity

The results of acute toxicity studies are summarized in Table 3. All these studies were conducted in compliance with GLP, and/or a QA statement was attached.

Table 3. Summary of acute toxicity studies with imazapyr

Species	Strain	Sex	Route	Batch no. and purity	LD_{50} (mg/kg bw) / LC_{50} (mg/L)	Reference
Rat	Sprague-Dawley Crl:CD®(SD)BR	M + F	Oral	AC 11574-27 98.8% w/w	> 5 000[a]	Lowe (1998)
Rabbit	New Zealand White	M + F	Oral	Not available	4 800 (probit analysis)	Kynoch (1983a)
Dog	Beagle	M + F	Oral	AC 4866-62 99.5%	> 5 000[b]	Fischer (1986)
Rat	Sprague-Dawley (HC/CFY)	M + F	Dermal	SCR 481/208 95.9%	> 2 000	Kynoch (1983b)
Rabbit	New Zealand White	M + F	Dermal	AC 4866-62 99.5%	> 2 000	Fischer (1990a)
Rat	Sprague-Dawley	M + F	Inhalation (4 h) MMAD ≤ 10 µm	AC 4361-97 93%	Actual gravimetric > 1.3 mg/L (nominal 5.1 mg/L)	Voss (1983)
Rabbit	New Zealand White	M	Skin Irritation	AC 4866-62 99.5%	Not irritating	Fischer (1990b)
Rabbit	New Zealand White	M	Eye irritation	AC 4866-62 99.5%	Irritating to eye	Fischer (1990c)
Guinea-pig	Hartley	M	Skin sensitization	AC4361-97 93%	Not sensitizing	Ledoux (1983)

F: female; LC_{50}, median lethal concentration; LD_{50}, median lethal dose; M: male; MMAD: mass median aerodynamic diameter; w/w: weight per weight

[a] The highest dose tested. The predominant clinical sign is salivation in male rats only.

[b] Signs of toxicity limited to emesis at 1–1.5 hours after dosing.

2.2 Short-term studies of toxicity

(a) Oral administration

Short-term studies of oral toxicity were conducted in rats and dogs.

Rats

In a range-finding study, imazapyr (purity 93%; lot no. 4096-27A) was administered to Charles River CD strain albino rats (4 weeks old; five of each sex per dose) via the diet at a concentration of 0, 1000, 5000 or 10 000 ppm (equal to 0, 135, 675 and 1395 mg/kg bw per day, respectively, averaged over both sexes) for 28 consecutive days. Clinical observations were made twice a day. Body weight, feed consumption and water consumption were recorded once a week. Clinical, haematological, gross pathological and histopathological examinations were carried out at the end of the study.

At 10 000 ppm, body weight gain of male rats was significantly suppressed. No test substance–related overt toxic effects were observed at any treatment level during the course of the study. All rats survived the 28-day dosing period.

No test substance–related changes in feed intake, haematological parameters, biochemical parameters or organ weights were observed that could be attributed to ingestion of imazapyr. There were no gross or microscopic pathological changes that were attributable to ingestion of imazapyr.

It was concluded that the no-observed-adverse-effect level (NOAEL) for this study was 5000 ppm imazapyr in the diet (equal to 675 mg/kg bw per day), based on the suppression of body weight gain in males at 10 000 ppm (equal to 1395 mg/kg bw per day).

The study was conducted prior to the implementation of GLP (Fischer, 1982).

In a 90-day dietary study, imazapyr (purity 97.2%; lot no. AC 4468-114) was administered to Charles River CD strain albino rats (Sprague-Dawley derived) at a concentration of 0, 1000, 5000 or 10 000 ppm in the diet (equal to 0, 88.6, 438.6 and 879.1 mg/kg bw per day, respectively, averaged over both sexes). At the start of the trial, the rats were approximately 4 weeks old, and the average body weight was in the range of 86–98 g for males and 80–93 g for females. Each dose group had 30 animals of each sex. The animals were observed for toxic signs and morbidity twice daily and given a thorough examination at each weekly weighing. Feed consumption and body weights were recorded weekly. In all animals, haematological and biochemical examinations as well as urine analysis were carried out. All animals were assessed gross pathologically and subjected to a histopathological examination.

No clinical signs of toxicity were observed at any treatment level during the course of the experiment. Some incidental findings occasionally seen among both control and treated rats were hair loss, body sores, malalignment of upper incisors, and reddish or black material on or around the eyes, nose and mouth. Feed intakes for both sexes at all dose levels were comparable. The body weight gains were somewhat decreased, although not significantly ($P < 0.05$), in both sexes at 5000 and 10 000 ppm. No treatment-related changes could be observed in haematological, biochemical or urine analysis parameters in test or control animals. Relative kidney weights were significantly ($P < 0.05$) increased in female rats at 10 000 ppm. Subsequently, histopathological examination failed to reveal any cause for this change. Therefore, the change was considered to be fortuitous. No other significant organ weight changes were observed in either sex at 10 000 ppm. Absolute and relative organ weights for both sexes at 1000 and 5000 ppm were unaffected by ingestion of imazapyr. No test substance–related gross or macroscopic changes were found at necropsy of the test animals.

In view of the above, the NOAEL was 10 000 ppm (equal to 879.1 mg/kg bw per day), the highest dose tested. The study was conducted as per GLP, and a QA statement was attached (Fischer, 1984).

Imazapyr (purity 99.3%; lot no. AC 4866-62) was administered to albino rats (Charles River CD rats, Sprague-Dawley derived) at a dietary concentration of 0, 15 000 or 20 000 ppm (equal to 0, 1336 and 1740 mg/kg bw per day, respectively, averaged over both sexes) in a 13-week oral toxicity study. The study comprised three groups, each containing 10 male and 10 female rats about 4.5 weeks

of age and weighing 100–130 g (males) or 102–120 g (females). The rats were observed daily for signs of overt toxicity, morbidity and mortality. Ophthalmological examinations were conducted prior to the study and at termination. Detailed clinical observations, individual body weights and individual feed consumption were recorded weekly. Haematological, clinical chemistry and urine analysis determinations were performed on all surviving animals at termination. At termination, all surviving animals were subjected to a gross necropsy, and selected organs were weighed. Samples of selected tissues were examined for histopathological evaluation from all test animals.

No overt clinical signs of toxicity or mortality were observed during the study period that could be attributed to administration of the test material. Feed consumption for both male and female rats at all dose levels was generally comparable to or in excess of those of the control group at most measurement intervals. Body weights were increased (not statistically significantly) during the 13-week study period for both sexes at all treatment levels in comparison with those of the control rats. Body weight gain for the treated groups was generally comparable to or in excess of those of the control groups, but total body weight gains for male and female rats that received imazapyr tended to be increased (2.5–6.5%) over those of the control group. Haematology, clinical chemistry and urine analysis parameters were unaffected by treatment with imazapyr. No changes were observed in absolute or relative (to body weight) organ weights that could be attributed to administration of the test material. There were no gross or microscopic pathological observations that were attributable to the treatment in any of the tissues evaluated.

The data for this study support a NOAEL in the rat of 20 000 ppm (equal to 1740 mg/kg bw per day), the highest dose tested.

The study was conducted in compliance with GLP, and a QA statement was attached (Fischer, 1992).

Dogs

In a 1-year study in Beagle dogs (aged 5–6 months and weighing around 6.2–8.4 kg for males and 6.3–8.4 kg for females), four groups of six males and six females were given diets containing imazapyr (purity 99.5%; lot no. AC 4866-62) at a concentration of 0 (control), 1000 (low dose), 5000 (middle dose) or 10 000 ppm (high dose). The study design and average imazapyr doses are shown in Table 4.

Table 4. Study design and doses of imazapyr ingested

Test group	Concentration in diet (ppm)	Dose per animal (study averages) (mg/kg bw per day)[a]		No. of animals assigned	
		Males	Females	Males	Females
1	0	0	0	6	6
2	1 000	30.4 ± 4.4	29.9 ± 3.9	6	6
3	5 000	141.2 ± 19.4	138.5 ± 21.2	6	6
4	10 000	282.1 ± 41.8	293.7 ± 27.8	6	6

[a] Calculated based on feed consumption and body weight data.
Source: Shellenberger, Nolen & Tegeris (1987)

Animals were examined daily for overt signs of toxicity. Body weights were determined prior to treatment, at the start of the study and weekly thereafter; feed consumption was measured daily. Blood biochemical and haematological parameters and urine analyses were determined prior to dosing, at 6 weeks and at 3, 6 and 12 months. Ophthalmological examinations were performed during the pretest period and at 6 and 12 months. All animals were necropsied for a complete gross pathological and histopathological evaluation and determination of absolute and relative organ weights.

No clinical signs were observed during the study that could be related to the test chemical. All clinical signs were seen in dogs of all dose groups and therefore were considered incidental. All dogs survived to terminal necropsy. Ophthalmological examinations revealed no ocular changes directly attributable to the test compound.

Mean weekly body weights of compound-treated males at all dose levels and of mid-dose (5000 ppm) females were similar to or exceeded those of the controls throughout the study. The only statistically significant differences were increased body weights, which were not toxicologically significant; mean body weights of low-dose (1000 ppm) and high-dose (10 000 ppm) females were slightly lower than those of the controls, but the differences were never statistically significant. Feed consumption of compound-treated males and females was essentially similar to or exceeded that of the controls, and the only statistically significant differences were increased consumption seen frequently in males. Blood biochemical and haematological parameters in males and females revealed no statistically significant differences that were considered to be compound related, as changes observed were not consistent. Occasional statistically significant differences between controls and compound-treated males and females were considered to be random occurrences, as mean values were generally within the normal expected range. Urine analysis parameters were considered to be similar between the control and compound-treated males and females throughout the study. There were no statistically significant differences in mean organ weights or in mean organ to body weight and organ to brain weight ratios between compound-treated and control males or females. The occasional distribution of gross lesions in males and females at termination indicated that these lesions were incidental to chemical treatment. Microscopic examination of all tissues revealed no changes attributable to the test chemical. Lesions observed at necropsy either occurred in near equal incidence in control and compound-treated animals or were found principally in control and mid-dose animals and were considered random occurrences.

In view of the above, the NOAEL in dogs in this 1-year study is 10 000 ppm (equal to 282.1 and 293.7 mg/kg bw per day for males and females, respectively), the highest dose tested.

A QA statement was attached (Shellenberger, Nolen & Tegeris, 1987).

(b) Dermal application

Rabbits

In a 21-day dermal toxicity study, imazapyr technical (purity 93%; lot no. AC 4361-97) was tested in New Zealand White rabbits (weight between 2.31 and 3 kg for males and 2.3 and 3.0 kg for females), with dermal doses of 0, 100, 200 and 400 mg/kg bw of the test substance applied to the back of each animal daily for a period of 6 hours.The area of test material application in both the treated and the control rabbits was covered with a gauze patch moistened with 5 mL of 0.9% saline and subsequently wrapped with an impervious plastic film, which was secured upon itself with an adhesive tape. After 6 hours of exposure, the rabbits were unwrapped, and all remaining test material was removed with soap and water. The rabbits were dried with a clean towel and then returned to their cages. This dosing procedure was repeated 5 days/week for 3 weeks.

No treatment-related clinical signs or mortalities were observed in this study. Two rabbits died with gross evidence of pneumonia, which was confirmed microscopically. There were no treatment-related effects on body weight or estimated feed consumption parameters measured in this study. Haematology, serum chemistry, clinical observation and histopathology revealed no consistent or distinct adverse effects associated with treatment.

In view of the above, there is no indication that imazapyr causes any systemic toxicity at levels of up to 400 mg/kg bw per day when applied topically for 5 days/week for 3 weeks to the back of the rabbit.

A QA statement was attached (Larson, 1983).

2.3 Long-term studies of toxicity and carcinogenicity

Mice

Imazapyr (purity 99.5%; lot no. AC 4866-062) was administered to CD-1 mice (42 days old, weighing about 27 g [males, mean] and 21 g [females, mean] at the initiation of the study) at a dietary concentration of 0, 1000, 5000 or 10 000 ppm for a period of approximately 18 months. The study design and the doses of imazapyr administered are shown in Table 5.

Table 5. Study design and doses of imazapyr administered in a long-term study in mice

	Dietary concentration (ppm)	Dose per animal (study averages) (mg/kg bw per day)		No. of animals assigned	
		Males	Females	Males	Females
1	0	0	0	65	65
2	1 000	126–254	151–303	65	65
3	5 000	674–1 194	776–1 501	65	65
4	10 000	1 301–2 409	1 639–3 149	65	65

Source: Auletta (1988)

The detailed physical examinations for signs of local or systemic toxicity, tests for pharmacological effects and palpation of tissue masses were undertaken pretest and weekly after. Body weights were measured twice pretest, weekly through 14 weeks, biweekly in weeks 16 through 26, monthly thereafter and terminally (after fasting). Feed consumption was estimated pretest, weekly through 14 weeks, biweekly in weeks 16 through 26 and monthly thereafter. For laboratory studies, the experimental outlines are as shown in Table 6.

Table 6. Experimental outlines of laboratory studies in a long-term study in mice

Group	Dietary concentration (ppm)	Number of animals									
		Total		Haematology[a]		Necropsy				Histopathology[b]	
						12 months		18 months			
		M	F	M	F	M	F	M	F	M	F
I	0 (control)[c]	65	65	10	10	10	10	28	36	65	65
II	1 000	65	65	10	10	10	10	35	37	65	65
III	5 000	65	65	10[d]	10	10	10	34	28	65	65
IV	10 000	65	65	10	10	10	10	33	30	65	65

F: female; M: male

[a] Haematology was performed at month 12 and month 18.

[b] Microscopic evaluations were performed on selected tissues for all animals dying accidentally or spontaneously, killed in moribund condition or at the month 12 and month 18 sacrifices.

[c] Control animals received standard laboratory diet.

[d] At the 18-month interval, one animal died accidentally during terminal blood collection; therefore, haematology parameters could be measured only for nine Group III males.

Source: Auletta (1988)

Evaluations of mortality, physical observations, body weights, feed consumption, haematology values and organ weight data revealed no evidence of any effect of test material administration.

Evaluations of gross and microscopic pathology findings revealed a variety of abnormalities commonly seen in old mice. No dose-related differences in incidence or severity of these findings were seen, and no effect of test material administration was apparent. Neoplasms occurred in all groups (control and treated); no effect of test material was seen.

The above data supported a NOAEL of 10 000 ppm (equal to 1301 mg/kg bw per day for males and 1639 mg/kg bw per day for females), the highest dose tested.

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

Rats

In a chronic toxicity study, imazapyr (purity 99.5%; lot no. AC 4866-062) was administered to Sprague-Dawley CD rats (65 of each sex per group) via a diet containing 1000, 5000 or 10 000 ppm (equal to 49.9, 252.6 and 503.0 mg/kg bw per day for males and 64.2, 317.6 and 638.6 mg/kg bw per day for females, respectively) for 2 years. Control animals (65 of each sex per group) received standard laboratory diet only. At the initiation of the treatment, the animals were about 44 days old and weighed in the range of 158–221 g for males and 121–174 g for females. The animals were housed singly under controlled conditions and received standardized diet and water ad libitum. Clinical observations, body weight and feed consumption measurements were performed on all animals pretest and at selected intervals during the treatment period. Ophthalmoscopic examinations were performed on all animals at month 12 and at termination of the study. Haematology, clinical chemistry and urine analysis evaluations were performed on 10 animals of each sex per group at months 3, 6, 12 and 18 and at study termination. After approximately 12 months of treatment, 10 animals of each sex per group were sacrificed; all remaining survivors were sacrificed after 24 months of treatment. Selected organs were weighed and organ to body weight ratio and organ to brain weight ratio were calculated for all animals sacrificed after 12 months of treatment and for 10 animals of each sex per group after 24 months of treatment. Complete gross postmortem examination and histopathological evaluation of selected tissues were conducted on all animals. The experimental outlines for the laboratory studies are shown in Table 7.

Table 7. Experimental outlines for laboratory studies in a long-term study in rats

Group	Dietary concentration (ppm)	Number of animals							
		Total		Clinical laboratory studies (months 3, 6, 12, 18 and termination)		Necropsy and histopathology			
						12 months[a]		24 months[b]	
		M	F	M	F	M	F	M	F
I	0 (control)[c]	65	65	10	10	13	14	52	51
II	1 000	65	65	10	10	13	10	52	55
III	5 000	65	65	10	10	12	12	53	53
IV	10 000	65	65	10	10	13	10	52	55

F: female; M: male

[a] Includes unscheduled deaths prior to month 12.

[b] Includes unscheduled deaths between month 12 and study termination.

[c] Control animals received standard laboratory diet.

Source: Daly (1988)

There were no differences in the number of deaths of either sex among the control and treated groups. Physical observations were of the type commonly seen in laboratory rodents. There were no treatment-related ocular findings noted at the 12-month or terminal ophthalmological examination. Body weight data did not reveal any compound-related changes. Although there were slight increases (some were statistically significant) in mean feed consumption noted in all treated female groups,

generally during the 1st year, they were not considered of toxicological significance. There was no treatment-related alteration noted in the haematology, clinical chemistry or urine analysis. There were no treatment-related findings noted in the mean organ weights, organ to body weight ratios or organ to brain weight ratios of the treated male or female animals at both interim and terminal sacrifices. The results of the gross postmortem examination revealed a random distribution of gross lesions in the treated and control groups. The gross lesions were considered to be incidental changes, with no apparent relationship to the test material. Microscopically, the high-dose males (5/65, 7.69%) showed a higher incidence of C-cell carcinomas of the thyroid glands when compared with the control (1/65, 1.53%), low-dose (1/65, 1.53%) and mid-dose (1/63, 1.58%) males. Among females, one high-dose and one control rat exhibited C-cell carcinomas of the thyroid glands. The incidences of C-cell carcinoma in various groups are reproduced in Table 8. However, incidences of C-cell proliferative lesions in males showed a lack of a stepwise dose–response relationship and a lack of progression from C-cell hyperplasia to adenoma to carcinoma. None of the incidence data in the treated groups was statistically significantly different from those of the control group ($P > 0.05$). A summary of the incidences of proliferative lesions of thyroid glands in male rats is shown in Table 9. The earliest C-cell carcinomas among males were detected microscopically in a control animal at 88 weeks (spontaneous death) and in a high-dose animal at 92 weeks (spontaneous death). The latest were in a mid-dose and a high-dose animal at 106 weeks, both killed at the terminal sacrifice.

Table 8. Incidence of C-cell carcinomas

	Males				Females			
	Group I 0 ppm	Group II 1 000 ppm	Group III 5 000 ppm	Group IV 10 000 ppm	Group I 0 ppm	Group II 1 000 ppm	Group III 5 000 ppm	Group IV 10 000 ppm
No. of animal tissues examined	65	65	63	65	65	65	65	64
C-cell carcinoma number (%)	1 (1.53%)	1 (1.53%)	1 (1.58%)	5 (7.69%)	1 (1.53%)	0 (0%)	0 (0%)	1 (1.56%)

Source: Daly (1988)

Table 9. Summary of the incidence[a] of proliferative lesions of thyroid glands in male rats

	Group I 0 ppm	Group II 1 000 ppm	Group III 5 000 ppm	Group IV 10 000 ppm
No. of thyroid glands examined	65	65	63	65
C-cell hyperplasia	15 (23.10%)	8 (12.31%)	13 (20.63%)	6 (9.23%)
C-cell adenoma	2 (3.10%)	3 (4.62%)	9 (14.29%)	4 (6.15%)
C-cell carcinoma	1 (1.53%)	1 (1.53%)	1 (1.58%)	5 (7.69%)
C-cell adenoma and carcinoma combined	3 (4.62%)	4 (6.15%)	10 (15.87%)	9 (13.85%)
C-cell hyperplasia, adenoma and carcinoma combined	17 (26.15%)	12 (18.46%)	21 (33.33%)	15 (23.08%)

[a] The differences between the incidences of all groups are not statistically significant.

Source: Daly (1988)

A private consultant (Brown, 1988) who reviewed 260 male thyroid glands also confirmed that there were no significant biological differences in the incidences of proliferative C-cell lesions between the control and compound-treated male rats.

Further, the historical control data compiled during the period of 1979–1988 for male Charles River albino CD® rats at Bio/Dynamics Inc. revealed that the overall average incidence of C-cell carcinomas was 6.1%, with a range between studies of 0–22.7%; this range encompasses the incidence of carcinomas (7.69%) for the high-dose males in the 2-year study. Also, the concurrent control value for carcinomas (1/65, 1.5%) was significantly lower than the average historical control value of 6.1% for spontaneous carcinomas in male rats for the periods 1979–1988. The summary of historical control data is shown in Table 10.

Table 10. Historical control data for Charles River albino CD® (Sprague-Dawley) rats: studies terminated between 1979 and 1988 in Bio/Dynamics Inc. laboratories

Thyroid tissue findings	Incidence of finding	
	Males	Females
C-cell adenoma		
- Overall average	5.0% (134/2702)	4.9% (133/2730)
- Average range	0–20.9% (0/99–14/67)	0–15% (0/98–12/80)
C-cell carcinoma		
- Overall average	6.1% (166/2702)	5.3% (144/2730)
- Average range	0–22.7% (0/73–15/66)	0–16.4% (0/73–11/67)

Source: Bio/Dynamics Inc. Laboratories (1991)

From the literature, Suzuki, Mohr & Kemmerle (1979) reported a much higher incidence of C-cell carcinoma in Sprague-Dawley rats: 79% (33/42) in males and 49% (19/39) in females. Other studies also indicated an incidence of 16–40% C-cell carcinomas in other strains of rats, including Long-Evans, Sprague-Dawley, Wistar and wild rats (*Rattus norvegicus*) (VonSchilling, Frohberg & Oettel, 1967; Lindsay, Nichols & Chaikoff, 1968; Boorman, 1976).

In view of the above, it can be concluded that no proliferative lesions of C-cells in this study were deemed to be treatment related. None of the incident data in the treated groups was statistically significantly different from those of the control group. Further, the data do not show any stepwise dose–response relationship, progression from hyperplasia to adenoma to carcinoma or decreased latency in the development of C-cell carcinoma. Taking into account the historical control data and other factors as stated above, these lesions are not considered to be related to the test material.

There was an increased incidence of astrocytomas (a brain tumour) in high-dose male rats in comparison with the controls. No microscopic findings, neoplastic or non-neoplastic, were considered to be related to the test material.

The study was conducted in compliance with GLP, and a QA statement was attached (Daly, 1988).

The above study (Daly, 1988) was extended by a histopathological examination of the brain of male rats. A light microscopic pathological evaluation of the brains (original sections of the forebrain, midbrain and hindbrain and additional sections from the original paraffin blocks) and forebrains (additional sections from new paraffin blocks) of male rats from Groups I and IV was undertaken.

A higher incidence of primary brain tumours (astrocytomas) was observed in Group IV males receiving 10 000 ppm in the diet compared with control, low-dose or mid-dose males. The incidence in Group IV males (7.7%) was not statistically significantly increased ($P > 0.05$) compared with

control males (3.1%) and was close to or within the range of what might be expected in 2-year-old Sprague-Dawley rats, based on a present-day approach to brain tissue evaluation. However, it is noted that 14 brain sections per animal were examined histopathologically in the high-dose group and the control group, whereas 9 sections per animal were examined in the low- and mid-dose groups. There was no evidence of preneoplastic lesions, and all brain tumours tended to be well differentiated and non-expansive beyond the outer contours of the brain. There was no decreased time to the appearance of tumours in treated compared with control males. The incidences of primary brain tumours are presented in Table 11.

Table 11. Incidences of primary brain tumours in male rats

	Group I[a] 0 ppm	Group II[b] 1 000 ppm	Group III[b] 5 000 ppm	Group IV[a] 10 000 ppm
No. of animals per group	65	65	65	65
Total no. of tumours per group	2 (3.1%)	1 (1.5%)	2 (3.1%)	5 (7.7%)
No. of astrocytomas per group	2 (3.1%)	0	1 (1.5%)	5* (7.7%)

*: $P < 0.05$ (Fischer's exact test)

[a] Fourteen brain sections per animal were examined histopathologically. Five of these sections represented two separate pieces of tissue from right and left forebrain.

[b] Nine brain sections per animal were examined histopathologically.

Source: Broxup (1992)

The incidence of astrocytoma (7.7%) in males at 10 000 ppm was slightly above the upper bound of the historical control range of benign astrocytoma (0–1.4%) in CD rats, but within the range of malignant astrocytoma (0–8.8%). No malignant astrocytoma was observed in the study. Recently, glial tumours, including astrocytoma, encountered in carcinogenicity studies have been recognized as malignant due to the difficulty in appreciating their true future biological behaviour (Kaufmann et al., 2012). Therefore, the increase was accepted to be within the historical control range of combined astrocytomas and considered not to be treatment related. One secondary tumour (pituitary adenoma) was observed in each of the control, low-dose and high-dose groups, and two were observed in the mid-dose group. Miscellaneous non-neoplastic lesions were seen. These appeared to be incidental or due to other findings (e.g. compression of the ventral diencephalon by an extracerebral lesion). In view of the above, it is concluded that a higher incidence of primary brain tumours was observed in Group IV males receiving 10 000 ppm compared with control, low-dose or mid-dose males, and there is a strong possibility that this finding is incidental. The summary of historical control data for astrocytomas in Charles River albino CD® (Sprague-Dawley) rats (studies terminated between 1979 and 1988) in Bio/Dynamics Inc. laboratories is shown in Table 12.

Table 12. Historical control data for brain astrocytomas in Charles River albino CD® (Sprague-Dawley) rats: studies terminated between 1979 and 1988 in Bio/Dynamics Inc. laboratories

Tissues/finding	Males	Females
Malignant astrocytoma		
- Overall average	1.2% (34/2769)	1.1% (32/2790)
- Average range	0–8.8% (0/80–6/68)	0–4% (0/75–2/50)
Benign astrocytoma		
- Overall average	0.04% (1/2769)	–
- Average range	0–1.4% (0/80–1/69)	–

Source: Bio/Dynamics Inc. Laboratories (1991)

This histopathological evaluation was conducted according to GLP (Broxup, 1992).

The study by Daly (1988) was further extended by a statistical analysis of adrenal medullary tumours for female rats. Statistical analysis was performed on the histopathological data for adrenal medullary neoplasms, adenoma and carcinoma (Richter, 1992). Fisher's exact test was used to compare each treatment group with the control. The incidences in several treatment groups were too small for a valid Cochran-Armitage test. Analysis was done for adenoma, carcinoma and adenoma combined with carcinoma (total medullary tumours).

The incidence of tumours evaluated is shown in Table 13.

Table 13. Adrenal medullary tumours evaluated in female rats

	0 ppm	1 000 ppm	5 000 ppm	10 000 ppm
No. examined	65	65	65	65
Adenoma	2	2	0	4
Carcinoma	0	1	0	2
Carcinoma + adenoma	2	3	0	6

Source: Richter (1992)

There was no statistically significant increase in adenomas, carcinomas or adenomas plus carcinomas in any treatment group when compared with the control group (Richter, 1992).

The chronic dietary toxicity and carcinogenicity study by Daly (1988) was further extended by a study concerning the proliferative lesions in the adrenal medulla of female rats. The criteria for diagnosis of adrenal medullary tumours were reviewed. The original incidence of these lesions as reported by the study pathologist, Dr. Saulog, is tabulated in Table 14.

Table 14. Proliferative lesions in the adrenal medulla of female rats

	0 ppm	1 000 ppm	5 000 ppm	10 000 ppm
No. examined	65	65	65	65
Hyperplasia	6	5	8	7
Adenoma	1	2	0	6
Carcinoma	0	0	0	1
Total tumours	1	2	0	7

Source: Saulog & Richter (1991)

The slides were submitted to a consulting pathologist, Dr W.R. Brown, by the sponsor. Following his review, there were some differences in diagnosis between the two pathologists. The criteria for diagnosis of adrenal medullary tumours were reviewed and agreed upon. The pathologists reached a consensus on all neoplastic diagnoses. The revised incidence of adrenal medullary proliferative lesions is shown in Table 15.

No neoplastic alterations were observed in the adrenal medulla of 5000 ppm females. The incidences of the medullary adenomas and carcinomas and medullary hyperplasias occurred sporadically in the control and treated groups. The proliferative lesions in the adrenal medulla of females are not deemed to be treatment related.

The study was conducted according to GLP (Saulog & Richter, 1991).

Table 15. Agreed proliferative lesions in the adrenal medulla of female rats (after review)

	0 ppm	1 000 ppm	5 000 ppm	10 000 ppm
No. examined	65	65	65	65
Hyperplasia	5	5	8	8
Adenoma	2	2	0	4
Carcinoma	0	1	0	2
Total tumours	2	3	0	6

Source: Saulog & Richter (1991)

In this 2-year chronic toxicity and carcinogenicity study in rats, no signs of toxicity or carcinogenicity were observed at doses up to 10 000 ppm. No compound-related tumours were observed. Hence, the NOAEL for toxicity and carcinogenicity is 10 000 ppm (equal to 503 mg/kg bw per day), the highest dose tested (Daly, 1988).

2.4 Genotoxicity

Imazapyr was tested for genotoxicity in five in vitro and four in vivo studies. In all studies, imazapyr was found to be negative. All studies complied with GLP, and QA statements were attached. On the basis of these studies, it is concluded that imazapyr is unlikely to be genotoxic. A summary of the studies described is given in Table 16.

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

In a two-generation reproductive toxicity study, imazapyr technical (purity 99.5%; lot no. AC 4866-062) was administered to groups of Sprague-Dawley (CD-CRL: COBS CD(SD) BR) rats (25 of each sex per group; 43 days old and weighing 187–240 g [males] and 128–166 g [females]) at a dietary concentration of 0, 1000, 5000 or 10 000 ppm. After the acclimatization period, F_0 parental animals continuously received the test substance throughout the entire study. Achieved intakes are shown in Table 17. The parental generation animals received a 64-day premating treatment period. On day 0 postpartum, the pups were weighed by sex in each litter of four males and four females, where possible. The F_{1a} pups were weaned on day 21 postpartum, given an external examination and killed on day 21, 22 or 23 postpartum. Any pups with external abnormalities were given an internal examination. Following weaning of the F_{1a} litters, the dams were rested for a minimum of 21 days, and then the mating procedure was repeated, to produce the F_{1b} litters. Those F_{1b} generation rats selected for adult observations were treated from weaning (21 days postpartum) for at least 78 days before placement for mating. The males were then treated after the end of the second mating period until necropsy, whereas the females were treated throughout the gestation, parturition and lactation periods. The F_2 generation pups were killed following weaning.

There were no deaths, clinical signs or pathological findings that were considered related to treatment with imazapyr.

The body weights in the treated groups of males in the F_0 generation were not significantly different from those of the controls. However, occasional statistically significant intergroup differences in body weight gains were observed, which are not attributed to treatment. In treated groups of females of the F_0 generation, neither body weight nor body weight gain differed significantly from values of the control group for the premating period or the gestation and lactation periods of both reproductive phases.

The feed intake in both sexes was unaffected by the treatment. The conception rate for the first mating period of the F_0 generation at the 10 000 ppm level was significantly ($P < 0.05$) decreased. However, taking values for the second mating period into consideration in combination

Table 16. Summary of genotoxicity studies on imazapyr

Study	Strain/species	Substance; concentration/dose	Purity (%)	Result	Reference
In vitro					
Bacterial reverse mutation assay (Ames test)	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537, TA1538; *Escherichia coli* WP2 uvrA	Imazapyr; 0, 50, 158, 500, 1 580, 5 000 µg/plate	93	Negative (±S9)	Allen (1983)
Bacterial reverse mutation assay (Ames test)	*S. typhimurium* TA98, TA100, TA1535, TA1537; *E. coli* WP2 uvrA	Imazapyr; 0, 3.3, 10, 33, 100, 333, 1 000, 2 500, 5 000 µg/plate	99.6	Negative (±S9)	Woitkowiak (2012)
Chromosomal aberration assay in mammalian cells	CHO cells	Imazapyr; 50, 170, 500, 1 700, 5 000 µg/mL	93	Negative (±S9)	Farrow & Cortina (1984)
Unscheduled DNA synthesis	Rat hepatocytes	50, 100, 500, 1 000, 5 000 µg/mL	93	Negative	Sernau & Farrow (1984)
Forward mutation assay in mammalian cells (HPRT test)	CHO cells	Imazapyr technical; 6, 9, 12 mg/mL	93	Negative (±S9)	Johnson & Allen (1984)
In vivo					
Chromosome analysis (micronucleus test)	NMRI mouse	Imazapyr technical; 0, 500, 1 000, 2 000 mg/kg bw (two administrations)	100	Negative	Schwind & Landsiedel (2006)
Chromosome analysis (micronucleus test)	NMRI mouse	Imazapyr technical; 0, 500, 1 000, 2 000 mg/kg bw (single administration)	99.1	Negative	Honarvar (2006)
Dominant lethal assay for male fertility	Rat, Charles River CD	Imazapyr technical; 0, 125, 250, 300, 1 000, 2 000 mg/kg bw per day (administration on days 1 through 5)	93	Negative	Salamon & Enloe (1983a)
Dominant lethal assay	Rat (male), Charles River	Imazapyr technical; 0, 250, 500, 1 000 mg/kg bw per day (administration on days 1 through 5)	93	Negative	Salamon et al. (1984)

CHO: Chinese hamster ovary; DNA: deoxyribonucleic acid; HPRT: hypoxanthine–guanine phosphoribosyltransferase; S9: 9000 × *g* supernatant fraction from rat liver homogenate

with values of the first mating period indicates this not to be of toxicological significance. There was no significant difference in fertility indices, day of mating or other parameters of parental performance (Tables 18–21).

Although there were marked intergroup variations in the incidence of dead pups at birth, which on occasion attained statistical significance, there was no consistent trend indicative of a relationship with treatment. Other parameters of maternal performance, including gestational index, length of gestation, numbers of live pups at birth and sex ratio, were similar to control values.

As far as the F_{1a}, F_{1b}, F_{2a} and F_{2b} pups are concerned, the viability, survival and lactation indices were unaffected, as was the clinical condition of the pups in treated groups. On all but one

Table 17. Study design and range of weekly achieved intakes of imazapyr

Test group	Dietary concentration (ppm)	Dose per animal (mg/kg bw per day)		No. of animals assigned	
		Males	Females	Males	Females
F_0 generation					
1	0	0	0	25	25
2	1 000	43.1–117.0	67.6–131.5	25	25
3	5 000	202.1–586.2	331.4–646.9	25	25
4	10 000	379.8–1 150.6	637.0–1 284.3	25	25
F_{1b} generation					
1	0	0	0	25	25
2	1 000	48.3–142.8	80.2–149.9	25	25
3	5 000	252.8–720.8	404.7–736.1	25	25
4	10 000	483.4–1 471.8	761.3–1 537.1	25	25

Source: Robinson et al. (1987)

Table 18. Group parental performance, F_0 generation, first mating phase

	No. of males	No. of females	No. of females failing to mate	Mean no. of days to mating (SD)	No. of pregnant females	Mating index (%)	Fertility index (%)	Conception rate (%)
Group 1	25	25	2	2.70	22	92	88	95.7
0 ppm				(2.120)				
Group 2	25	25	0	3.40	21	100	84	84
1 000 ppm				(2.739)				
Group 3	25	25	0	3.61	21	100	84	84
5 000 ppm				(2.904)				
Group 4	25	25	0	2.44	18	100	72	72*
10 000 ppm				(1.044)				

SD: standard deviation; *: $P < 0.05$ (Fisher's)
Source: Robinson et al. (1987)

Table 19. Group parental performance, F_0 generation, second mating phase

	No. of males	No. of females	No. of females failing to mate	Mean no. of days to mating (SD)	No. of pregnant females	Mating index (%)	Fertility index (%)	Conception rate (%)
Group 1	25	25	2	2.86	17	92	68	73.9
0 ppm				(2.587)				
Group 2	25	25	2	2.78	21	92	84	91.3
1 000 ppm				(1.704)				
Group 3	25	25	1	2.04	18	96	72	75
5 000 ppm				(0.955)				
Group 4	25	25	1	2.54	21	96	84	87.5
10 000 ppm				(1.141)				

SD: standard deviation
Source: Robinson et al. (1987)

Table 20. Group parental performance, F_0 generation, first and second mating phases

	No. of males/females	No. of males producing at least one pregnancy	No. of females pregnant at least once	No. of males producing two pregnancies	No. of females pregnant twice
Group 1 0 ppm	25	22	22	16	16
Group 2 1 000 ppm	25	23	23	19	19
Group 3 5 000 ppm	25	22	22	16	17
Group 4 10 000 ppm	25	22	22	16	16

Source: Robinson et al. (1987)

Table 21. Group parental performance, F_{1b} generation, first and second mating phases

	No. of males/females	No. of males producing at least one pregnancy	No. of females pregnant at least once	No. of males producing two pregnancies	No. of females pregnant twice
Group 1 0 ppm	25	23	23	19	19
Group 2 1 000 ppm	25	21	22	18	18
Group 3 5 000 ppm	25	23	23	21	21
Group 4 10 000 ppm	25	22	22	17	17

Source: Robinson et al. (1987)

occasion, the pup body weights in the treated groups were not significantly different from control values. There were no pathological findings related to the treatment.

In view of the above, it can be concluded that treatment of groups of male and female rats of both the F_0 and F_{1b} generations with imazapyr at a dose of 1000, 5000 or 10 000 ppm in the diet did not cause any significant effects upon mortality, clinical condition, body weight, feed consumption or pathological status. There were also no significant adverse effects upon reproductive performance or development of the pups in the F_{1a}, F_{1b}, F_{2a} or F_{2b} generation.

Based on the results of this two-generation reproductive toxicity study with imazapyr technical in Wistar rats, the NOAEL for parental, reproductive and offspring toxicity was considered to be 10 000 ppm (equal to about 1471.8 mg/kg bw per day), the highest dose tested.

A formal GLP compliance and QA statement was included in the report (Robinson et al., 1987).

(b) Developmental toxicity

Rats

Imazapyr (purity 93%; lot no. AC 4361-97) was administered orally by gavage to groups of 25 pregnant Sprague-Dawley rats from day 6 through day 15 postcoitum. Dose levels of 100, 300 and

1000 mg/kg bw per day were administered as a test article/0.1% Tween 80 deionized water mixture at a dosing volume of 10 mL/kg bw. Dosing volumes were calculated based on each animal's body weight on gestation day 6. Dosage materials were given employing a syringe equipped with a 16 gauge ball-tipped gavage needle. Vehicle control group animals were treated in a similar manner with a 0.1% Tween 80 deionized water solution. Polygamous cohabitation among rats was initiated following completion of the quarantine period. On the following day, the females were examined for signs of copulation (determined by the presence of a copulatory plug in the vagina or sperm-positive results in the vaginal smear). Those females for which copulation was confirmed were assigned to a treatment group, and that day was taken as day 0.

The study design and pregnancy outcomes are shown in Table 22.

Table 22. Design of a developmental toxicity study in rats

Test group	Dose for females (mg/kg bw per day)	Number of bred females	Number of resulting pregnancies[a]
1	0	25	22
2	100	25	24
3	300	25	23
4	1 000	25	22

[a] Only data from gravid females were used for analysis.
Source: Salamon & Enloe (1983b)

All animals were observed at least twice daily for mortality, morbidity and overt signs of toxicity. Body weights were recorded for all females assigned to this study on gestation days 0, 6, 9, 12, 15 and 20 (final sacrifice). Feed consumption was monitored by visual observation on a daily basis. All females were sacrificed on gestation day 20. The gravid uterus was excised, weighed and examined to determine the numbers of implantation sites, resorption sites and fetuses. The number of corpora lutea was also recorded. The thoracic and abdominal organs of each female were examined for gross morphological change. Maternal tissues were retained for possible later histopathological examination.

Each fetus was removed from the chorion and examined for external developmental anomalies. Approximately two thirds of the fetuses from each litter were retained in ethyl alcohol for subsequent examination of their skeletal development. The remaining fetuses were retained in Bouin's fixative and subsequently examined for visceral development.

All statistical analyses of maternal and litter data were performed with the levels of significance at $P < 0.05$ and $P < 0.01$ using analysis of variance (ANOVA), analysis of covariance (ANCOVA) as well as the chi-squared test, Kruskal-Wallis test and Dunnett's multiple comparison test.

All females assigned to this study survived to final sacrifice. Maternal body weights and body weight gains obtained for the treated groups' dams were similar to those in the control group. Salivation, which occurred during the dosing period, was observed in 6/22 gravid females in the 1000 mg/kg bw per day group, as shown in Table 23.

This effect was observed only at variable gestation days and appeared to be a treatment-related effect. However, because the observation was transient in nature and not found in other studies as well as other doses, it was not considered to be an adverse effect. It might be a result of the high dose and the irritating effect of the compound.

No other noteworthy observations were noted for the treated dams. No gross pathological changes were noted that were considered to be a result of the maternal exposure to imazapyr. Reproductive data obtained for the treated dams were similar to those of the controls (Table 24).

Table 23 Maternal observation of salivation

Serial no.	Animal no.	Gestation day on which salivation was observed
1	AE 3673	8
2	AE 3666	9
3	AE 3752	11, 13, 15
4	AE 3653	10
5	AE 3740	13, 14
6	AE 3765	14

Source: Salamon & Enloe (1983b)

Table 24. Reproduction data in a developmental toxicity study in rats [a]

Test group dose (mg/kg bw per day)	No. of pregnant animals		No. of corpora lutea	No. of implantation sites	No. of resorptions		No. of viable fetuses	No. of dead fetuses
					Early	Late		
0	22	Mean	16.3	14.7	0.4	0.0	14.3	0.0
		SD	2.00	2.41	0.50	0.21	2.32	
100	24	Mean	17.4	15.0	0.7	0.0	14.3	0.0
		SD	2.89	3.76	0.92	0.20	3.71	
300	23	Mean	15.9	14.3	0.4	0.0	13.9	0.0
		SD	2.70	2.62	0.66	0.21	2.71	
1 000	22	Mean	14.8	13.8	0.9	0.0	12.9	0.0
		SD	3.12	3.41	1.48		3.60	

SD: standard deviation

[a] No statistically significant intergroup differences were noted.

Source: Salamon & Enloe (1983b)

Mean fetal body weight and crown–rump length data obtained for the imazapyr group fetuses were comparable to those for the control group fetuses. Examinations of the fetal external, skeletal and visceral development revealed no aberrant structural changes that appeared to be the result of maternal exposure to the test article.

Imazapyr was not found to be teratogenic with this test system/study design under the ambient conditions at this facility.

In view of the above, the NOAEL for maternal toxicity was 300 mg/kg bw per day, based on salivation observed in 6/22 gravid females at 1000 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 1000 mg/kg bw per day, the highest dose tested.

The study was GLP compliant (Salamon & Enloe, 1983b).

Rabbits

A teratology pilot study was conducted in New Zealand White albino rabbits to establish the exposure levels of imazapyr to be employed in the conduct of a teratology study using animals of the same species. Young female rabbits weighing 3.84–4.26 kg (group mean) (five per group) were treated daily by oral gavage with imazapyr (purity 93%; lot no. AC 4361-97) at 0, 250, 500, 1000 or 2000 mg/kg bw on days 6 through 18 of gestation. The test article was administered as a test article/0.1% Tween 80 deionized water mixture. Immediately after copulation, the bred doe was given

an intravenous injection of luteinizing hormone (approximately 1 mg/kg bw) in the marginal ear vein. The study design was as shown in Table 25.

Table 25. Design of a teratology pilot study in rabbits

Test group	Dose for females (mg/kg bw per day)	Number of bred females	Number of resulting pregnancies
1 (vehicle control)	0	5	4
2 (Test I)	250	5	5
3 (Test II)	500	5	3
4 (Test III)	1 000	5	5
5 (Test IV)	2 000	5	5

Source: Salamon & Enloe (1983c)

The animals were examined for mortality, signs and symptoms twice daily. Body weights were recorded on defined gestation days. Feed consumption was monitored by visual observation on a daily basis. On day 28 postcoitum, all females were killed by cervical dislocation and the fetuses removed by caesarean section. Postmortem examinations, including gross macroscopic examination of all internal organs, with emphasis upon the uterus, uterine contents, position of fetuses in the uterus and number of corpora lutea, were performed, and the results were recorded. The uteri (and contents) of all pregnant females were examined to determine the numbers of implantation sites, resorption sites and fetuses. Tissues were retained in 10% neutral buffered formalin as needed to confirm gross findings.

All statistical analyses of maternal data were performed with the levels of significance at $P < 0.05$ and $P < 0.01$ using ANOVA, Kruskal-Wallis test and Dunnett's multiple comparison test.

Two of the five animals in the 250 mg/kg bw per day dose group, four of five animals in the 1000 mg/kg bw per day dose group and all five animals in the 2000 mg/kg bw per day dose group died prior to final sacrifice. Necropsy examinations of these animals revealed stomach lesions (discolorations/depressions) in four animals in the 1000 mg/kg bw per day dose group, two of which were classified by the pathologist as erosive lesions and two as gastric ulcers. Gastric mucosal changes classified by the pathologist as erosive lesions were noted for four of the five animals in the 2000 mg/kg bw per day dose group. The remaining 2000 mg/kg bw per day dose group female that died exhibited gastric and pyloric mucosal discolorations. One of the 250 mg/kg bw per day dose group females that died exhibited fluid in the trachea, and the pathologist noted chronic non-suppurative pneumonia involving 50% of the lung; the other 250 mg/kg bw per day dose group mortality exhibited pulmonary exudate and discoloration, gastric mucosal depressions and ulcers, as noted by the pathologist. Body weight data and observations noted for the treated group does that survived to final sacrifice (gestation day 28) were similar to those for the control group. The mean numbers of corpora lutea, implantation sites, resorption sites and viable fetuses obtained for the sacrificed does revealed no intergroup differences that appeared to be related to the exposure to imazapyr. Gross necropsy examinations performed upon the does that survived to final sacrifice revealed no alterations that could be correlated with the exposure to the test article.

The imazapyr exposure levels of 1000 and 2000 mg/kg bw per day resulted in maternal death. This toxicity appears to have been due to local effects of the compound on the gastrointestinal tract. There were no indications of any exaggerated pharmacological or embryocidal effects at the imazapyr exposure level of 250 or 500 mg/kg bw per day. In view of the above, the maternal NOAEL in this pilot study was 500 mg/kg bw per day, based on the maternal deaths at 1000 mg/kg bw per day and above.

The study was GLP compliant, and a QA statement was attached (Salamon & Enloe, 1983c).

In a study of developmental toxicity, groups of 18 female New Zealand White albino rabbits were given imazapyr (purity 93%; lot no. 4361-97) at a dose of 0, 25, 100 or 400 mg/kg bw per day by gavage on gestation days 6–18. The test article was administered as a test article/0.1% Tween 80 deionized water mixture orally via gavage. At the time of procurement, the rabbits were young, with a group mean weight of 3.92–4.07 kg. Each female was placed in the male's cage (1 : 1 pair) and allowed to remain until successful copulation was observed or until the female's rejection of the male was apparent. Immediately following copulation, the bred doe was given an intravenous injection of luteinizing hormone (approximately 1 mg/kg bw) in the marginal ear vein. The study design was as shown in Table 26.

Table 26. Design of a developmental toxicity study in rabbits

Test group	Dose for females (mg/kg bw per day)	Number of bred females	Number of resulting pregnancies
1 (vehicle control)	0	18	17
2 (TI)	25	18	18
3 (TII)	100	18	16
4 (TIII)	400	18	17

Source: Salamon, Stephen & Enloe (1983)

The animals were observed twice daily for mortality, morbidity and overt signs of toxicity. Body weights were recorded on defined gestation days. Feed consumption was monitored by visual observation on a daily basis. All females were killed on gestation day 28. The gravid uterus was excised, weighed and examined to determine the numbers of implantation sites, resorption sites and fetuses. The number of corpora lutea was also recorded. The thoracic and abdominal organs of each female were examined for gross morphological change. Maternal tissues were retained for possible later histopathological examination.

Each fetus was removed from the chorion and examined for external developmental anomalies. Each fetus was examined for internal development, sexed and eviscerated. The heads were removed from approximately one third of the fetuses per litter, retained in Bouin's fixative and subsequently examined. The carcasses of these fetuses as well as all remaining fetuses per litter were preserved in ethyl alcohol and subsequently examined for skeletal structure development.

All statistical analyses of maternal and litter data were performed with the levels of significance at $P < 0.05$ and $P < 0.01$ using ANOVA and ANCOVA as well as the chi-squared test, Kruskal-Wallis test and Dunnett's multiple comparison test.

Maternal body weight data obtained (0, 6, 12, 15, 18, 24 and 28 days of gestation) for the treated groups' does were comparable with those of the control does. No untoward antemortem observations were noted that could be correlated with the exposure to imazapyr. Two animals from the control group and two from the 400 mg/kg bw per day group died during the dosing period; gross pathological findings for these does were limited to pulmonary changes. At study termination, the surviving test animals were found to have a slightly higher incidence of common and expected pulmonary and hepatic changes (Table 27). This increased incidence was not judged to be test article related.

Maternal reproductive data and fetal weight and crown–rump length data obtained for the imazapyr-treated groups were comparable with those for the controls. The examinations of fetal external development revealed that one fetus in the 25 mg/kg bw per day group and four fetuses in the 400 mg/kg bw per day group exhibited anomalies. The 25 mg/kg bw per day group fetus exhibited a short tail. The anomalies noted for the 400 mg/kg bw per day group were one fetus with a kink at the tip of its tail, two fetuses from the same litter with talipes and one anurous fetus with talipes and spina bifida. The incidences of spina bifida in this study were 1/144 fetuses (0.7%) and 1/16 litters (6.3%).

This study was conducted in New Zealand White albino rabbits in 1983. Unfortunately, corresponding historical control data are not available for the laboratory where the study was conducted. However, in two sets of recent historical control data from studies conducted from 2000 to 2003, the spina bifida incidences were in the range of 0–0.5% per fetus and 0–4.2% per litter.

Table 27. Gross pathological findings of lungs and liver of rabbits that died during the dosing period and at study termination

Group/ dose (mg/kg bw per day)	Gross pathological findings of lungs[a]			Gross pathological findings of liver[a]		
	No. of dead showing lesion	No. of sacrificed showing lesion/total no. sacrificed	Findings	No. of dead showing lesion	No. of sacrified showing lesion/ total no. sacrificed	Findings
0	2	2/4	Discoloration, diffuse, red, ~50–75% involved, multiple focal	0	1/4	Discoloration, white surface (multiple focal)
TI: 25	0	4/7	Discoloration, diffuse, red, ~50–75% involved, multiple focal, consolidation	0	2/7	Discoloration, white surface (multiple focal) Exaggerated lobular pattern, diffuse, pale surface
TII: 100	0	4/6	Discoloration, diffuse, red, ~50–75% involved, multiple focal, consolidation	0	1/6	Depression, solitary, white surface, firm, ~0.2 cm
TIII: 400	2	4/7	Discoloration, diffuse, red, ~50–75% involved, multiple focal, consolidation	0	2/7	Discoloration, multiple focal / diffuse, pale, ~75–100%

[a] "Dead" refers to rabbits that died during the dosing period; "sacrificed" refers to rabbits that died at study termination.

Source: Salamon, Stephen & Enloe (1983)

In addition, the left eye of a fetus in the 25 mg/kg bw per day group appeared larger than normal; however, this eye appeared normal in size during the internal examination of the head. The remaining fetuses obtained were judged to be free of grossly apparent external anomalies. There were no consistent, deleterious findings noted during the evaluations of the fetal internal, skeletal or internal head development that were considered to be a result of exposure to imazapyr. Statistical evaluations of the fetal anomaly data revealed no statistically significant differences in the numbers of treated groups' litters with fetuses exhibiting external, internal, skeletal or internal head anomalies when compared with the controls (Table 28).

In view of the above, imazapyr was not found to be teratogenic with this test system/study design under ambient conditions. Accordingly, the NOAEL for maternal and embryo/fetal toxicity for imazapyr was 400 mg/kg bw per day, the highest dose tested.

The study was GLP compliant (Salamon, Stephen & Enloe, 1983).

Table 28. Fetal developmental findings in rabbits[a]

Findings	0 mg/kg bw per day		25 mg/kg bw per day		100 mg/kg bw per day		400 mg/kg bw per day	
	No. of fetuses	No. of litters	No. of fetuses	No. of litters	No. of fetuses	No. of litters	No. of fetuses	No. of litters
Runt[b]	1/108 (0.9%)	1/13 (7.7%)	6/152 (3.9%)	5/17 (29.4%)	3/147 (2%)	3/16 (18.8%)	3/144 (2.1%)	3/16 (18.8%)
Haematoma[b]	0/108	0/13	0/152	0/17	0/147	0/16	2/144 (1.4%)	1/16 (6.3%)
Left eye larger than normal	0/108	0/13	1/152[c] (0.7%)	1/17 (5.9%)	0/147	0/16	0/144	0/16
Short tail	0/108	0/13	1/152 (0.7%)	1/17 (5.9%)	0/147	0/16	0/144	0/16
Anurous	0/108	0/13	0/152	0/17	0/147	0/16	1/144 (0.7%)	1/16 (6.3%)
Slight kink, tip of tail	0/108	0/13	0/152	0/17	0/147	0/16	1/144 (0.7%)	1/16 (6.3%)
Spina bifida	0/108	0/13	0/152	0/17	0/147	0/16	1/144 (0.7%)	1/16 (6.3%)
Talipes								
- Both posterior appendages	0/108	0/13	0/152	0/17	0/147	0/16	1/144 (0.7%)	1/16 (6.3%)
- Both anterior appendages	0/108	0/13	0/152	0/17	0/147	0/16	2/144 (1.4%)	1/16 (6.3%)
13th rib bilateral[b]	37/108 (34.3%)	8/13 (61.5%)	51/152 (33.6%)	14/17 (82.4%)	45/147 (30.6%)	12/16 (75%)	38/144 (26.4%	15/16 (93%)
13th rib unilateral[b]	10/108 (9.3%)	6/13 (46.2%)	23/152 (33.6%)	14/17 (82.4%)	14/147 (9.5%)	8/16 (50%)	13/144 (9%)	9/16 (56.3%)
Absent cauda vertebra	0/108	0/13	0/152	0/17	0/147	0/16	1/144 (0.7%)	1/16 (6.3%)
Assymetrical ossification (sternum)	0/108	0/13	0/152	0/17	1/147 (0.7%)	1/16 (6.3%)	0/144	0/16
Dual ossification centre								
- Sternebrae	0/108	0/13	2/152 (1.3%)	2/17 (11.0%)	2/147 (1.4%)	2/16 (12.5%)	1/144 (0.7%)	1/16 (6.3%)
- Vertebrae	0/108	0/13	0/152	0/17	1/147 (0.7%)	1/16 (6.3%)	0/144	0/16
Irregular ossification pattern								
- Frontal	0/76	0/13	3/108 (2.8%)	1/17 (5.9%)	1/147 (0.7%)	1/16 (6.3%)	0/102	0/16
- Parietal	2/76 (2.6%)	2/13 (15.4%)	5/108 (4.6%)	3/17 (17.6%)	5/105 (4.8%)	4/16 (25%)	1/102 (1%)	1/16 (6.3%)

Findings	0 mg/kg bw per day		25 mg/kg bw per day		100 mg/kg bw per day		400 mg/kg bw per day	
	No. of fetuses	No. of litters	No. of fetuses	No. of litters	No. of fetuses	No. of litters	No. of fetuses	No. of litters
Misshapen vertebra	0/108	0/13	0/152	0/17	0/147	0/16	1/144 (0.7%)	1/16 (6.3%)
Misshapen sternum	0/108	0/13	0/152	0/17	1/147 (0.7%)	1/16 (6.3%)	0/144	0/16
Misshapen underdeveloped sternebra	0/108	0/13	2/152 (1.3%)	2/17 (11.8%)	2/147 (1.4%)	2/16 (12.5%)	1/144 (0.7%)	1/16 (6.3%)
Misaligned caudal vertebra	0/108	0/13	0/152	0/17	1/147 (0.7%)	1/16 (6.3%)	0/144	0/16
Misaligned compressed sacral vertebra	0/108	0/13	0/152	0/17	0/147	0/16	1/144 (0.7%)	1/16 (6.3%)
Extra thoracic vertebral body	0/108	0/13	0/152	0/17	0/147	0/16	1/144 (0.7%)	1/16 (6.3%)
Fused ribs	0/108	0/13	0/152	0/17	0/147	0/16	1/144 (0.7%)	1/16 (6.3%)
Spiny process from lumbar vertebra	0/108	0/13	0/152	0/17	1/147 (0.7%)	1/16 (6.3%)	0/144	0/16
Incompletely ossified								
- Frontal[b]	4/76 (5.3%)	1/13 (7.7%)	6/108 (5.6%)	4/17 (23.5%)	7/105 (6.7%)	4/16 (25%)	7/102 (6.9%)	5/16 (31.3%)
- Frontal and parietal[b]	0/76	0/13	2/108 (1.9%)	2/17 (11%)	4/105 (3.8%)	4/16 (25%)	3/102 (2.9%)	2/16 (12.5%)
- Interparietal[b]	0/76	0/13	2/108 (1.9%)	2/17 (11%)	1/105 (1%)	1/16 (6.3%)	0/102	0/16
- Parietal[b]	1/76 (1.3%)	1/13 (7.7%)	1/108 (0.9%)	1/17 (5.9%)	1/105 (1%)	1/16 (6.3%)	1/102 (1%)	1/16 (6.3%)
- Sternebrae[b]	108/108 (100%)	13/13 (100%)	151/152 (99.3%)	17/17 (100%)	145/147 (98.6%)	16/16 (100%)	143/144 (99.3%)	16/16 (100%)
- Vertebrae[b]	1/108 (0.9%)	1/13 (7.7%)	1/152 (0.7%)	1/17 (5.9%)	0/147	0/16	1/144 (0.7%)	1/16 (6.3%)
Non-ossified								
- Sternebrae[b]	52/108 (48.1%)	9/13 (69.2%)	62/152 (40%)	13/17 (76.5%)	52/147 (35.4%)	14/16 (87.5%)	56/144 (38.9%)	13/16 (81.3%)
- Vertebrae[b]	0/108	0/13	1/152 (0.7%)	1/17 (5.9%)	0/147	0/16	1/144 (0.7%)	1/16 (6.3%)

[a] Nine out of the 100 mg/kg bw per day group fetuses were improperly fixed, and only the head and ribs were examined for developmental alteration. Therefore, those nine fetuses were omitted from those incidences/percentages of skeletal findings that do not include the head or ribs.
[b] Considered to be incidental findings.
[c] Appeared normal during internal examination.
Source: Salamon, Stephen & Enloe (1983)

2.6 *Special studies*

(a) Neurotoxicity

To ascertain the possibility of acute neurotoxic effects of imazapyr, groups of 10 male and 10 female Wistar (Crl:WI(Han)) rats (aged about 49 days and weighing between 195.3 and 238.5 g for males and between 45.0 and 187.3 g for females at dosing) were exposed to imazapyr (purity 99.4%; batch no. 9129H01HA) in a single oral administration by gavage at a dose level of 0 (drinking-water containing 1% carboxymethyl cellulose served as vehicle control; test group 0), 200 (test group 1), 600 (test group 2) or 2000 mg/kg bw (test group 3). The administration volume was 10 mL/kg bw. The animals were observed up to 2 weeks after dosing. The general state of health of the rats was examined daily. Body weight determinations, functional observational batteries and motor activity measurements were carried out 7 days prior to and on the day of the administration as well as 7 and 14 days after administration. At the end of the study, five animals of each sex per test group were fixed by in situ perfusion under deep anaesthesia with isoflurane and subjected to neuropathological examinations. The remaining animals were killed using carbon dioxide without further examination.

For clinical examinations, Dunnett's test (two-sided) was used for comparison of each dose group with the control group for body weight and body weight change, and non-parametric one-way analysis using the Kruskal-Wallis test (two-sided) was used for comparison of each dose group with the control group for faeces, rearing, gripping intensity of front extremities, gripping intensity of hind extremities, foot splay test and motor activity. For neuropathology, non-parametric one-way analysis using the Kruskal-Wallis test (two-sided) was used for comparison of each dose group with the control group for weight parameters.

Clinical examinations, functional observational battery as well as motor activity measurements revealed no test substance–related effects in male and female Wistar rats on the day of test substance administration or on study days 7 and 14 at any dose level. Regarding neuropathology, brain weight determination, necropsy and neuropathology examinations by light microscopy did not reveal any treatment-related neuropathological findings.

From the above findings, it can be concluded that imazapyr when administered as a single oral gavage dose caused no test substance–related adverse effects. Therefore, under the conditions of the present study, the NOAEL was 2000 mg/kg bw, the highest dose tested, for male and female Wistar rats.

The study was GLP compliant, and a QA statement was attached (Buesen et al., 2011a).

In a subchronic neurotoxicity study in Wistar (Crl:WI(Han)) rats, imazapyr (purity 99.4%; batch N 9129HO1HA) was administered to groups of 10 rats (approximately 49 days old) of each sex per dose at a dietary concentration of 0, 100, 300 or 1000 ppm (equal to 0, 89, 272 and 924 mg/kg bw per day for males and 0, 92, 283 and 933 mg/kg bw per day for females, respectively) for at least 91 days (13 weeks). Each group was subdivided into two subsets (A and B) in order to balance the groups for functional observational battery and motor activity measurements. For functional observational battery and motor activity measurements, the animals were tested in randomized order. It was ensured that all animals were examined within the same day after start of dosing so that the time of testing for all animals remained identical. The examinations of all subsets were performed at the same time of day to avoid potential diurnal effects.

Functional observational battery and motor activity measurements were carried out prior to the test substance administration on days −7, 1, 22, 50 and 85. At the end of the study, five animals of each sex per dose group were fixed by in situ perfusion under deep anaesthesia with isoflurane and subjected to neuropathological examinations. The remaining animals were killed using carbon dioxide without further examination.

Clinical examinations, functional observational battery and motor activity measurements revealed no test substance–related neurobehavioural effects at any dose level on study days 1, 22, 50

and 85. Regarding neuropathology, brain weight determination, necropsy and neuropathology examinations by light microscopy did not reveal any treatment-related neuropathological findings.

In view of the above, imazapyr administered at dose-adjusted concentrations over a period of 3 months revealed no adverse neurobehavioural effects in Wistar rats. No test substance–related findings were observed in the neurohistopathology investigation at any dose level.

Under the conditions of this study, the NOAEL for neurotoxicity was 924 mg/kg bw per day for Wistar rats, the highest dose tested.

The study was GLP compliant, and a QA statement was attached (Buesen et al., 2011b).

(b) Immunotoxicity

To determine the immunotoxic potential of imazapyr, imazapyr (purity 99.4%; batch no. 9129H01HA) was administered to female C57BL/6 J Rj mice (49 ± 1 days old) at a dose level of 0 (control; test group 0), 500 (test group 1), 1500 (test group 2) or 5000 ppm (test group 3), equal to 0, 155, 525 and 1668 mg/kg bw per day (mean intake), respectively, via the diet over a period of 4 weeks. At the same time, a positive control group (test group 4) including eight female C57BL/6 J Rj mice received cyclophosphamide monohydrate (purity 100%; batch no. 1362353) by gavage at 10 mg/kg bw per day. All animals were immunized 6 days before blood sampling and necropsy using 0.5 mL sheep red blood cells (4×10^8 sRBC/mL) administered intraperitoneally.

Feed consumption and body weight were determined weekly. The animals were examined for signs of toxicity or mortality at least once a day. Detailed clinical examinations in an open field were conducted prior to the start of the administration period and weekly thereafter. Measurement of the sRBC immunoglobulin M (IgM) antibody titres was performed at the end of the study. Additionally, all animals were assessed by gross pathology, and the organ weights of spleen and thymus were measured.

During the clinical examinations, imazapyr did not cause signs of general systemic toxicity in any test group. No animal died prematurely in the present study. The administration of cyclophosphamide monohydrate as the positive control substance (10 mg/kg bw per day) led to expected effects on feed consumption and body weight. With regard to clinical pathology, the sRBC IgM antibody titres in female mice dosed with the test substance were not affected by treatment. In contrast, the mice dosed with the positive control compound had significantly reduced sRBC IgM antibody titres, which is indicative of immunotoxicity.

Regarding pathology, the test substance led to no pathological findings in female mice. The administration of the positive control substance (10 mg/kg bw per day) led to effects indicative of immunotoxicity (i.e. reduced absolute and relative spleen and thymus weights).

The oral administration of the positive control substance cyclophosphamide monohydrate (10 mg/kg bw per day) led to findings indicative of immunotoxicity. This was represented by lower sRBC IgM antibody titres as well as reduced spleen and thymus weights. Thus, assay sensitivity was verified in the present immunotoxicity study performed in female mice.

Under the conditions of the study, imazapyr did not reveal any signs of immunotoxicity when administered via the diet over a period of 4 weeks to female mice.

Thus, the NOAEL for general systemic toxicity as well as immunotoxicity was identified to be 5000 ppm (equal to 1668 mg/kg bw per day).

The study was GLP compliant, and a QA statement was attached (Buesen et al., 2011c).

(c) Studies on metabolites

Plant metabolism studies with imazapyr were conducted in six different crops: soya bean, maize, sugar cane, oil palm, clover and Bermuda grass. In each crop, it was found that parent imazapyr was the only major compound found. Minor metabolites, including 2,3-pyridine

dicarboxylic acid (PDC), were identified only in clover and Bermuda grass, which are consumed only by livestock. It has been shown that PDC is of comparable acute toxicity (LD_{50}) to imazapyr by the oral and dermal routes of administration. However, a greater number of clinical signs were observed post-dosing for the metabolite than for the parent. As regards skin and mucous membrane irritation, there was not much difference between parent and metabolite. Additionally, PDC is also negative in the in vivo mouse micronucleus test.

Acute toxicity

The acute toxicity of PDC is summarized in Table 29.

Table 29. Acute toxicity of imazapyr metabolite PDC

Species	Strain	Sex	Route	Batch no. / purity	LD_{50} (mg/kg bw)	Reference
Rat	Crl:CD(SD)BR	Male + female	Oral	AC 5561-131 99.1%	> 5 000[a]	Lowe (1990)
Rat	Crl:CD(SD)BR	Male + female	Oral	AC 5561-131 9.1%	> 5 000[a]	Lowe (1997)
Rabbit	New Zealand White	Male + female	Dermal	AC 5561-131 99.1%	> 2 000	Fischer (1990d)
Rabbit	New Zealand White	Male	Skin irritation	AC 5561-131 99.1%	Slightly irritating to rabbit skin	Fischer (1990e)
Rabbit	New Zealand White	Male	Eye irritation	AC 5561-131 99.1%	Irreversibly irritating to rabbit eye	Lowe (1991)

[a] Toxic signs such as decreased activity, epistaxis, diuresis, diarrhoea, urine staining, brown staining of anogenital area, anorexia, chromodacryorrhoea, bloating and piloerection were observed. There was complete recovery by day 6.

Genotoxicity

The genotoxicity of PDC is summarized in Table 30.

Table 30. Genotoxicity of imazapyr metabolite PDC

Study	Test system	Dose (mg/kg bw)	Purity (%)	Results	Reference
In vivo mouse micronucleus test	Crl:NMRI mouse, male (treated twice through oral gavage at a 24 h interval, and samples of bone marrow were taken 24 h after the last treatment; vehicle: corn oil)	500, 1 000 and 2 000	99.7	Negative ±S9	Schulz & Landsiedel (2008)

S9: 9000 × *g* supernatant fraction of rat liver homogenate

Combined repeated-dose toxicity study

To study and detect possible effects of PDC on the integrity and performance of the reproductive system of both sexes of rats, including gonadal function, mating behaviour, conception, gestation and parturition, and also to obtain information about the general toxicological profile,

including target organs and the NOAEL after repeated oral administration, a combined repeated-dose toxicity study of PDC with the reproductive/developmental toxicity screening test in Wistar rats was conducted through oral administration (gavage).

PDC (purity 99.7%; batch no. 080319) was administered orally by gavage to groups of 10 male and 10 female Wistar rats (Crl:WI(Han)), aged about 8–12 weeks (F_0 animals), at a dose level of 0 mg/kg bw per day (drinking-water containing 1% carboxymethyl cellulose served as vehicle control; test group 0), 50 mg/kg bw per day (test group 1), 250 mg/kg bw per day (test group 2) or 1000 mg/kg bw per day (test group 3) (Table 31).

Table 31. Design of a combined repeated-dose toxicity study with reproductive/developmental toxicity screening test on PDC in rats

Test group	Dose (mg/kg bw per day)	Concentration (mg/100 mL)	Dosing volume (mL/kg bw)	Number of animals	
				Males	Females
0	0	0	10	10	10
1	50	500	10	10	10
2	250	2 500	10	10	10
3	1 000	10 000	10	10	10

Source: Buesen et al. (2010)

On clinical examination, signs of general systemic toxicity were observed only in male parental animals at a dose level of 1000 mg/kg bw per day during the premating period, as there were significantly lower body weights accompanied by reduced feed consumption, but not by reduced general condition. Male animals of the 50 and 250 mg/kg bw per day test groups did not show any findings. No test substance–related findings were observed for female animals at any dose level during the entire study period. When compared with the control group, the terminal body weights of F_0 males were significantly decreased to 99%, 98% and 94% ($P < 0.01$) of the control group values at 30, 250 and 1000 mg/kg bw per day, respectively.

One female animal (No. 135) in test group 3 (1000 mg/kg bw per day) was found dead in the 2nd week of the study. This finding was considered to be incidental and not treatment related, as no pathological findings could explain the premature death. In addition, all other animals of test group 3 (1000 mg/kg bw per day) did not show any severe clinical findings.

Concerning clinical pathology, no treatment-related adverse effects were found.

During histopathological examination, male and female animals of test group 3 (1000 mg/kg bw per day) revealed a minimal to moderate increase in incidence of apoptosis in the epithelium of the caecum. In addition, male animals of test groups 2 and 3 (250 and 1000 mg/kg bw per day) showed minimal to mild desquamation of the caecal epithelial cells (Table 32). These findings were not regarded to be treatment related.

The reduction in body weight was possibly related to these caecum findings. Here, males revealed desquamation of the caecal epithelial cells. This finding could have led to disturbances in digestion and/or water retention, which, in turn, may have led to weight loss or reduced weight gain. Furthermore, the caecum of males and females of test group 3 (1000 mg/kg bw per day) revealed an increased incidence of apoptosis. This cell loss (desquamation) and programmed cell death (apoptosis) may not be regarded to be induced by the test substance and adverse in nature. Female animals exclusively showed apoptosis and no shedding/desquamation of the epithelial cells. In the thymus of some test substance–treated animals, an increase of starry sky cells (uptake of apoptotic cells/debris by macrophages) was observed. As there was no clear dose–response relationship (males: 0/2/0/3, females: 0/3/1/2), a treatment-related effect is regarded to be very unlikely.

Table 32. Histopathological findings in caecum in F_0 parental animals administered PDC

	Males				Females			
	0 mg/kg bw per day	50 mg/kg bw per day	250 mg/kg bw per day	1 000 mg/kg bw per day	0 mg/kg bw per day	50 mg/kg bw per day	250 mg/kg bw per day	1 000 mg/kg bw per day
No. of organs examined	10	10	10	10	10	10	10	10
Desquamation of epithelium	0	0	4	7	0	0	0	0
- Grade 1	–	–	4	4	–	–	–	–
- Grade 2	–	–	–	3	–	–	–	–
Apoptosis increased	0	1	0	5	0	0	0	7
- Grade 1	–	–	–	1	–	–	–	1
- Grade 2	–	1	–	3	–	–	–	5
- Grade 3	–	–	–	1	–	–	–	1

Source: Buesen et al. (2010)

The animal that was found dead (animal no. 135) revealed the most severe increase in starry sky cells in the thymus. The appearance of the starry sky cells is known to occur during involution of the thymus, starving or stress. In this animal, the increase in starry sky cells is regarded to have been due to the stress during agony.

Fertility indices for male and female animals were not impaired by test substance administration, even at a dose level of 1000 mg/kg bw per day. In addition, live birth and viability indices of pups in all test groups were not influenced.

Under the conditions of this combined repeated-dose toxicity study with the reproductive/ developmental toxicity screening test in Wistar rats, the NOAEL for systemic toxicity, reproductive performance and fertility in male and female Wistar rats was 1000 mg/kg bw per day, the highest PDC dose tested. The NOAEL for embryo and fetal toxicity was 1000 mg/kg bw per day, the highest PDC dose tested.

The study was GLP compliant, and a QA statement was attached (Buesen et al., 2010).

3. Observations in humans

3.1 Medical surveillance on manufacturing plant personnel

No information was provided.

3.2 Direct observation

No data on exposure of the general public or epidemiological studies are available from the manufacturer, nor have any epidemiological studies performed by third parties been identified.

Comments

Biochemical aspects

Imazapyr is quickly and extensively absorbed following oral administration to rats. There were no substantial sex differences in the absorption, elimination or distribution of radioactivity in rats receiving an oral dose of radiolabelled imazapyr. The majority of the administered dose was

excreted in urine (68–95%) and, to a lesser extent, in faeces (5.5–33%). Most of the elimination occurred within the first 24 hours after dosing (57–91% in urine; 3–24% in faeces). The half-life of imazapyr in the rat was less than 1 day. Imazapyr was excreted mostly unchanged. Trace levels of polar and non-polar metabolites were formed and excreted in urine and faeces. Only trace amounts of tissue residues were detected in the liver and kidneys of the high-dose group, indicating no bioaccumulation.

Toxicological data

The oral LD_{50}s were greater than or equal to 5000 mg/kg bw in rats, rabbits and dogs, but some clinical signs were noted in dogs immediately after dosing. The dermal LD_{50}s in rats and rabbits were greater than 2000 mg/kg bw. Imazapyr appears to be of low acute inhalation toxicity, but characterization by this route was limited due to the high median particle sizes. Imazapyr was irritating to the eye but not irritating to the skin in rabbits. It was not a dermal sensitizer in guinea-pigs.

Repeated-dose toxicity studies in rats and dogs indicate no effects except for reduced body weight gain in a 28-day oral toxicity study in rats, in which there was decreased body weight gain in males at the highest dose tested, 10 000 ppm (equal to 1395 mg/kg bw per day). However, in two 13-week studies in rats, no treatment-related effects were observed up to 20 000 ppm (equal to 1740 mg/kg bw per day), and in a 1-year study in dogs, up to 10 000 ppm (equal to 282.1 mg/kg bw per day).

In two long-term oral toxicity and carcinogenicity studies in mice and rats (18 months in mice, 2 years in rats), no substance-related effects were observed. In the mouse study, no treatment-related effects occurred up to the highest dose tested (10 000 ppm, equal to 1301 mg/kg bw per day). Imazapyr did not show any carcinogenic potential. In rats, the NOAEL was the highest dose tested, 10 000 ppm (equal to 503 mg/kg bw per day). No compound-related tumours were observed. Three supplementary evaluations (two histopathological and one statistical) supported the results of the 2-year rat study.

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

Imazapyr was tested for genotoxicity in an adequate range of assays, both in vitro and in vivo. It showed no evidence of genotoxicity.

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

In a two-generation dietary reproductive toxicity study in rats, the NOAEL for parental and offspring toxicity was 10 000 ppm (equal to about 1471.8 mg/kg bw per day), the highest dose tested. There was also no effect on reproduction at the highest dose tested.

In a study of developmental toxicity in rats treated by gavage, salivation observed in 6/22 gravid females at 1000 mg/kg bw per day was considered to be due to the high dose and the irritating effect of the compound. The NOAEL for embryo and fetal toxicity was 1000 mg/kg bw per day, the highest dose tested.

In a pilot study for developmental toxicity in rabbits, treatment-related maternal toxicity, including death, was observed at 1000 mg/kg bw per day and above. This toxicity appears to have been due to local effects of the compound on the gastrointestinal tract. In the main study, the NOAEL for maternal and embryo/fetal toxicity was 400 mg/kg bw per day, the highest dose tested.

The Meeting concluded that imazapyr is not teratogenic in rats or rabbits.

In acute and subchronic neurotoxicity studies with imazapyr, there was no indication of neurotoxicity (including functional observational battery and motor activity measurements), with NOAELs of 2000 mg/kg bw and 924 mg/kg bw per day, respectively. Both doses were the highest doses tested.

The Meeting concluded that imazapyr is not neurotoxic.

In a 4-week dietary study in mice, no immunotoxic effects were seen up to the highest dose tested, 1668 mg/kg bw per day.

Toxicological data on metabolites and/or degradates

PDC, a minor plant metabolite of imazapyr, is of low acute toxicity by the oral (LD_{50} > 5000 mg/kg bw) and dermal (LD_{50} > 2000 mg/kg bw) routes of administration. However, a greater number of clinical signs were observed post-dosing for the metabolite than for the parent. PDC is negative in the in vivo mouse micronucleus test. In a combined repeated-dose toxicity/reproductive and developmental toxicity study, in which rats were treated by gavage from 2 weeks premating through to 1 week post-mating in males and up to day 4 of lactation in females, the NOAEL for systemic and embryo and fetal toxicity was 1000 mg/kg bw per day, the highest dose tested.

Human data

No information is available on adverse health effects or poisoning in manufacturing plant personnel or in operators and workers exposed to imazapyr.

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

Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) of 0–3 mg/kg bw, derived from a NOAEL of 282 mg/kg bw per day, the highest dose tested, from the 1-year study of oral toxicity in dogs. A safety factor of 100 was applied. Although imazapyr is generally of low toxicity, the Meeting concluded that an ADI was necessary because effects were observed at high doses in the 28-day oral toxicity study in rats.

The Meeting concluded that it was not necessary to establish an acute reference dose (ARfD) for imazapyr 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 imazapyr

Species	Study	Effect	NOAEL	LOAEL
Mouse	Two-year study of toxicity and carcinogenicity[a]	Toxicity	10 000 ppm, equal to 1 301 mg/kg bw per day[b]	–
		Carcinogenicity	10 000 ppm, equal to 1 301 mg/kg bw per day[b]	–
Rat	Short-term studies of toxicity[a,c]	Toxicity	5 000 ppm, equal to 675 mg/kg bw per day	10 000 ppm, equal to 1 395 mg/kg bw per day
	Two-year studies of toxicity and carcinogenicity[a,c]	Toxicity	10 000 ppm, equal to 503 mg/kg bw per day[b]	–
		Carcinogenicity	10 000 ppm, equal to 503 mg/kg bw per day[b]	–

Species	Study	Effect	NOAEL	LOAEL
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	10 000 ppm, equal to 1 471.8 mg/kg bw per day[b]	–
		Parental toxicity	10 000 ppm, equal to 1 471.8 mg/kg bw per day[b]	–
		Offspring toxicity	10 000 ppm, equal to 1 471.8 mg/kg bw per day[b]	–
	Developmental toxicity study[d]	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[d]	Maternal toxicity	400 mg/kg bw per day[b,e]	–
		Embryo and fetal toxicity	400 mg/kg bw per day[b]	–
Dog	One-year study of toxicity[a]	Toxicity	10 000 ppm, equal to 282.1 mg/kg bw per day[b]	–

LOAEL: lowest-observed-adverse-effect level; NOAEL: no-observed-adverse-effect level
[a] Dietary administration.
[b] Highest dose tested.
[c] Two or more studies combined.
[d] Gavage administration.
[e] Maternal deaths occurred at higher doses in the pilot study, likely due to local effects.

Estimate of acceptable daily intake

0–3 mg/kg bw

Estimate of acute reference dose

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 imazapyr

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid; absorption is ~80%
Dermal absorption	No data
Distribution	Widely distributed
Potential for accumulation	None
Rate and extent of excretion	Rapid, 68–90% within 24 h, mainly via urine
Metabolism in animals	Minimal
Toxicologically significant compounds in animals, plants and the environment	Imazapyr

Acute toxicity	
Rat, LD_{50}, oral	> 5 000 mg/kg bw
Rat, LD_{50}, dermal	> 2 000 mg/kg bw
Rat, LC_{50}, inhalation	No reliable data
Rabbit, dermal irritation	Not irritating
Rabbit, ocular irritation	Irritating
Dermal sensitization	Non-sensitizing (Magnusson and Kligman test)
Short-term studies of toxicity	
Target/critical effect	No effect at highest dose tested
Lowest relevant oral NOAEL	282 mg/kg bw per day, the highest dose tested (dog)
Lowest relevant dermal NOAEL	400 mg/kg bw per day, the highest dose tested (rabbit)
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	No effects at highest dose tested
Lowest relevant NOAEL	503 mg/kg bw per day, the highest dose tested (rat)
Carcinogenicity	Not carcinogenic
Genotoxicity	
	Not genotoxic
Reproductive toxicity	
Reproduction target/critical effect	No reproductive effects
Lowest relevant parental NOAEL	1 471.8 mg/kg bw per day, the highest dose tested (rat)
Lowest relevant offspring NOAEL	1 471.8 mg/kg bw per day, the highest dose tested (rat)
Lowest relevant reproductive NOAEL	1 471.8 mg/kg bw per day, the highest dose tested (rat)
Developmental toxicity	
Developmental target/critical effect	None (but maternal deaths observed in pilot study, likely due to local effects)
Lowest relevant maternal NOAEL	400 mg/kg bw per day, the highest dose tested (rabbit)
Lowest relevant embryo/fetal NOAEL	400 mg/kg bw per day, the highest dose tested (rabbit)
Neurotoxicity	
	Not neurotoxic
Other toxicological studies	
Immunotoxicity	Not immunotoxic
Medical data	
	No information on health effects in manufacturing personnel

LC_{50}: median lethal concentration; LD_{50}: median lethal dose; NOAEC: no-observed-adverse-effect concentration; NOAEL: no-observed-adverse-effect level

Summary

	Value	Study	Safety factor
ADI	0–3 mg/kg bw	One-year oral toxicity study (dogs)	100
ARfD	Unnecessary	—	—

ADI: acceptable daily intake; ARfD: acute reference dose

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ISOXAFLUTOLE

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Explanation

Isoxaflutole is the International Organization for Standardization–approved name for 5-cyclopropyl-4-(2-methylsulfonyl-4-trifluoromethylbenzoyl)-isoxazole (International Union of Pure and Applied Chemistry), with Chemical Abstracts Service No. 141112-29-0. The company code for isoxaflutole is RPA 201772. Isoxaflutole is an isoxazole herbicide that is used as a pre-emergent or early post-emergence broadcast treatment for the control of broadleaf and grass weeds. Its primary target in plants is the enzyme 4-hydroxyphenylpyruvate dioxygenase (HPPD); inhibition of the enzyme results in the bleaching of weeds due to the blockage of phenylquinone biosynthesis.

Isoxaflutole 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 daily intake

1. Biochemical aspects

Absorption, distribution, metabolism and excretion of isoxaflutole following a single gavage low dose, high dose and repeated dosing (14 days) have been studied in rats. Fig. 1 shows the radiolabelling position of isoxaflutole used in the absorption, distribution, metabolism and excretion studies in rats.

Fig. 1. [^{14}C]Isoxaflutole (RPA 201772)

* denotes the position of the uniformly labelled phenyl ring

1.1 Absorption, distribution and excretion

In an absorption, distribution, excretion and metabolism study, [^{14}C]isoxaflutole (purity 98.7%) was administered to groups of male and female Sprague-Dawley (CD) rats (five of each sex per dose) by gavage at a single low oral dose (1 mg/kg body weight [bw]), repeated low oral dose (1 mg/kg bw in a 14-day repeated-dose series) and a single high dose (100 mg/kg bw). In addition, pharmacokinetics in blood was investigated using two groups of rats (five of each sex per dose) that received a single oral dose of [^{14}C]isoxaflutole at 1 or 100 mg/kg bw. Urine and faeces were collected at intervals of 0–24, 0–48, 0–96, 0–120, 0–144 and 0–168 hours, and tissues were collected at 168 hours post-dosing. Metabolite analysis was performed on the urine and faeces from animals of all dose groups and on the liver samples of male and female rats in the two low-dose groups (Filaquier, 1994). A separate time course tissue distribution study was conducted in Sprague-Dawley rats. In this study, [^{14}C]isoxaflutole (purity 98.7%) was administered to groups (16 of each sex per dose) of male and female Sprague-Dawley (CD) rats by gavage at a single low oral dose (1 mg/kg bw) and a single high oral dose (100 mg/kg bw). Four rats of each sex per dose were killed at 1, 24, 96 and 168 hours post-dosing, and isoxaflutole-derived radioactivity was measured in various tissues (Valles, 1999).

The total recovery of radioactivity in male and female rats ranged from 97.3% to 100.4% of the administered dose (Table 1). The mean total recovery of radioactivity from the males and females was 98.09%. As there were no data available for isoxaflutole from intravenous administration or bile duct–cannulated rats, the extent of absorption is interpolated from available urinary excretion data. Based on urinary elimination, it appears that about 60%, 67% and 36% of the administered dose were absorbed in the low-dose, repeated low-dose and high-dose groups, respectively (Table 1) in 168 hours. The mean maximum estimated proportion of the dose absorbed was calculated from the radioactivity detected in the urine, cage washes and tissues, which yielded about 73%, 75% and 39% for the low-dose, repeated low-dose and high-dose groups, respectively. The urine was the major route of elimination for the low-dose groups (about 69–74% of the dose), whereas faeces was the major route of elimination for the high-dose group (about 55–63% of the dose). These results suggest

that significant absorption of the test material occurred at the low dose; as the dose increased, there was saturation of absorption, resulting in a major portion of the parent compound being excreted unchanged.

The blood pharmacokinetic parameters for isoxaflutole are shown in Table 2. The maximal concentrations in blood (C_{max}) were achieved between 0.5 and 1 hour post-dosing. The results indicate a direct dose–response relationship with the maximal concentrations of [^{14}C]isoxaflutole found in the whole blood, with an indication of higher levels in the male rats. The time of maximal concentration (T_{max}) appeared shorter for the females, and the elimination phase half-lives were very similar between sexes and dose regimens. The results from blood/plasma pharmacokinetic groups indicated that [^{14}C]isoxaflutole and/or its metabolites have a mean β-phase elimination half-life of about 60 hours, irrespective of the dose level.

The tissue distribution results from the single and repeated low-dose groups indicated that only very low radioactive residue concentrations remained in the tissues 168 hours after dose administration (Table 3).

The liver and the kidney were the only two tissues containing mean residue concentrations above 0.22 µg equivalents (eq)/g, reaching maximum mean levels of approximately 4.6 µg eq/g in the liver and about 3.8 µg eq/g in the kidneys (Table 4). The high-dose group results indicated that the highest mean radioactive residue levels (both sexes) were found in the blood (7.7 µg eq/g), plasma (6.3 µg eq/g), liver (4.6 µg eq/g) and kidneys (3.4 µg eq/g).

Table 1. Recovery of radioactivity in rats in 168 hours after administration of [^{14}C]isoxaflutole

	% of radioactive dose recovered					
	Single low dose		Repeated low dose		Single high dose	
	Males	Females	Males	Females	Males	Females
Tissues	4.33	3.36	2.62	1.44	1.48	1.79
Cage wash	7.68	11.34	6.12	6.4	1.48	0.63
Urine	61.16	58.80	66.65	67.41	31.37	41.20
Faeces	26.06	26.94	24.04	24.72	63.00	55.23
Total recovery	99.23	100.44	99.43	99.97	97.33	98.85
Urinary excretion (urine + cage wash)	68.84	70.14	72.77	73.81	32.85	41.83
Estimated oral absorption (urine + cage wash + tissues)	73.17	73.50	75.39	75.25	34.33	43.62

Source: Filaquier (1994)

Table 2. Blood pharmacokinetic parameters for isoxaflutole in the rat based upon total radioactivity measurements.

Dose (mg/kg bw)	Sex	C_{max} (µg eq/g)		T_{max} (h)		Elimination half-life (h)	
		Mean	SD	Mean	SD	Mean	SD
100	Males	48.10	12.2	0.98	0.40	59.23	2.6
	Females	25.19	5.9	0.67	0.05	60.04	3.9
1	Males	0.50	0.10	1.03	0.35	61.05	6.1
	Females	0.27	0.05	0.52	0.04	59.49	8.4

C_{max}: maximum concentration in blood; eq: equivalents; SD: standard deviation; T_{max}: time to reach C_{max}
Source: Filaquier (1994)

Table 3. Mean percentage of administered radioactivity found in the tissues at 168 hours after administration of isoxaflutole

Group	Mean % of administered radioactivity	
	Males	Females
Single oral high dose	1.48	1.79
Single oral low dose	4.33	3.36
Repeated oral low dose	2.62	1.44

Source: Filaquier (1994)

Table 4. Distribution of radioactivity in rat tissues/organs at 168 hours after administration of [^{14}C]isoxaflutole

Tissue/organ	Isoxaflutole distribution (µg eq/g tissue, or ppm)					
	Single low dose		Multiple low dose		Single high dose	
	Males	Females	Males	Females	Males	Females
Liver	0.498	0.388	0.427	0.172	4.53	4.59
Kidneys	0.223	0.498	0.213	0.221	2.93	3.78
Heart	0.001	0.001	0.001	n.d.	1.85	3.19
Lungs	0.006	0.001	0.004	0.001	2.46	4.00
Brain	n.d.	n.d.	n.d.	n.d.	0.26	0.38
Spleen	n.d.	0.001	0.001	n.d.	1.52	1.91
Muscle	n.d.	n.d.	n.d.	n.d.	1.18	1.44
Fat	0.001	0.001	0.002	0.001	1.71	1.62
Gonads	n.d.	n.d.	n.d.	n.d.	0.80	2.36
GI tract + contents	0.004	0.005	0.007	0.004	2.03	1.60
Bone and marrow	n.d.	n.d.	n.d.	n.d.	0.84	1.09
Adrenal	0.002	0.002	n.d.	n.d.	2.32	2.69
Uterus	–	0.001	–	0.001	–	2.57
Eyes	n.d.	n.d.	n.d.	n.d.	0.65	0.74
Harderian gland	n.d.	n.d.	n.d.	n.d.	1.06	1.66
Residual carcass	n.d.	n.d.	n.d.	n.d.	0.72	0.93
Skin and fur	0.012	0.023	0.015	0.020	0.40	0.56
Blood	0.002	0.004	0.004	0.003	6.28	9.08
Plasma	0.001	0.002	0.005	0.004	5.22	7.28

eq: equivalents; GI: gastrointestinal; n.d.: not detected; ppm: parts per million
Source: Filaquier (1994)

The results from the tissue kinetic study (Valles, 1999) reflected those seen in the absorption, distribution, metabolism and excretion study (Filaquier, 1994); radioactivity was widely distributed in the tissues, with a predominance in the liver and kidney. The distribution of the absorbed radioactivity was found to be similar for both dose levels used (1 and 100 mg/kg bw), with the higher concentrations being observed in the gastrointestinal tract (at early sacrifice times), liver, kidney, plasma and cardiac blood. The levels observed in the liver and kidney remained among the highest at all sampling times in both dose groups and both sexes. The highest tissue concentrations were found at 1 hour post-administration at the low dose, whereas they were observed at 24 hours post-administration at the high dose, reflecting a rapid absorption in the former case. The distribution of radioactivity was comparable in males and females at both dose levels (Valles, 1999).

In the low-dose and repeated low-dose groups, [^{14}C]isoxaflutole was primarily excreted in the urine of rats (about 58.8–67.4% of the administered dose). In the two low-dose groups, approximately 24.0–26.8% of the administered dose was excreted in the faeces. In the high-dose group, the mean recoveries were mainly in the faeces, indicating saturation of absorption (Table 5). The majority of radioactivity was eliminated in the first 24 hours for the two low-dose groups and within the first 48 hours for the high-dose group. There was no observed sex-related difference in the elimination pattern in either the single or the repeated low-dose groups.

Table 5. Time course excretion of isoxaflutole-derived radioactivity in the urine and faeces following single low dose, repeated low dose and single high dose in rats

Route of excretion / time interval (h)	% of the administered dose					
	Single low dose		Repeated low dose		Single high dose	
	Males	Females	Males	Females	Males	Females
Urine						
0–24	55.40	48.71	60.27	61.98	23.95	29.98
0–48	57.86	52.79	63.67	64.30	29.29	37.83
0–72	59.20	54.91	64.64	65.41	30.24	39.50
0–96	59.93	56.34	65.33	66.29	30.67	40.16
0–120	60.46	57.43	65.85	66.78	30.97	40.61
0–144	60.83	58.22	66.30	67.13	31.19	40.92
0–168	61.16	58.80	66.65	67.41	31.37	41.20
Faeces						
0–24	20.03	18.10	18.50	22.61	46.26	30.83
0–48	24.46	24.74	22.75	23.78	59.06	50.01
0–72	24.92	25.68	23.50	24.17	60.84	53.14
0–96	25.15	26.16	23.74	24.44	61.66	53.93
0–120	25.37	26.53	23.87	24.58	62.23	54.45
0–144	25.51	26.76	23.97	24.66	62.66	54.91
0–168	26.05	26.94	24.04	24.72	63.00	55.23

Source: Filaquier (1994)

1.2 Biotransformation

The metabolism of [^{14}C]isoxaflutole was qualitatively and quantitatively similar in both sexes of rats and was not influenced by repeated administration. The compound was rapidly and extensively metabolized, as seen by the lack of parent material in the urine of rats in the two low-dose groups; only traces were detected in the urine of rats in the high-dose group. Unchanged parent compound was detected in the faeces of rats from the high-dose group, indicating saturation of absorption of the compound. After oral administration, 9 metabolites were detected in the urine, and 11 metabolites were detected in the faecal extracts. Independent of the time period and the elimination route, the major metabolite detected in the urine (UMET/5) and faecal (FMET/7) extracts for the three dose groups was identified as RPA 202248 (3-cyclopropyl-2-[2-mesyl-4-trifluoromethylbenzoyl]-3-oxopropane nitrile). It is a diketonitrile derivative of the parent compound, which represented 70% of the radioactivity excreted in the urine and faeces. The metabolites UMET/1 and FMET/1 (more polar metabolite) were identified as RPA 203328 (2-mesyl-4-trifluoromethylbenzoic acid; minor metabolite); the metabolites UMET/2 and FMET/3 were not clearly identified, but suggested the presence of a strongly acidic proton or the presence of an unstable conjugate. The metabolites UMET/7 and FMET/9 were identified as an amine derivative from RPA 202248. The minor metabolites UMET/8 and FMET/10 were possibly the Des-SO_2Me derivatives from the parent compound. The unchanged isoxaflutole was detected as UMET/9 and FMET/11. There was no

indication of any metabolites resulting from Phase II (conjugation) reactions. The liver samples from the males and females from the two low-dose groups contained TMET/1 (RPA 202248) as a major metabolite, representing 33–77.91% of the initial radioactivity measured. TMET/2 (unidentified) was detected as a minor metabolite (0.28–2.15% of the initial radioactivity) (Table 6).

***Table 6. Mean percentage of administered radioactivity associated with the radiolabelled components characterized/identified in the urine and faeces of rats following single oral low dose, repeated oral low dose and single oral high dose of isoxaflutole*[a]**

	Met. ID	**RPA 203328 (UMET/1 & FMET/1)**						**RPA 207048 (FMET/5)**					
	Structure												
	Group	SOHD		SOLD		ROLD		SOHD		SOLD		ROLD	
Sample	Sex	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Urine	% dose	1.2	0.56	1.0	–	0.7	2.0	–	–	–	–	–	–
Faeces	% dose	2.4	2.0	0.99	0.58	0.58	0.57	1.9	1.3	–	–	–	–
Total	% dose	3.6	2.6	2.0	0.58	1.3	2.6	1.9	1.3	–	–	–	–
	Met. ID	**RPA 202248 (UMET/5, FMET/7 & TMET/1)**						**RPA 205834 (UMET/7 & FMET/9)**					
	Structure												
	Group	SOHD		SOLD		ROLD		SOHD		SOLD		ROLD	
Sample	Sex	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Urine	% dose	28.2	36.2	60.1	58.8	63.8	63.9	0.79	2.3	–	–	–	–
Faeces	% dose	41.8	43.7	19.4	18.8	20.6	21.3	1.5	2.0	–	–	0.03	–
Total	% dose	70.0	79.9	79.5	77.6	84.4	852	2.29	4.3	–	–	0.03	–
	Met. ID	**RPA 205568 (UMET/8 & FMET/10)**						**Isoxaflutole (UMET/9 & FMET/11)**					
	Structure												
	Group	SOHD		SOLD		ROLD		SOHD		SOLD		ROLD	
Sample	Sex	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Urine	% dose	0.11	0.42	–	–	–	–	0.22	0.05	–	–	–	–
Faeces	% dose	1.9	0.88	–	–	–	–	8.0	5.6	–	–	–	–
Total	% dose	2.0	1.3	–	–	–	–	8.2	5.7	–	–	–	–

–: not detected; ID: identification; Met.: metabolite; SOHD: single oral high dose; SOLD: single oral low dose; ROLD: repeated oral low dose

[a] The data are presented in terms of percentage of administered dose and are means over the 0–168 h post-dosing period for both sexes.

Source: Adapted from Shipp (2012).

The proposed metabolic pathway for isoxaflutole in rats is shown in Fig. 2.

Fig. 2. Proposed metabolic pathway for isoxaflutole in rats

Isoxaflutole

RPA 205568
Minor metabolite in urine and faeces

RPA 205834
Minor metabolite detected in urine and faeces

RPA 202248
Major metabolite detected in urine, faeces and liver

RPA 207048
Minor metabolite detected in faeces.

RPA 203328
Minor metabolite detected in urine and faeces

Source: Shipp (2012)

1.3 Dermal absorption

In a dermal absorption study, [^{14}C]isoxaflutole was dermally applied to male Crl:CD®BR rats at 0.865, 7.32 or 79 µg/cm^2. Four animals per dose were exposed for 0.5, 1, 2, 4, 10 and 24 hours. Carboxymethyl cellulose in water (1%) was used as a vehicle for dermal application. Small amounts of radioactivity were detected in/on the skin of the application site, accounting for 0.92–12.0% of the applied radioactivity. The amounts of radioactivity found in the blood, eliminated in the excreta and retained in the carcass were considered to be the direct dermal absorption of isoxaflutole by rats. The highest direct absorption was at the longest exposure time (24 hours after dose). It accounted for

4.49% (0.494 µg eq), 0.92% (0.846 µg eq) and 0.2% (1.97 µg eq) at 0.865, 7.32 and 79 $\mu g/cm^2$, respectively. The amount of dermal absorption of isoxaflutole was not proportional to the dose (Cheng, 1996).

2. Toxicological studies

2.1 Acute toxicity

The results of acute toxicity studies with isoxaflutole (including skin and eye irritation and dermal sensitization studies) are summarized in Table 7.

Table 7. Acute toxicity of isoxaflutole

Species	Strain	Sex	Route	Purity; vehicle	Result	Reference
Rat	Sprague-Dawley	M + F	Oral	98.7%; 0.5% CMC	LD_{50} > 5 000 mg/kg bw	Allen (1993b)
Rat	Wistar	F	Oral	98.6%; 2% aqueous Cremophor EL	LD_{50} > 2 000 mg/kg bw	Eiben (2005b)
Rat	Wistar	M + F	Dermal	98.6%; wet gauze	LD_{50} > 2 000 mg/kg bw	Eiben (2005a)
Rabbit	New Zealand White	M + F	Dermal	98.7%; 0.5% CMC	LD_{50} > 2 000 mg/kg bw	Allen (1993d)
Rat	Sprague-Dawley	M + F	Inhalation (whole body)	98.3%	LC_{50} (4 h) > 5.23 mg/L	Jackson (1994)
Rabbit	New Zealand White	M + F	Skin irritation	98.7%; 0.5% CMC	Non-irritating	Allen (1993c)
Rabbit	New Zealand White	F	Skin irritation	98.6%; moistened with water	Non-irritating	Schüngel (2005b)
Rabbit	New Zealand White	M + F	Eye irritation	98.7%	Minimally irritating	Allen (1993a)
Rabbit	New Zealand White	F	Eye irritation	98.6%	Minimally irritating	Schüngel (2005a)
Guinea-pig	Dunkin Hartley	M + F	Skin sensitization (Buehler method)	99.4%; propylene glycol	Non-sensitizing	Rees (1992)
Guinea-pig	Hartley	M + F	Skin sensitization (Magnusson-Kligman)	99.6%; propylene glycol	Non-sensitizing	Rees (1996)
Guinea-pig	Hartley	F	Skin sensitization (Buehler method)	98.6%; polyethylene glycol	Non-sensitizing	Vohr (2005)

CMC; carboxymethyl cellulose; F: female; LC_{50}: median lethal concentration; LD_{50}: median lethal dose; M: male

(a) Oral administration

Rats

One male and one female fasted Sprague-Dawley rat were treated orally, by gavage, with isoxaflutole (purity 98.7%) in 0.5% carboxymethyl cellulose at a single dose of 5000 mg/kg bw and observed for 5 days. A second group of five fasted male and five fasted female Sprague-Dawley rats was similarly treated at a single gavage dose of 5000 mg/kg bw. Animals were observed for mortality and clinical signs several times for the 1st day and once daily thereafter for 14 days. Body weights were recorded on days 0, 7 and 14. A gross necropsy was performed on all animals.

No deaths or clinical signs of an adverse reaction to treatment occurred, and there were no effects on body weight or macroscopic findings at necropsy in any animal. Based on these results, the acute oral median lethal dose (LD_{50}) was estimated to be greater than 5000 mg/kg bw (Allen, 1993b).

In a separate study, three fasted female Wistar rats were treated orally, by gavage, with isoxaflutole (purity 98.6%) in 2% aqueous Cremophor EL at a single dose of 2000 mg/kg bw and observed for 14 days. Another group of three female rats was similarly treated.

No deaths, clinical signs, effects on body weight or gross pathological findings at necropsy were observed. Based on these results, the acute oral LD_{50} was estimated to be greater than 2000 mg/kg bw (Eiben, 2005b).

(b) Dermal application

Rats

In an acute dermal lethality study, a group of five male and five female Wistar rats was treated with isoxaflutole (purity 98.6%) topically at a dose of 2000 mg/kg bw. The compound was applied to a wet gauze patch, then to the shorn skin using stretch tape and a jacket. After a 24-hour exposure period, the application sites were cleaned and evaluated according to the Draize method.

No deaths or systemic clinical signs of an adverse reaction to treatment occurred. There were no local signs of an effect of treatment at the application site, and there were no macroscopic findings at necropsy in any animal. All animals gained weight during the study. Based on these results, the acute dermal LD_{50} was estimated to be greater than 2000 mg/kg bw (Eiben, 2005a).

Rabbits

A group of five male and five female New Zealand White rabbits was treated with isoxaflutole (purity 98.7%) dispersed in aqueous solution of 0.5% carboxymethyl cellulose at a dose of 2000 mg/kg bw once for 24 hours by topical administration. Each application site was covered with surgical gauze and secured with adhesive and an elasticated corset. After a 24-hour exposure period, the application sites were cleaned and evaluated according to the Draize method. Animals were observed for mortality and clinical signs several times for the 1st day and once daily thereafter for 13 days. Body weights were recorded on days 0, 7 and 14. After 14 days of observation post-treatment, the animals were subjected to necropsy and postmortem examination.

No deaths or clinical signs of an adverse reaction to treatment occurred. A very slight erythema at the treatment site was noted in two male rabbits and one female rabbit at 1 day after treatment and persisted in one of the male rabbits through the 2nd day after dosing. There were no macroscopic findings at necropsy in any animal. All animals gained weight during the study. Based on these results, the acute dermal LD_{50} was estimated to be greater than 2000 mg/kg bw (Allen, 1993d).

(c) Exposure by inhalation

Rats

A group of five male and five female Sprague-Dawley rats was exposed once for 4 hours, by whole-body exposure, to a dust atmosphere of isoxaflutole (purity 98.3%) at the maximum technically achievable concentration of 5.23 mg/L air. The animals were observed for 14 days post-treatment. Body weights were recorded on day 0 and weekly thereafter. All animals were subjected to necropsy and postmortem examination.

The mass median aerodynamic diameter of isoxaflutole in the chamber air was 3.1 µm, the standard geometric deviation was 1.95 and over 88% of the particles were 7 µm or less. No deaths occurred during the exposure or observation period. Clinical signs observed during exposure were limited to partial closing of the eyes and accumulation of the test compound on the fur. During the post-exposure observation period, there were no treatment-related clinical signs except for the presence of residual test compound on the fur immediately following exposure. Body weight gain was comparable to the control values at termination. There were no macroscopic findings at necropsy, except for the lungs of one male rat, which were slightly congested. Based on these results, the acute (4-hour) median lethal concentration (LC_{50}) was estimated to be greater than 5.23 mg/L air (Jackson, 1994).

(d) Dermal irritation

Rabbits

In a study of primary dermal irritation, one female and five male New Zealand White rabbits were dermally exposed to 0.5 g of isoxaflutole (purity 98.7%) moistened with 0.5 mL of 0.5% carboxymethyl cellulose and placed onto the shorn skin on the back of each rabbit under a 2.5 cm^2 gauze patch secured in position with surgical adhesive tape. The test material was in contact with the skin for 4 hours. After removal of the patch, the treated application site was washed off with water. Dermal irritation was scored at 1, 24, 48 and 72 hours after the removal of the patch using the Draize method. The animals were observed for 14 days post-treatment.

A very slight erythema was noted on one animal at 1 hour after removal of the patches. No other animals were noted with erythema, eschar or oedema at any time point. Based on the results of this study, isoxaflutole was not irritating to the skin of rabbits (Allen, 1993c).

In a separate primary dermal irritation study, three gauze patches, each containing 0.5 g isoxaflutole (purity 98.6%) moistened with water, were applied to the shorn skin of a female New Zealand White rabbit and secured with non-irritating tape. The first patch was removed after 3 minutes, and, in the absence of serious skin reactions, the second patch was removed after 1 hour. As observations of the animal indicated no hazard to animal welfare, the third patch was removed after 4 hours. Additionally, 0.5 g of isoxaflutole was applied to the shorn skin of two other rabbits under gauze patches secured with non-irritating tape for 4 hours.

No erythema, eschar or oedema was observed at any observation time point. Under the conditions of the study, isoxaflutole is not an irritant to the skin of the rabbit (Schüngel, 2005b).

(e) Eye irritation

Rabbits

In a primary eye irritation study, a volume of 0.1 mL (approximately 99 mg) of isoxaflutole (purity 98.7%) was instilled into the right conjunctival sac of three male and three female New Zealand White rabbits. The eyes were not washed. The ocular irritation was assessed approximately 1, 24, 48 and 72 hours after treatment.

Iridial inflammation was noted in the treated eye of one animal at 1 hour after treatment. Minimal to moderate conjunctival irritation was noted in all treated eyes at 1 hour after treatment. All

treated eyes appeared normal at the 24-hour observation. Based on the results of this study, isoxaflutole is minimally irritating to rabbit eyes (Allen, 1993a).

In a second primary eye irritation study, a volume of 0.1 mL (approximately 99 mg) of isoxaflutole (purity 98.6%) was instilled into the right conjunctival sac of three female New Zealand White rabbits. The eyes were not washed for 24 hours. The ocular irritation was assessed approximately 1, 24, 48 and 72 hours after treatment.

All three animals showed slight redness of the conjunctivae in the treated eye at 1 hour after compound application. Two animals had conjunctival redness that persisted through 24 hours after treatment. Based on the results of this study, isoxaflutole is minimally irritating to rabbit eyes (Schüngel, 2005a).

(f) Dermal sensitization

Guinea-pigs

The skin sensitization potential of isoxaflutole (purity 99.4%) was investigated in 10 male and 10 female Dunkin Hartley guinea-pigs using the modified Buehler method. The control group included five male and five female guinea-pigs. The shaven left flanks of 10 male and 10 female guinea-pigs were subjected to a 6-hour occluded topical application of 50% weight per volume (w/v) isoxaflutole in propylene glycol on days 1, 3, 5, 8, 10, 12, 15, 17 and 19 of the test period. On day 29, all test and control animals were challenged by 6-hour occluded topical applications of 50% and 10% w/v isoxaflutole in propylene glycol and propylene glycol alone to their shaven right flanks. Dermal responses to the challenge procedure were assessed approximately 24 and 48 hours after application of the occlusive dressings.

Applications of 50% w/v isoxaflutole in propylene glycol caused intermittent, very faint erythema throughout the induction period. There were no reactions in either the test or control animals after the challenge application of isoxaflutole at either 10% or 50% in propylene glycol. Based on the lack of response in the challenge phase, isoxaflutole is not considered to be a sensitizer using the modified Buehler method (Rees, 1992).

In a second study, the skin sensitization potential of isoxaflutole (purity 99.6%) was investigated in 10 male and 10 female Dunkin Hartley guinea-pigs using the maximization test. A concurrent positive control group was not included. Concentrations of 10% and 50% isoxaflutole in propylene glycol were used for intradermal induction, topical induction and challenge phases. Skin reactions to the challenge applications were evaluated 24 and 48 hours after patch removal.

Intradermal injection of 10% isoxaflutole in propylene glycol caused some slight erythema and skin discoloration, whereas inclusion of Freund's complete adjuvant caused slight to moderate erythema, skin discoloration and pallor. Topical application of isoxaflutole at 50% in propylene glycol caused isolated cases of exfoliation and eschar formation. Challenge application of isoxaflutole at either 50% or 10% in propylene glycol did not cause any dermal reaction in any animal at either 24 or 48 hours after removal of the material. Under the conditions of the guinea-pig maximization study, there was no indication of delayed contact hypersensitivity induced by isoxaflutole (Rees, 1996).

In a third study, the skin sensitization potential of isoxaflutole (purity 98.6%) was investigated in 20 female Crl:HA guinea-pigs using the maximization test. Ten females were used as controls. Isoxaflutole was applied to female guinea-pigs in polyethylene glycol 400 at concentrations of 5% for intradermal injection, 50% for topical induction and 50% for challenge application. Isoxaflutole in polyethylene glycol was injected into the skin either with or without Freund's complete adjuvant. Topical application of isoxaflutole was then conducted for a 48-hour period starting on study day 8. Finally, 3 weeks after the initial intradermal injection, new areas were shorn of hair, and isoxaflutole at 50% in polyethylene glycol 400 was applied for a 24-hour period. The

application sites were assessed at 24 and 48 hours after removal of the test item and scored for severity of reaction.

After the intradermal induction, animals in the control group and the test group showed strong effects up to encrustation at the injection sites of the first induction. No skin reactions were observed in the treated and control groups after the challenge phase. Under the conditions of the guinea-pig maximization study, there was no indication of delayed contact hypersensitivity induced by isoxaflutole (Vohr, 2005).

2.2 Short-term studies of toxicity

(a) Oral administration

Mice

In a 28-day preliminary dietary study, groups of 10 male and 10 female CD-1 mice received isoxaflutole (purity 99.9%) via dietary administration at a concentration of 0, 175, 700, 2800 or 7000 parts per million (ppm) daily for 28 days. The dose levels were equal to 0, 29.4, 120.7, 474.6 and 1140.1 mg/kg bw per day for males and 0, 34.7, 142.9, 534.4 and 1347.4 mg/kg bw per day for females, respectively.

There were no treatment-related mortalities, clinical or ophthalmoscopic observations or treatment-related effects on body weight, body weight gain or feed consumption. The liver appeared to be the target organ. Significant treatment-related clinical chemistry findings included increased activities of alanine aminotransferase (ALAT) at 700 ppm and above and aspartate aminotransferase (ASAT) at 2800 ppm and above; alkaline phosphatase (AP) activity was increased at 7000 ppm in males, but not significantly (Table 8).

Table 8. Treatment-related clinical chemistry findings in the mouse 28-day study with isoxaflutole

Observation	Males					Females				
	0 ppm	175 ppm	700 ppm	2 800 ppm	7 000 ppm	0 ppm	175 ppm	700 ppm	2 800 ppm	7 000 ppm
Total bilirubin (µmol/L)	3.91	3.26**	2.22**	1.40**	1.14**	2.49	2.19	1.87	1.21**	1.00**
Total protein (g/L)	54.80	55.80	57.10	56.44	60.90**	54.50	54.10	55.00	56.80	61.40**
ASAT (IU/L)	41.40	40.30	44.80	59.89**	79.60**	58.80	58.60	58.50	67.70	65.30
ALAT (IU/L)	21.3	22.0	25.5	76.5**	133.5**	21.70	24.00	28.40*	36.40*	48.40**
AP (IU/L)	61.0	51.3	52.5	55.9	124.7	72.9	80.20	71.10	77.0	72.40
Creatinine (µmol/L)	39.10	32.30*	28.30**	31.22**	26.60**	35.30	26.30*	23.90**	22.70**	17.89**
Liver weight (g)	1.30	1.45*	1.57**	2.13**	2.82**	0.86	0.96	1.00**	1.30**	1.91**
Liver weight (% of body weight)	3.99	4.35*	4.72**	6.52**	8.79**	3.72	4.08*	4.23**	5.47**	7.86**

ALAT: alanine aminotransferase; AP: alkaline phosphatase; ASAT: aspartate aminotransferase; IU: International Units; *: $P < 0.05$; **: $P < 0.01$
Source: Esdaile & Dange (1994)

Increased liver weights were observed at all dose levels in both sexes. Significant treatment-related gross pathological observations were noted in the liver and included enlarged liver (males at 700, 2800 and 7000 ppm and females at 2800 and 7000 ppm) and white striations (both sexes at 7000 ppm). Treatment-related histopathological findings in the liver included centrilobular hepatocellular hypertrophy (both sexes at 700 ppm and above) and hepatocellular necrosis (males at 2800 and 7000 ppm and females at 7000 ppm). Other histopathological findings included increased extramedullary haematopoiesis in spleen (both sexes at 7000 ppm) and X-zone cell vacuolation in the adrenal glands (females at 7000 ppm).

In the absence of any other significant findings at 175 ppm, the increased liver weights were considered a minor adaptive change; therefore, the no-observed-adverse-effect level (NOAEL) was 175 ppm (equal to 29.4 mg/kg bw per day), based on increases in liver enzymes (ALAT and ASAT), clinical chemistry changes (decreased bilirubin and creatinine levels) and increased liver weight at 700 ppm (equal to 120.7 mg/kg bw per day) (Esdaile & Dange, 1994).

In a 90-day preliminary dietary study, groups of 12 CD-1 mice of each sex per dose level received 0, 50, 1000 or 2000 ppm isoxaflutole (purity 983 g/kg) via dietary administration daily for 13 weeks and 5 days. The dose levels were equal to 0, 7.6, 170.0 and 324.1 mg/kg bw per day for males and 0, 8.7, 181.2 and 376.2 mg/kg bw per day for females, respectively.

There were no treatment-related mortalities, clinical, ophthalmoscopic or gross pathological observations, or treatment-related effects on body weight, body weight gain, feed consumption or feed efficiency. The liver appeared to be the target organ. Significant treatment-related clinical chemical observations were increased ASAT and ALAT activities at 2000 ppm in both sexes (Table 9).

Table 9. Potentially treatment-related clinical chemistry parameters and liver weights in the mouse 90-day study with isoxaflutole

Observation	Males				Females			
	0 ppm	50 ppm	1 000 ppm	2 000 ppm	0 ppm	50 ppm	1 000 ppm	2 000 ppm
ALAT (IU/L)	42	36	53	126***	37	34	38	47*
ASAT (IU/L)	71	66	92	112*	75	73	78	93*
CPK (IU/L)	122	137	189	114	69	70	76	154***
Terminal body weight (g)	37.3	38.3	34.4	35.5	27.2	28.7	28.1	27.7
Liver weight (g)	1.64	1.80*	1.90*	2.28**	1.33	1.32	1.37	1.54**
Liver weight (% of body weight)	4.409	4.176	5.543**	6.402**	4.890	4.539	4.890	5.569**

ALAT: alanine aminotransferase; ASAT: aspartate aminotransferase; CPK: creatine phosphokinase; IU: International Units; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$
Source: Chase (1994c)

Increased absolute liver weights were observed in males at 50 ppm and above (Table 9). Increased relative liver weights were observed in males at 1000 ppm and above. Macroscopic examination revealed that one male at 2000 ppm had a large liver, and two further males at 2000 ppm had pale livers. Periacinar hepatocytic hypertrophy was observed at 1000 ppm and above. At 50 ppm, the lowest dose tested, the only significant observation was an increase in absolute liver weight in males.

In the absence of a similar increase in females and in the absence of any biochemical, gross pathological or histopathological observations at 50 ppm in either sex, this was considered to be a minor adaptive change; therefore, the NOAEL was 50 ppm (equal to 7.6 mg/kg bw per day), based on increased ALAT and ASAT activities, increased absolute and relative liver weights and increased incidence of periacinar hepatocytic hypertrophy at 1000 ppm (equal to 170.0 mg/kg bw per day) (Chase, 1994c).

Rats

In a short-term dietary study, groups of 10 male and 10 female CD rats received isoxaflutole (purity 99.4%) via dietary administration at a constant dose of 0, 25, 100, 400 or 1000 mg/kg bw per day for 6 weeks followed by a 7-week reversibility period (without test article). All control animals were killed after 17 weeks.

No mortality was observed during the study. An increased incidence of unilateral or bilateral opaque eyes was noted in all male groups beginning in week 3, without a dose–response relationship. Opacities persisted through the 1st week of reversibility; however, most of them had resolved by the 2nd week of the reversibility phase of the study. At ophthalmology, focal corneal opacities were seen in all treated groups (apart from females receiving 25 mg/kg bw per day) from week 3 of treatment, but their incidence was not dose related. The males were more affected than the females. The severity of each lesion tended to increase throughout the treatment period; this sometimes included an increase in size and opaqueness, with an associated keratitis and/or vascularization. After 6 days of reversibility, the majority of lesions had nearly completely recovered, leaving only a slight corneal haze and/or ghost vessels. Within 20 days of the reversibility phase, all of the lesions had regressed.

During the treatment period, the body weight gain for males and females receiving 1000 mg/kg bw per day and females receiving 400 mg/kg bw per day was low compared with that of their controls (81% and 67% for males and females receiving 1000 mg/kg bw per day and 82% for females receiving 400 mg/kg bw per day). During the reversibility phase, treated animals gained more weight than did the controls. Body weight gain for animals receiving 25 or 100 mg/kg bw per day and males receiving 400 mg/kg bw per day was considered to be unaffected by treatment. Feed consumption was decreased in females at 1000 mg/kg bw per day during the treatment period, but similar to that of controls during the reversibility phase. For the treatment period, the overall feed conversion efficiency of males and females receiving 400 or 1000 mg/kg bw per day was low compared with that of the controls. For the reversibility period, the feed conversion efficiency was greater for these animals than for their controls. Urine analysis after 5 weeks of treatment showed decreased pH in males at the top three doses and the presence of total reducing substances in six males and six females receiving 1000 mg/kg bw per day and one male and one female at each of 400 and 100 mg/kg bw per day (Table 10). After 4 weeks of treatment, there were slight decreases in some red cell parameters and total white cell counts in males or females and an increase in prothrombin times in males (Table 10). In clinical chemistry, there were decreases in a number of parameters, primarily related to the effects of isoxaflutole on the liver, in both males and females.

No treatment-related effects were observed at necropsy of treated animals. No treatment-related effects were observed on organ weights. Histopathology of the eyes from 10 treated animals revealed epithelial thickening and vacuolation, subepithelial fibroblastic reaction and active stromal vascularization in some animals.

In conclusion, the dietary administration of isoxaflutole at 25, 100, 400 and 1000 mg/kg bw per day produced liver toxicity and corneal lesions in the eye visible by both gross inspection and ophthalmological examination. The corneal lesions were resolved by 3 weeks after the end of the treatment, and liver weights were comparable to control values in 7 weeks after the end of the treatment. Therefore, the lowest-observed-adverse-effect level (LOAEL) was 25 mg/kg bw per day, based on corneal opacities and effects on the liver observed at all doses (Chase, 1994a).

Table 10. Treatment-related haematology, clinical chemistry and urine analysis parameters in the 6-week dietary/7-week reversibility study with isoxaflutole in the rat

Observation	Males					Females				
	0 mg/kg bw per day	25 mg/kg bw per day	100 mg/kg bw per day	400 mg/kg bw per day	1 000 mg/kg bw per day	0 mg/kg bw per day	25 mg/kg bw per day	100 mg/kg bw per day	400 mg/kg bw per day	1 000 mg/kg bw per day
PCV (%)	45	44	46	43	43	43	44	44	44	41**
Hb (g%)	15.1	15.8	16.1	15.5*	15.3**	15.6	15.8	15.7	15.9	15.0
RBC (10^6/cm^2)	8.05	7.86	7.98	7.80	7.89	7.83	8.02	7.89	8.10	7.83
MCHC (%)	36	36	35*	36	36	36	36	36	36	37
MCV (μm^3)	55	56	57	55	54	55	55	55	55	52**
MCH (pg/cell)	20	20	20	20	19	20	20	20	20	19*
Lymphocytes (1000/cm^2)	18.0	18.5	14.7*	14.7	14.4*	13.9	13.1	12.9	12.5	11.2*
Prothrombin time (s)	12.1	13.2*	12.4	13.4*	15.4***	12.5	13.1*	12.8	12.6	12.3
AP (IU/L)	159	133*	113***	110***	91***	96	88	75***	68***	70***
ALAT (IU/L)	34	34	30	35	30	24	25	22	31	18
ASAT (IU/L)	92	80**	71***	72***	63***	70	69	70	77	59
Urine pH	7.4	7.2	6.9***	7.0**	6.6***	6.5	6.3	6.1**	6.5	6.4
Total reducing substances	0/10	0/10	1/10	1/10	6/10	0/10	0/10	1/10	1/10	6/10
Terminal body weight (g)	593.9	525.7	528.5	524.1	505.3	329.7	308.7	315.0	319.3	298.8
Liver weight (g)	24.0	20.6	22.1	21.8	21.5	12.5	11.7	12.6	12.3	11.8
Liver weight (% of body weight)	4.04	3.91	4.18	4.16	4.27	3.78	3.77	3.99	3.85	3.93

ALAT: alanine aminotransferase; AP: alkaline phosphatase; ASAT: aspartate aminotransferase; Hb: haemoglobin; IU: International Units; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; MCV: mean cell volume; PCV: packed cell volume; RBC: red blood cells; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$
Source: Chase (1994a)

In a 90-day dietary study, groups of 10 CD rats of each sex per dose received isoxaflutole (purity 99.4%) at 0, 1.0, 3.0, 10 or 100 mg/kg bw per day via dietary administration daily for 13 weeks and 3 days.

There were no treatment-related mortalities and no treatment-related effects on body weight, body weight gain, feed consumption or feed efficiency. There were no clinical signs of toxicity except opaque eyes observed during the treatment period for four males and four females receiving 100 mg/kg bw per day and two males at 10 mg/kg bw per day. The eyes of some males receiving the highest dosage were also noted to be dull. There were no significant treatment-related effects on haematological, clinical chemistry or urine analysis parameters examined. Treatment-related findings in males at 100 mg/kg bw per day included increased absolute and relative liver weights, with an associated increased incidence of periacinar hepatocytic hypertrophy; these were considered to be an adaptive response and not adverse. Absolute and relative kidney weights were increased in males at 100 mg/kg bw per day. Significant treatment-related ophthalmoscopic, gross pathological and histopathological findings were observed in the eye in males at 10 mg/kg bw per day and in both sexes at 100 mg/kg bw per day. Clinical and ophthalmoscopic observations included increased incidences of bilateral and unilateral opaque eyes and focal corneal opacity, respectively. Focal

corneal opacity was first apparent during week 3 and persisted throughout the study. Significant gross pathological observations included an increased incidence of corneal opacity (unilateral and bilateral) in both sexes at 100 mg/kg bw per day. The overall incidence of corneal lesions was similar in both sexes, although the severity of the lesions was more significant in the males. The most notable histopathological findings included vacuolation (males and females) and superficial exfoliation of the epithelial cells (males and females), epithelial thickening (males), necrosis and inflammation (males and females), subepithelial fibroblastic reaction (males and females) and vascularization of the stroma (males and females). The changes were considered by the study author to be reversible after a short recovery period following cessation of treatment in the previous study (Chase, 1994a), with the exception of residual evidence of tissue repair detectable only by histopathological examination. There were no treatment-related effects at 1.0 or 3.0 mg/kg bw per day; therefore, the NOAEL for isoxaflutole was 3.0 mg/kg bw per day, based on haematological changes, corneal opacity and liver toxicity observed at 10 mg/kg bw per day (Chase, 1994b).

The tyrosine concentrations in the plasma samples taken at 13 weeks from the 13-week toxicity study by dietary administration to CD rats described above (Chase, 1994b) were determined in a separate study (Table 11). The mode of action of isoxaflutole is the inhibition of the HPPD enzyme, which results in an increase in tyrosine levels in the blood and other tissues.

Table 11. Plasma tyrosine concentrations in the rat 90-day study with isoxaflutole

	Males					Females				
	0 mg/kg bw per day	1.0 mg/kg bw per day	3.0 mg/kg bw per day	10 mg/kg bw per day	100 mg/kg bw per day	0 mg/kg bw per day	1.0 mg/kg bw per day	3.0 mg/kg bw per day	10 mg/kg bw per day	100 mg/kg bw per day
Tyrosine concentration (nmol/mL)	516.8	725.7	922.6	1 061	1 330	511.3	423.3	890.6	1 023	1 308
Fold increase	–	1.4	1.8	2.1	2.6	–	0.8	1.7	2.0	2.6

Source: Little (1993a)

Plasma tyrosine levels were increased for males in all dose groups and for females from 3.0 mg/kg bw per day and above (Little, 1993a).

Dogs

In a pilot study, isoxaflutole (purity 99.9%) was administered orally in gelatine capsules to one male and one female Beagle dog in order to determine the maximum tolerated dosage. Doses increased from 100 mg/kg bw per day for the first 3 days to 300, 550 and then 1000 mg/kg bw per day for 3 succeeding days at each dose.

There were no clinical findings, effects on body weight or feed consumption, or observations at gross necropsy that could be related to treatment. The maximum tolerated dose was therefore set at greater than 1000 mg/kg bw per day in the dog when administered over a 3-day period (Mondot, 1992a).

In a second pilot study, isoxaflutole (purity 99.9%) was administered orally in gelatine capsules to one male and one female Beagle dog to evaluate potential toxicity. One dog from each sex received a dose of 1000 mg/kg bw per day for 14 days.

There were no deaths, clinical signs, effects on body weight or feed consumption or ophthalmological findings. At necropsy, no detectable changes or variation from normal was noted in the treated dogs. In conclusion, isoxaflutole was well tolerated when administered orally in capsules to Beagle dogs at a dose of 1000 mg/kg bw per day (Mondot, 1992b).

In a third pilot study, one male and one female Beagle dog were administered isoxaflutole (purity 97%) orally in capsules at a dose of 1000 mg/kg bw per day for 6 weeks. After a washout period of 8 days with no dosing, both dogs were then administered isoxaflutole in the diet at 25 000 ppm for 14 days.

There was no effect of treatment on mortality, clinical signs, body weight, feed consumption, ophthalmological findings, urine analysis, haematology, clinical chemistry or macroscopic findings. AP increased in both sexes during the dosing periods and decreased during the washout period. Relative liver weight was slightly increased in both animals; in the female, histopathological findings included occasional medullary foci of mineralization in the kidney and congestion and minimal centrilobular rarefaction of hepatocytes in the liver.

In conclusion, the study results indicate that isoxaflutole is not markedly toxic to dogs after short-term administration of 1000 mg/kg bw per day (Brooker, 1994b).

In a 1-year toxicity study, isoxaflutole (purity 98.7%) was administered to five Beagle dogs of each sex per dose in the diet at a dose level of 0, 240, 1200, 12 000 or 30 000 ppm (equal to 0, 8.56, 44.81, 453 and 1265 mg/kg bw per day for males and 0, 8.41, 45.33, 498 and 1254 mg/kg bw per day, for females, respectively) for 52 weeks. The treated males in the 30 000 ppm treatment group were sacrificed after 26 weeks due to severe chronic reaction to the test substance.

No animals died during the study. No treatment-related ophthalmological abnormalities were noted. All males in the 30 000 ppm treatment group were killed, for humane reasons, after 26 weeks of treatment due to apparent anaemia, suspected from pallor of the gums and confirmed by haematology. Pale gums were recorded for one male and one female in the 12 000 ppm treatment group during weeks 28–51 and 23–27, respectively. There was a statistically significant decrease in mean body weight gain of females in the 12 000 and 30 000 ppm treatment groups over 52 weeks of treatment (Table 12). Mean body weight gain of males in the 12 000 ppm treatment group was lower than that of the controls, although the difference did not attain statistical significance. Weight gains at 26 weeks were 65% and 56% of control gains in males and females in the 12 000 ppm group, respectively. Feed consumption was comparable between the control and treatment groups.

Table 12. Body weight and body weight gain in the dog 52-week dietary study with isoxaflutole

Week(s)	Males					Females				
	0 ppm	240 ppm	1 200 ppm	12 000 ppm	30 000 ppm	0 ppm	240 ppm	1 200 ppm	12 000 ppm	30 000 ppm
0	9.3	9.5	9.4	9.6	9.3	9.0	9.2	9.0	8.7	9.0
13	10.6	11.1	10.7	10.6	9.6	10.6	11.1	10.3	9.4	9.5
26	11.0	11.5	10.8	10.7	9.5	10.8	11.7	10.8	9.7	9.6
52	11.4	11.8	11.3	10.8	–	11.2	12.2	11.2	9.9	9.8
0–26	1.7	2.0	1.4	1.1	0.2*	1.8	2.6	1.7	1.0*	0.6**
0–52	2.0	2.3	1.8	1.2	–	2.2	3.0	2.2	1.2*	0.8**

*: $P < 0.05$; **: $P < 0.01$
Source: Brooker (1994a)

Females in the 12 000 and 30 000 ppm treatment groups generally showed a significant decrease in mean red cell indices (haematocrit, red blood cells and haemoglobin) compared with controls from week 13 through week 52 (Table 13). Males in the 12 000 ppm treatment group did not generally exhibit these decreases on the basis of mean value, although individual animals did show decreased red cell indices from week 22. The 30 000 ppm group males exhibited a marked reduction in red cell indices through the 26-week treatment period. Occasional reduction in red cell parameters in females in the 1200 ppm treatment group was only slight and sporadic and was not statistically significant. An increase in platelet count was recorded for males and females receiving 12 000 ppm and females receiving 30 000 ppm isoxaflutole, with the differences compared with controls considered statistically significant at both doses on all occasions, with the exception of week 39. No treatment-related changes were found in bone marrow smears.

Male and female Beagles in the 12 000 ppm treatment group, females in the 30 000 ppm treatment group and males in the 30 000 ppm treatment group at week 26 had lower serum albumin levels from week 13 onwards compared with controls (Table 14). This also resulted in corresponding reductions in total protein and a lower albumin to globulin ratio. Both sexes in the 12 000 ppm group and females in the 30 000 ppm group had significantly higher group mean plasma ALAT and AP levels than controls. Females in the 30 000 ppm treatment group exhibited a consistently low plasma urea level. From week 13 on, a treatment-related lowering of serum calcium was observed in both sexes in the 12 000 and 30 000 ppm treatment groups.

There were no meaningful differences in urinary pH; any statistically significant differences were considered to be of no toxicological importance. In males at 30 000 ppm, at week 26, urine specific gravity and protein were increased. Ketones and total reducing substances were increased in weeks 13 and 26. In males in all dose groups, urine specific gravity was increased relative to controls during week 36. Urine specific gravity and urine protein were increased in females at 30 000 ppm, although these findings were not always increased in a statistically significant manner. Urine specific gravity was occasionally increased in males and females at 12 000 ppm from week 39 of the study. Increased urine ketones were noted in males and females at 12 000 ppm and in females at 30 000 ppm from week 13 and in males and females at 1200 ppm only in week 26. The incidence of total reducing substances was increased in both males and females at 12 000 ppm and in females at 30 000 ppm from week 39. The increases in urinary ketones and total reducing substances are considered to be related to the excretion of the active ingredient and/or tyrosine metabolites in the urine and are thus markers of exposure rather than of toxicity.

Absolute and relative liver weights were elevated for two males in the 240 ppm treatment group, three males and one female in the 1200 ppm treatment group and all animals in the 12 000 and 30 000 ppm treatment groups. The increases in liver weights at 240 and 1200 ppm were small in magnitude and were considered as adaptive responses by the study author. Increases in kidney weights (relative and absolute) compared with controls were seen in the 1200 and 12 000 ppm treatment groups and in females receiving 30 000 ppm. Increased adjusted thyroid weights were noted for both sexes in the 12 000 ppm treatment group and in the 30 000 treatment group females. Treatment-related gross pathological changes were limited to friable surfaces of livers from male and female dogs in the 12 000 and 30 000 ppm treatment groups. An increased incidence and degree of hypertrophy of thyroid follicular epithelium were seen in males receiving 12 000 ppm (trace to minimum) and males and females receiving 30 000 ppm (trace to moderate). Liver changes were characterized by hepatocellular swelling and/or clumping and margination of cytoplasm. Centrilobular necrosis and fibrosis were also seen in males receiving 12 000 and 30 000 ppm. Dilated centrilobular sinusoids were seen in one male receiving 30 000 ppm. Occasional vacuolated hepatocytes were seen in females receiving 12 000 and 30 000 ppm. This change was also seen in one female receiving 240 ppm, but this is considered unlikely to be of toxicological significance.

In conclusion, the NOAEL was 1200 ppm (equal to 44.81 mg/kg bw per day), based on reduced weight gains, increase liver weight, histopathological findings in the liver and changes in haematological and clinical chemistry parameters at 12 000 ppm (equal to 453 mg/kg bw per day) (Brooker, 1994a).

Table 13. Treatment-related haematology parameters in the dog 52-week dietary study with isoxaflutole

Observation	Week	Males					Females				
		0 ppm	240 ppm	1 200 ppm	12 000 ppm	30 000 ppm	0 ppm	240 ppm	1 200 ppm	12 000 ppm	30 000 ppm
PCV (%)	−2	40	41	41	42	40	44	41	40*	40	42
	−1	41	41	41	40	38	43	43	41	39	41
	13	46	49	50	50	40	56	51	48*	49*	50*
	22	40	–	–	40	27	49	–	–	41	43
	24	43	–	–	46	31	51	–	–	41	44
	26	47	49	49	47	28	55	52	51	46**	46**
	39	47	48	47	47	–	51	51	48	44*	46*
	52	49	52	52	53	–	58	53	53	49*	54*
Hb (g/dL)	−2	12.7	12.9	12.9	13.0	12.7	13.8	13.5	12.6	12.7	13.4
	−1	13.0	13.3	13.0	12.4	12.3	13.6	13.8	13.5	12.7	12.9
	13	12.9	14.1	13.8	14.2	11.1	15.6	14.6	13.8	14.3	14.5
	22	13.5	–	–	13.5	8.8	16.3	–	–	13.9	14.3
	24	14.2	–	–	14.8	9.5	16.4	–	–	13.5	13.9
	26	14.2	15.1	14.9	14.1	8.0	16.8	15.9	15.7	14.2*	14.0**
	39	14.9	15.0	14.6	14.4	–	15.9	16.1	15.1	13.7*	14.0*
	52	15.4	15.7	15.3	15.4	–	17.5	15.7	15.8	14.3*	15.5*
RBC (× 10^6/mm^3)	−2	5.1	5.1	5.3	5.2	5.1	5.6	5.3	5.1*	5.0*	5.3
	−1	5.2	5.2	5.2	4.9	5.0	5.6	5.5	5.3	4.9*	5.2
	13	5.4	5.7	5.9	6.0	4.9	6.9	5.9*	5.8*	6.1*	6.4*
	22	5.5	–	–	5.5	3.6	6.7	–	–	5.6	6.1
	24	5.7	–	–	6.0	3.9	6.7	–	–	5.4	5.9
	26	5.8	6.0	6.2	5.8	3.3	6.9	6.3	6.4	5.8*	6.0**
	39	6.2	6.1	6.2	6.0	–	6.7	6.5	6.1	5.7	6.0
	52	6.2	6.3	6.6	6.6	–	7.2	6.4*	6.5*	6.0*	6.9*
MCHC (%)	−2	31.5	31.6	31.4	31.2	31.8	31.6	32.5**	31.9	32.2	32.2
	−1	31.5	32.0	31.5	31.4	32.1	31.8	32.4	32.7*	32.7*	31.8
	13	27.7	28.7	27.9	28.6	27.6	27.9	28.5	28.8*	29.0*	28.8*
	26	30.5	30.9	30.4	29.6	28.0	30.5	30.5	31.1	30.8	30.7
	39	31.6	31.3	31.0	30.7	–	31.2	31.3	31.6	30.8	30.5
	52	29.7	30.4	29.4	29.1	–	29.9	29.9	30.1	29.2	28.9*
Platelets (×10^3/mm^3)	−2	421	389	366	400	437	288	389	374	391	408*
	−1	371	363	317	365	383	345	374	356	367	350
	13	361	337	352	407	423	297	320	320	419*	426*
	26	357	353	380	476	334	308	324	334	459*	421*
	39	359	358	365	401	–	328	319	306	439	425
	52	345	396	357	411	–	305	356	320	471*	414*

Hb: haemoglobin; MCHC: mean corpuscular cell haemoglobin; PCV: packed cell volume; RBC: red blood cells; *: $P < 0.05$; **: $P < 0.01$

Source: Brooker (1994a)

Table 14. Treatment-related clinical chemistry parameters in the dog 52-week dietary study with isoxaflutole

End-point	Week	Males					Females				
		0 ppm	240 ppm	1 200 ppm	12 000 ppm	30 000 ppm	0 ppm	240 ppm	1 200 ppm	12 000 ppm	30 000 ppm
Albumin (g/dL)	−2	2.6	2.7	2.7	2.7	2.7	2.7	2.6	2.6	2.7	2.6
	−1	2.6	2.6	2.6	2.5	2.6	2.6	2.7	2.6	2.6	2.6
	13	2.7	2.7	2.6	2.3**	2.1**	2.9	2.8	2.7	2.3**	2.2**
	26	2.7	2.8	2.6	2.3*	2.0**	2.9	3.0	2.9	2.4**	2.3**
	39	2.8	2.9	2.6	2.3*	–	2.9	3.0	2.8	2.3**	2.3**
	52	2.8	2.8	2.6	2.3**	–	3.0	2.8	2.8	2.2**	2.3**
Globulin (g/dL)	−2	2.5	2.5	2.4	2.6	2.5	2.6	2.4	2.3*	2.4	2.5
	−1	2.4	2.4	2.4	2.4	2.4	2.4	2.4	2.3	2.2	2.4
	13	2.7	2.6	2.6	2.7	3.1	2.7	2.4	2.4	2.6	2.7
	26	3.0	2.6	2.9	2.8	3.3	2.8	2.6	2.7	2.9	2.7
	39	2.8	2.8	3.1	3.0	–	2.8	2.8	2.7	3.0	2.9
	52	2.8	2.6	2.8	2.9	–	2.8	2.9	2.8	3.4	3.0
Albumin/ globulin ratio	−2	1.05	1.06	1.10	1.04	1.06	1.03	1.11	1.13	1.10	1.08
	−1	1.09	1.11	1.07	1.05	1.06	1.06	1.13	1.16	1.16	1.09
	13	1.03	1.05	0.97	0.83	0.71**	1.08	1.20	1.11	0.88**	0.81**
	26	0.90	1.09	0.92	0.82	0.62**	1.05	1.15	1.11	0.87	0.85
	39	0.99	1.03	0.89	0.78	–	1.04	1.07	1.06	0.82*	0.79**
	52	0.98	1.08	0.95	0.81	–	1.08	0.98	1.04	0.69**	0.77**
Total protein (g/dL)	−2	5.2	5.2	5.1	5.2	5.2	5.3	5.0	5.0*	5.1	5.1
	−1	5.0	5.0	5.0	4.9	5.0	5.0	5.0	4.9	4.8	5.1
	13	5.4	5.3	5.2	5.0	5.2	5.6	5.2	5.1*	4.9**	5.0**
	26	5.7	5.4	5.5	5.1*	5.3*	5.7	5.6	5.6	5.3	5.0*
	39	5.6	5.6	5.7	5.4	–	5.7	5.7	5.5	5.3	5.2
	52	5.6	5.4	5.4	5.2	–	5.7	5.6	5.6	5.6	5.2
AP (mU/mL)	−2	239	240	249	249	297	306	224	208	244	275
	−1	235	245	235	241	266	286	218	205	240	268
	13	175	183	188	468**	636**	192	142	169	354**	490**
	26	217	160	167	610**	1 100**	169	141	158	540**	744**
	39	124	156	165	646**	–	146	141	150	479**	813**
	52	105	129	145	502**	–	136	117	155	498**	1 029**
ALAT (mU/mL)	−2	20	21	22	20	25	23	23	24	25	21
	−1	19	20	21	20	22	22	22	23	22	22
	13	21	28	23	31	72**	26	32	26	40*	50*
	26	21	27	24	28	38*	25	27	27	43*	40*
	39	22	29	24	31	–	24	27	24	39*	43*
	52	18	27	23	33**	–	25	25	22	27	29

Table 14 (continued)

End-point	Week	Males					Females				
		0 ppm	240 ppm	1 200 ppm	12 000 ppm	30 000 ppm	0 ppm	240 ppm	1 200 ppm	12 000 ppm	30 000 ppm
GGT (mU/mL)	26	3	3	3	4	5	3	3	2	3	4**
	39	2	< 3	< 2	3	–	< 2	< 2	< 2	3	4*
	52	3	< 3	< 3	3	–	3	< 1	< 2	2	3
5′-Nucleotidase (mU/mL)	26	< 0.4	< 0.4	0.2	< 1.6	2.6**	< 0.5	< 0.5	< 0.3	1.5*	3.3**
	39	0.1	0.2	0.5	2.6**	–	0.5	0.2	0.7	1.7*	3.5**
	52	< 0.2	< 0.2	< 0.2	1.3**	–	< 0.4	< 0.1	< 0.3	1.0*	3.1**
Calcium (meq/L)	−2	5.8	5.7	5.6	5.8	5.7	5.8	5.7	5.6	5.7	5.9
	−1	5.7	5.6	5.6	5.6	5.6	5.7	5.7	5.5	5.6	5.6
	13	5.3	5.4	5.3	5.1*	5.0**	5.5	5.3*	5.3*	5.1**	5.0**
	26	5.4	5.3	5.3	5.1*	5.0**	5.5	5.5	5.4	5.1**	5.1**
	39	5.2	5.1	5.1	5.0*	–	5.3	5.2	5.1	5.0	5.0
	52	5.1	5.1	5.1	4.9*	–	5.4	5.1*	5.2*	4.9**	4.9**
Urea (mg/dL)	−2	23	22	21	23	20	20	20	21	27	20
	−1	27	27	26	21*	25	23	26	24	26	23
	13	33	33	30	26	28	35	34	33	30	26
	26	29	26	24	20	30	27	28	27	25	21
	39	29	25	23	22	–	30	26	26	27	20**
	52	33	27*	27*	21**	–	30	24	27	24*	21**

ALAT: alanine aminotransferase; AP: alkaline phosphatase; eq: equivalents; GGT: gamma-glutamyltransferase; U: units; *: $P < 0.05$; **: $P < 0.01$
Source: Brooker (1994a)

(b) Dermal application

Rats

In a 21-day dermal toxicity study, eight CD rats of each sex per group were treated topically with isoxaflutole (purity not reported) at a dose of 0, 10, 100 or 1000 mg/kg bw per day, 8 hours/day, for 21 days. The test material was applied in 0.5% w/v methyl cellulose in purified water daily at a dosing volume of 2 mL/kg bw.

There were no deaths or signs of systemic toxicity observed during the study. Dermal irritation, including slight erythema and slight exfoliation on days 3 and 4, was observed in one female at 1000 mg/kg bw per day. Because of the isolated nature of the incidence, this finding was attributed to incidental causes. Mean body weights, body weight gains (days 0–20), mean feed consumption and mean feed efficiency of the treated animals did not significantly differ from those of the controls. There were no treatment-related changes in the haematology or clinical chemistry parameters. The lymphocyte counts of treated animals were slightly lower than those of the control animals, the values were within the normal ranges and there was no dose–response relationship. No treatment-related effects were noted. There were no treatment-related changes on gross necropsy examination of the animals. At 1000 mg/kg bw per day, the absolute liver weights of female rats and relative liver weights of male and female rats were higher (> 10%) than those of the control animals. This finding was considered to be treatment related but an adaptive response. Although increases (7%) in the liver weights were also noted at 100 and 10 mg/kg bw per day, the differences were not statistically significant. The only findings on histopathology involved the skin. These were confined to encrustations seen in one female rat from the 10 mg/kg bw per day group, two females from the

100 mg/kg bw per day group and one male from the 1000 mg/kg bw per day group at necropsy. These changes were of slight and minimal degree and showed no relationship to treatment.

In conclusion, based on lack of systemic toxicity, the NOAEL was 1000 mg/kg bw per day, the highest dose tested (Cummins, 1994).

(c) Exposure by inhalation

No studies are available.

2.3 Long-term studies of toxicity and carcinogenicity

Mice

In a 78-week carcinogenicity study, isoxaflutole (purity 98.7%) was given as a dietary admix to 64 or 76 mice of each sex per dose at 0, 25, 500 or 7000 ppm daily (equal to 0, 3.2, 64.4 and 977.3 mg/kg bw per day for males and 0, 4.0, 77.9 and 1161.1 mg/kg bw per day for females, respectively). Interim sacrifices were made at 26 weeks (12 mice of each sex at the 0 and 7000 ppm doses) and at 52 weeks (12 mice of each sex at all dose levels).

There was no treatment-related effect on mortality. The survival rates of the treated groups were not significantly different from those of the controls. There were no clinical signs, including palpable swellings, that differed between control and treated animals. Body weights and body weight gains were significantly lower than those of controls for male mice in the 500 and 7000 ppm treatment groups and for female mice in the 7000 ppm treatment groups. Feed consumption was unaffected by treatment; however, feed efficiency was lower in both sexes compared with controls at 7000 ppm during the first 14 weeks of the study (not determined after week 14). No treatment-related ocular changes were observed during the study. Blood from the control and 7000 ppm treatment groups was examined only for differential leukocyte counts by Romanowsky stain and direct visual count. No treatment-related differences in this parameter were observed. Blood from the 25 and 500 ppm treatment groups was not examined. Absolute and relative liver weights of male and female mice in the 7000 ppm treatment group were significantly higher (24–207%, $P < 0.01$) than those of the controls after 26, 52 and 78 weeks of treatment. There were significant differences in the absolute and relative adrenal weights of females in the 7000 ppm treatment group at 26 weeks and of males in the 7000 ppm treatment group at 52 weeks. However, in the absence of any histopathological findings, effects on adrenal weights were not considered as adverse. Macroscopic examination revealed a higher incidence of enlarged or swollen livers and/or liver masses and/or "areas of change" on the livers of male and female mice in the 7000 ppm treatment group. No other treatment-related gross postmortem differences were observed between mice in the 7000 ppm and the control groups.

Significant increases in microscopic liver abnormalities were observed in both sexes as early as the 26-week interim kill and were detected with increasing frequency at longer sacrifice intervals. At the 26-week kill, all of the high-dose males (12/12, $P < 0.001$) and females (12/12, $P < 0.001$) had developed periacinar hepatocytic hypertrophy. Other significant liver lesions at the 26-week kill of high-dose animals were hepatocyte necrosis (males 10/12, $P < 0.001$; females 7/12, $P < 0.01$) and pigmented Kupffer cells (males 10/12, $P < 0.001$). There were also non-significant incidences in high-dose males of periacinar hepatocytic fatty vacuolation (2/12) and pigment-laden hepatocytes (3/12), compared with none in controls. At 52 weeks, there were significant increases in periacinar hepatocytic hypertrophy in males and females, and periacinar hepatocytic fatty vacuolation decreased in males and increased in females ($P < 0.001$ and $P < 0.01$, respectively) in the 7000 ppm group. In addition, the high-dose males showed significant increases in other hepatocyte abnormalities. A significant increase in spleen extramedullary haematopoiesis (7/12, $P < 0.01$) was observed in high-dose males killed at 52 weeks. Male mice in the 500 ppm group exhibited a significant increase in periacinar hepatocytic hypertrophy (7/12, $P < 0.01$, versus none in controls).

Notable non-neoplastic lesions observed in mice in the 78-week terminal group are summarized in Table 15. Findings from animals sacrificed at termination and those having unscheduled deaths are presented separately, as each category was subjected to separate statistical

Table 15. Incidences of treatment-related non-neoplastic lesions in mice fed isoxaflutole for 78 weeks

Site and lesion	No. observed/no. examined			
	0 ppm	25 ppm	500 ppm	7 000 ppm
Males				
Liver				
Periacinar hepatocytic hypertrophy	0/37[a]	0/31	2/37	14***/36
	0/15[b]	*0/21*	*0/15*	*4/16*
Individual hepatocyte necrosis	4/37	5/31	12*/37	25***/36
	1/15	*0/21*	*1/15*	*8*/16*
Pigment-laden hepatocytes	0/37	0/31	0/37	6*/36
	0/15	*0/21*	*0/15*	*5*/16*
Erythrocyte-containing hepatocytes	0/37	1/31	4/37	11***/36
	NR	*NR*	*NR*	*NR*
Pigment-laden Kupffer cells	1/37	2/31	4/37	31***/36
	1/15	*0/21*	*1/15*	*12***/16*
Periacinar hepatocytic fatty vacuolation	17/37	17/31	14/37	9/36
	2/15	*0/21*	*1/15*	*2/16*
Basophilic foci	1/37	2/31	0/37	9**/36
	0/15	*0/21*	*0/15*	*1/16*
Clear cell foci	5/37	0/31	3/37	11/36
	NR	*NR*	*NR*	*NR*
Increased ploidy	0/37	0/31	2/37	10***/36
	0/15	*0/21*	*0/15*	*2/16*
Spleen				
Extramedullary haematopoiesis	3/37	4/6	4/8	11*/36
	4/15	*8/21*	*7/15*	*7/15*
Thyroid				
Amyloidosis	2/37	–/0	–/0	12**/36
	3/15	*2/21*	*0/15*	*6/16*
Females				
Liver				
Periacinar hepatocytic hypertrophy	0/43	0/39	1/39	17***/46
	0/9	*0/13*	*2/13*	*1/6*
Individual hepatocyte necrosis	0/43	1/39	0/39	8**/46
	1/9	*0/13*	*0/13*	*0/6*
Pigment-laden hepatocytes	0/43	0/39	0/39	0/46
	0/9	*0/13*	*1/13*	*0/6*
Erythrocyte-containing hepatocytes	0/43	1/39	1/39	9**/46
	NR	*NR*	*NR*	*NR*
Pigment-laden Kupffer cells	7/43	4/39	2/39	10/46
	1/9	*1/13*	*2/13*	*1/6*
Periacinar hepatocytic fatty vacuolation	13/43	9/39	7/39	26*/46
	0/9	*1/13*	*1/13*	*1/6*
Basophilic foci	0/43	0/39	0/39	0/46
	0/9	*0/13*	*0/13*	*0/6*

Table 15 (continued)

Site and lesion	No. observed/no. examined			
	0 ppm	25 ppm	500 ppm	7 000 ppm
Clear cell foci	0/43	0/39	0/39	0/46
	NR	*NR*	*NR*	*NR*
Increased ploidy	0/43	0/39	0/39	0/46
	0/9	*0/13*	*0/13*	*0/6*
Spleen				
Extramedullary haematopoiesis	3/43	3/8	4/10	12*/46
	6/9	*8/13*	*5/13*	*5/6*
Thyroid				
Amyloidosis	1/43	–/0	–/0	14***/46
	0/9	*0/13*	*0/13*	*0/6*

NR: not reported; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$
[a] Data are from the mice killed on schedule at 78 weeks.
[b] Data in italics are from unscheduled deaths occurring throughout the 78-week study period.
Source: Chase (1995b)

analysis. Male and female mice in the 7000 ppm treatment group exhibited an increased incidence of periacinar hepatocytic hypertrophy, necrosis of individual hepatocytes and erythrocytes in hepatocytes, pigment-laden hepatocytes (only in males) and Kupffer cells, compared with the controls; male mice also exhibited basophilic foci of hepatocellular alteration and increased ploidy. Male mice in the 500 ppm group exhibited necrosis of individual hepatocytes (12/37, $P < 0.05$). In addition, mice in the 7000 ppm treatment group had a higher incidence of systemic amyloidosis, involving the kidneys, small intestine, stomach, thyroid, mesenteric lymph nodes and heart. Both sexes were significantly affected at the high dose. Amyloidosis of the thyroid occurred in 12/36 ($P < 0.01$) terminal kill males, 6/16 unscheduled male deaths and 14/46 ($P < 0.001$) terminal kill females at the high dose.

No neoplastic lesions that were considered to have been test article related were observed among males or females sacrificed at 26 weeks or among females in the 52-week interim group. At the 52-week sacrifice, 7/12 ($P < 0.05$) males at 7000 ppm showed an increased incidence of adenomas; only 1/12 males in the 500 ppm group developed carcinoma. For the 78-week terminal groups, Table 16 summarizes the data for unscheduled deaths and scheduled sacrifices, respectively, and the overall incidence. The tumour incidence among unscheduled deaths was similar to that observed in the scheduled sacrifices. There was a significant increase in the occurrence of adenomas (7/16, $P < 0.05$) in males at 7000 ppm. In animals sacrificed on schedule, significant ($P < 0.01$) increases in the incidence of adenomas and carcinomas were observed in the 7000 ppm males, and adenomas were increased significantly ($P < 0.001$) in the 7000 ppm females. The overall incidences of adenomas and carcinomas in both sexes at 7000 ppm were each considerably higher than the maxima of the historical control ranges. In males at 500 ppm, adenomas and carcinomas occurred with frequencies of 17% and 15%, higher than the corresponding mean historical control values of 15% and 6%. Statistical analysis of neoplastic tissue data indicated significant positive treatment-related trends in the incidence of benign, malignant and combined benign/malignant liver tumours. Pair-wise comparisons between the 7000 ppm treatment group and the controls for benign and combined tumour incidences were statistically significant.

No liver tumours were observed in mice sacrificed at 26 weeks. Adenomas appeared at approximately 1 year in controls and all dose groups of males. Carcinoma appearance, however, showed a clear trend from 78 days in controls to 47 days in the 7000 ppm dose group (the data were not analysed statistically). In females, no adenomas were observed before 77 weeks in any group. Carcinomas were not seen in any control or treated female through 500 ppm; in the 7000 ppm group,

Table 16. Overall incidence of liver tumours in mice fed isoxaflutole (78-week terminal phase; scheduled + unscheduled sacrifices)

	0 ppm	25 ppm	500 ppm	7 000 ppm	Historical controls[a]	
					Mean	Range
Unscheduled deaths						
Males						
No. examined	15	21	15	16	–	–
Hepatocellular adenoma	1 (7%)	1 (7%)	1 (7%)	7* (44%)	–	–
Hepatocellular carcinoma	1 (7%)	3 (14%)	3 (20%)	4 (25%)	–	–
Adenomas and/or carcinomas combined	2 (13%)	4 (19%)	4 (27%)	10[b] (63%)	–	–
Females						
No. examined	9	13	13	6	–	–
Hepatocellular adenoma	0 (0%)	0 (0%)	0 (0%)	2 (33%)	–	–
Hepatocellular carcinoma	0 (0%)	0 (0%)	0 (0%)	1 (17%)	–	–
Adenomas and/or carcinomas combined	0 (0%)	0 (0%)	0 (0%)	3 (50%)	–	–
Scheduled sacrifice						
Males						
No. examined	37	31	37	36	–	–
Hepatocellular adenoma	8 (22%)	9 (29%)	8 (22%)	20** (56%)	–	–
Hepatocellular carcinoma	3 (8%)	2 (6%)	5 (14%)	13** (36%)	–	–
Adenomas and/or carcinomas combined	11 (30%)	11 (35%)	10[b] (27%)	28[b] (78%)	–	–
Females						
No. examined	43	39	39	46	–	–
Hepatocellular adenoma	0 (0%)	1 (3%)	1 (3%)	13*** (28%)	–	–
Hepatocellular carcinoma	0 (0%)	0 (0%)	0 (0%)	3 (7%)	–	–
Adenomas and/or carcinomas combined	0 (0%)	1 (3%)	1 (3%)	15[b] (33%)	–	–
Unscheduled deaths and scheduled sacrifice combined						
Males (n = 52/group)						
Hepatocellular adenoma	9 (17%)[†]	10 (19%)	9 (17%)	27* (52%)	15.0%	3.8–23.1%
Hepatocellular carcinoma	4 (8%)[†]	5 (10%)	8 (15%)	17* (33%)	6.28%	1.9–11.5%
Adenomas and/or carcinomas combined	13 (25%)[†]	15 (29%)	14 (27%)	38* (73%)	Data not provided	

Table 16 (continued)

	0 ppm	25 ppm	500 ppm	7 000 ppm	Historical controls[a]	
					Mean	Range
Females (n = 52/group)						
Hepatocellular adenoma	0 (0%)†	1 (2%)	1 (2%)	15* (29%)	0.27%	0–2.0%
Hepatocellular carcinoma	0 (0%)‡	0 (0%)	0 (0%)	4 (8%)	0.55%	0–2.0%
Adenomas and/or carcinomas combined	0 (0%)†	1 (2%)	1 (2%)	18* (35%)	Data not provided	

*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ for unscheduled deaths and scheduled sacrifice group; *: $P < 0.001$ (pair-wise comparison); †: $P < 0.001$; ‡: $P < 0.05$ (trend test) for combined group

[a] Historical control data obtained from Chase (1995b); incidence data from seven studies showed that of the 366 males and 366 females examined, 55 males (15.03%) and 1 female (0.27%) had adenomas and 23 males (6.28%) and 2 females (0.55%) had carcinomas.

[b] Some animals developed both adenoma and carcinoma.

Source: Chase (1995b)

the onset of carcinomas was 60 weeks. Only 1/12 males at 500 ppm among unscheduled deaths in the 52-week interim sacrifice developed hepatocellular carcinoma.

In conclusion, the NOAEL for this study was 25 ppm (equal to 3.2 mg/kg bw per day), based on increased liver weights and increased histopathological changes (increased incidence of periacinar hepatocytic hypertrophy and necrosis of the individual hepatocytes) at 500 ppm (equal to 64.4 mg/kg bw per day). The NOAEL for carcinogenicity was 500 ppm (equal to 64.4 mg/kg bw per day), based on an increased incidence of hepatocellular adenomas and carcinomas in both sexes at 7000 ppm (equal to 977.3 mg/kg bw per day) (Chase, 1995b).

Rats

In a combined chronic toxicity and carcinogenicity study, isoxaflutole (purity 98.3–99.2%) was continuously administered to 75 Sprague-Dawley rats of each sex per dose at a dietary level of 0, 0.5, 2, 20 or 500 mg/kg bw per day for 104 weeks. An additional 20 rats of each sex per group were treated for 52 weeks, after which 10 rats of each sex per group were killed and the remainder were held for a maximum of 8 weeks without treatment in order to assess reversibility of treatment-related changes.

There was no treatment-related effect on mortality. Mortality was statistically significantly reduced at 500 mg/kg bw per day in both males and females in the oncogenicity phase of the study. Significant treatment-related clinical findings were observed in both sexes at 500 mg/kg bw per day and included opaque eyes, thin body build, abnormal gait and limited use of hindlimbs. From the 1st week of the study, mean body weight gains in the 500 mg/kg bw per day group rats were lower than those of controls; from weeks 0 through 104, body weight gains were decreased by 36% and 49% ($P < 0.01$) in males and females, respectively. These decreased body weight gains were considered to be treatment related. During the 6-week recovery phase (following 52 weeks of treatment), the 500 mg/kg bw per day group male and female rats had 104% and 59% weight gain increases, respectively, over the controls ($P < 0.01$ in males only), indicating partial recovery. Body weight gains of animals in the 0.5, 2 and 20 mg/kg bw per day groups were unaffected by treatment. Feed consumption in the 500 mg/kg bw per day group females (104-week terminal phase) was decreased (4–17%) at four consecutive 6-month intervals; overall feed consumption was 12% lower than in controls. No adverse effects on feed consumption were seen in males. Feed consumption for treated animals was similar to that of control rats during the 6-week recovery period. Feed conversion efficiency in the 104-week terminal phase rats, calculated through week 14, was lower in the 500 mg/kg bw per day group males

(12%) and females (19%) compared with controls. Ophthalmoscopic examinations (Table 17) revealed treatment-related corneal lesions, ranging from small focal superficial opacities to large corneal opacities with associated vascularization and iritis in males at 20 mg/kg bw per day and in both sexes at 500 mg/kg bw per day, with the rate of incidence greater in females and severity of the lesions greater in males.

Table 17. Number of rats with treatment-related corneal lesions[a]

Study week	Number observed/number examined				
	0 mg/kg bw per day	0.5 mg/kg bw per day	2 mg/kg bw per day	20 mg/kg bw per day	500 mg/kg bw per day
Males					
5	0/45	0/45	0/45	0/45	28/45 (62%)
11	0/45	0/45	0/45	0/45	27/45 (60%)
23	0/45	0/45	0/45	2/45 (4%)	29/45 (64%)
37	0/44	0/45	0/45	1/45 (2%)	32/44 (73%)
49	0/44	0/45	0/45	4/45 (9%)	33/44 (75%)
75	0/25	0/25	0/25	2/25 (8%)	18/25 (72%)
101	0/25	0/25	0/25	4/25 (16%)	20/25 (80%)
Females					
5	0/45	0/45	0/45	0/45	30/45 (67%)
11	0/45	0/45	0/45	0/45	38/45 (84%)
23	0/45	0/45	0/45	0/45	42/45 (93%)
37	0/44	0/44	0/45	1/45 (2%)	39/44 (89%)
49	0/43	0/44	0/45	1/45 (2%)	39/44 (89%)
75	0/25	0/25	0/25	0/25	23/25 (92%)
101	0/25	0/25	0/25	0/25	16/25 (64%)

[a] Expressed as the number of animals with the reported finding in one or both eyes.
Source: Chase (1995a)

During 102 weeks of treatment, a few changes in platelet count as well as erythrocyte count and mean haemoglobin values were seen in the 500 mg/kg bw per day group rats. These differences, although occasionally statistically significant, were generally minor and not dose related and therefore were judged not to be of toxicological concern. These changes were no longer apparent after a 7-week recovery period (following 52 weeks of treatment). There were treatment-related clinical chemistry and urine analysis findings in both sexes at 20 and 500 mg/kg bw per day; however, after a 7-week recovery period, there were no findings that were considered to be biologically or toxicologically significant in either sex. Significant ($P < 0.05$, 0.01 or 0.001) decreases in AP (decreased 3–49%), ALAT (decreased 14–53%) and ASAT (decreased 14–53%) activities and changes in levels of urea (increased 16–31%), glucose (decreased 0–18%), potassium (increased 6–17%), chloride (decreased 2–5%) and total plasma protein (increased 7–18%) were seen in one or both sexes primarily in the 500 mg/kg bw per day dose group in the 104-week study. Cholesterol levels were increased throughout the dosing period in the 500 mg/kg bw per day dose group animals (32–87% increases over controls; $P < 0.05$). In the 20 mg/kg bw per day males, cholesterol was elevated (29–54%; $P < 0.05$). Levels were also increased at the 6-, 50-, 78- and 102-week intervals in the 20 mg/kg bw per day dose group females (21–48%; $P < 0.05$ or 0.01). The overall evidence is inconclusive with respect to the biological importance of the elevated cholesterol levels. Urine analysis at 50 weeks indicated changes in pH (decreased 4–22%), urinary output (decreased 40% only in week 77 in females; male urinary output slightly increased [about 20%] in both week 50 and week 77), specific gravity (increased 1–3%), total reducing substances, ketones and colour. These findings were detected primarily in the 500

mg/kg bw per day group. In females at 20 mg/kg bw per day, specific gravity was increased at 77 weeks of treatment. After 101 weeks of treatment, significant differences were seen only in urinary pH (decreased 6%) in males and specific gravity (increased 2%) in females. Urine was positive for total reducing substances after 50 weeks for rats receiving 500 mg/kg bw per day, but not at 101 weeks. Furthermore, after the 6-week recovery period, the urinary parameters in the control and treated rats were similar.

Among animals sacrificed at 52 weeks, in males at 20 mg/kg bw per day and in both males and females at 500 mg/kg bw per day, absolute and relative liver weights were increased relative to controls. Absolute and relative thyroid weights were increased in males and relative thyroid weight was increased in females at 500 mg/kg bw per day (Table 18). After the 8-week recovery period (following 52 weeks of treatment), absolute and relative thyroid weights remained increased in the high-dose females. Relative kidney weight in males at 500 mg/kg bw per day was 19% higher than in the controls. In animals sacrificed at 104 weeks, in males at 20 mg/kg bw per day and in both males and females at 500 mg/kg bw per day, absolute and relative liver weights were increased relative to controls. In males at 500 mg/kg bw per day, absolute and relative kidney weights were increased compared with control animals. In females at 500 mg/kg bw per day, the absolute and relative weights of the uterus and cervix weighed together were increased relative to controls. Treatment-related gross pathological findings at 20 and 500 mg/kg bw per day included swollen livers (males at 20 and 500 mg/kg bw per day), "areas of change" on lungs (males at 500 mg/kg bw per day), masses (males and females at 500 mg/kg bw per day) in the liver, opaque eyes (males at 20 and 500 mg/kg bw per day) and dark enlarged thyroids with masses (males at 20 and 500 mg/kg bw per day). At 20 mg/kg bw per day, the incidence of enlargement of the thyroid was increased in males only.

Table 18. Terminal body weight and organ weights in the rat oncogenicity study with isoxaflutole

Observation	Males					Females				
	0 mg/kg bw per day	0.5 mg/kg bw per day	2 mg/kg bw per day	20 mg/kg bw per day	500 mg/kg bw per day	0 mg/kg bw per day	0.5 mg/kg bw per day	2 mg/kg bw per day	20 mg/kg bw per day	500 mg/kg bw per day
12 months										
N	10	10	10	10	10	7	9	10	10	10
Terminal body weight (g)	835.3	852.5	770.0	759.8	680.3*	414.7	428.5	412.8	449.6	303.1
Liver weight (g)	22.8	26.1	24.8	28.5*	40.8**	14.0	15.1	13.5	15.7	18.8**
Liver weight (% of body weight)	2.75	3.01	3.25	3.78**	6.11**	3.46	3.50	3.27	3.48	6.38**
Thyroid weight (g)	0.027	0.033	0.031	0.030	0.047*	0.021	0.021	0.020	0.027	0.023
Thyroid weight (% of body weight)	0.003 2	0.003 9	0.004 0	0.004 0	0.006 9**	0.005 3	0.004 8	0.004 8	0.006 0	0.007 9*
12 months + 8 weeks of reversibility										
N	9	9	10	10	9	10	10	10	10	8
Terminal body weight (g)	803.4	856.6	812.1	868.7	737.6	445.2	485.8	488.5	446.7	384.1
Liver weight (g)	25.1	28.1	23.8	31.0*	24.9	14.8	16.4	15.6	15.2	14.6

Observation	Males					Females				
	0 mg/kg bw per day	0.5 mg/kg bw per day	2 mg/kg bw per day	20 mg/kg bw per day	500 mg/kg bw per day	0 mg/kg bw per day	0.5 mg/kg bw per day	2 mg/kg bw per day	20 mg/kg bw per day	500 mg/kg bw per day
Liver weight (% of body weight)	3.11	3.28	2.93	3.58	3.40	3.34	3.37	3.21	3.40	3.83
Thyroid weight (g)	0.029	0.033	0.034	0.043	0.035	0.020	0.020	0.021	0.021	0.028*
Thyroid weight (% of body weight)	0.003 7	0.003 8	0.004 2	0.004 9	0.004 9	0.004 5	0.004 3	0.004 4	0.004 7	0.007 3**
24 months										
N	34	25	34	37	46	35	23	25	22	48
Terminal body weight (g)	802.0	850.8	870.9	798.1	568.7**	548.7	551.1	564.5	543.6	347.0**
Liver weight (g)	27.4	25.0	30.3	31.4*	42.8**	20.5	21.7	19.2	22.0	25.5**
Liver weight (% of body weight)	3.55	2.99*	3.58	4.08	7.62**	3.81	3.95	3.43*	4.11	7.51**
Thyroid weight (g)	0.051	0.055	0.046	0.063	0.055	0.046	0.031	0.042	0.034	0.029
Thyroid weight (% of body weight)	0.006 1	0.006 5	0.005 3	0.008 2	0.009 5**	0.009 5	0.005 8	0.006 9	0.006 4	0.008 5
Kidney weight (g)	5.47	5.06	5.84	5.77	6.37*	3.96	3.82	3.66	3.67	3.26**
Kidney weight (% of body weight)	0.723	0.616	0.695	0.744	1.137**	0.767	0.728	0.661	0.697	0.952**
Uterus weight (g)	–	–	–	–	–	0.85	1.00	0.78	0.84	1.10*
Uterus weight (% of body weight)	–	–	–	–	–	0.163	0.191	0.141	0.165	0.334**

*: $P < 0.05$; **: $P < 0.01$
Source: Chase (1995a)

At terminal sacrifice, treatment-related non-neoplastic findings in the liver included periacinar hepatocytic hypertrophy (both males and females at 20 and 500 mg/kg bw per day), focal cystic degeneration (males at 20 and 500 mg/kg bw per day), midzonal foamy hepatocytes (males at 20 mg/kg bw per day and both males and females at 500 mg/kg bw per day), portal tract senile changes in bile duct (both males and females at both 20 and 500 mg/kg bw per day), basophilic and clear cell foci (females at 500 mg/kg bw per day) and pigment-laden hepatocytes (females at 500 mg/kg bw per day). Treatment-related lesions of the eye were observed in males and included increased incidences of keratitis (2, 20 and 500 mg/kg bw per day), vascularization of the stroma (500 mg/kg bw per day), epithelial thickening (20 and 500 mg/kg bw per day) and superficial exfoliated epithelial cells (500 mg/kg bw per day). Other treatment-related non-neoplastic findings included increased incidences of thyroid cystic follicular hyperplasia, axonal and myelin sciatic nerve degeneration, and focal degeneration and inflammation of the thigh muscle in males at 20 and 500 mg/kg bw per day and in females at 500 mg/kg bw per day.

There were no treatment-related neoplastic lesions detected in the animals at the interim kill or on completion of the recovery period. The incidences of selected neoplastic lesions detected in animals that were killed or died during the study and at the 104-week terminal sacrifice are presented in Table 19. Treatment-related neoplastic lesions were detected in the livers and thyroid glands. In the 500 mg/kg bw per day group rats (both sexes), there were significant increases in the incidences of hepatocellular adenomas, hepatocellular carcinomas, and combined adenomas plus carcinomas (41% and 62% for males and females, respectively). In both sexes at 500 mg/kg bw per day, the incidence of carcinomas (23% and 32% for males and females, respectively) contributed to the overall increase in liver tumour incidence; animals with carcinomas accounted for over half of the total number of animals bearing adenomas and/or carcinomas. Thyroid follicular cell adenomas showed a significantly increased incidence ($P < 0.01$) in males at 500 mg/kg bw per day, but not females, although there were positive trends for both sexes ($P < 0.05$). There were no treatment-related neoplastic lesions detected in the 0.5, 2 or 20 mg/kg bw per day dose group rats.

Table 19. Overall incidence of liver and thyroid tumours in rats fed isoxaflutole (104-week terminal phase)[a]

Site/tumour	0 mg/kg bw per day	0.5 mg/kg bw per day	2 mg/kg bw per day	20 mg/kg bw per day	500 mg/kg bw per day	Historical controls[b]	
						Mean (%)	Range (%)
Males							
Liver							
Hepatocellular adenoma	2/75 (2.7)[‡c]	3/75 (4.0)	5/75 (6.7)	6/75 (8.0)	14**/75 (18.7)	2.51	0–10
Hepatocellular carcinoma	5/75 (6.7)[‡]	1/75 (1.4)	4/75 (5.3)	2/75 (2.7)	17**/75 (22.7)	2.28	0–6
Hepatocellular adenomas and/or carcinomas combined	7/75 (9.3)[†]	4/75 (5.3)	9/75 (12)	8/75 (10.7)	31***/75 (41.3)	Data not provided	
Thyroid							
Follicular cell adenoma	3/74 (4.1)[†]	1/72 (1.3)	5/74 (6.8)	7/75 (9.3)	15**/75 (20)	3.04	0–6.4
Follicular cell carcinoma	0/74	1/72	2/74	1/75	3/75	–	–
Females							
Liver							
Hepatocellular adenoma	4/75[†] (5.3)	2/75 (2.7)	1/75 (1.3)	0/75 (0)	29***/74 (39.2)	1.19	0–3.6
Hepatocellular carcinoma	0/75[†] (0)	0/75 (0)	1/75 (1.3)	0/75 (0)	24***/74 (32.4)	0.00	0–0
Hepatocellular adenomas and/or carcinomas combined	4/75[†] (5.3)	2/75 (2.7)	2/75 (2.7)	0/75 (0)	46***/74 (62.2)	Data not provided	
Thyroid							
Follicular cell adenoma	1/74[‡] (1.4)	0/73 (0)	1/73 (1.4)	4/74 (5.4)	3/73 (4.1)	0.72	0–2.0
Follicular cell carcinoma	0/74 (0.0)	1/73 (1.4)	1/73 (1.4)	0/74 (0.0)	2/73 (2.7)	–	–

**: $P < 0.01$; ***: $P < 0.001$ (pair-wise analysis); †: $P < 0.001$; ‡: $P < 0.05$ (trend analysis)

[a] Includes scheduled kills and unscheduled deaths.

[b] Historical control incidence data from eight studies, 440 males and 420 females.

[c] Percentage of animals with specific lesions.

Source: Chase (1995a)

In males, the earliest adenoma was observed at the 52-week interim sacrifice (approximately 365 days). Otherwise, there was no indication of a treatment-related decrease in the latency period in males. In females at 500 mg/kg bw per day, the first liver adenoma and carcinoma appeared considerably earlier (427 and 426 days, respectively) than did these tumours in controls (728 days at the terminal kill). The first thyroid tumour appeared in the 20 and 500 mg/kg bw per day females somewhat sooner than in controls, 576 and 623 days, respectively, versus 714 days for controls.

In conclusion, the NOAEL was 2 mg/kg bw per day, based on liver, thyroid, ocular and nervous system toxicity in males and liver toxicity in females seen at 20 mg/kg bw per day. An increased incidence of adenomas and carcinomas of the liver was found in male and female rats at 500 mg/kg bw per day. In male rats, an increase of thyroid follicular cell adenomas was also observed at 500 mg/kg bw per day (Chase, 1995a).

2.4 Genotoxicity

The results of studies of genotoxicity with isoxaflutole are summarized in Table 20. All the studies, either in vitro or in vivo, were negative. Isoxaflutole is not considered to possess any mutagenic or genotoxic potential.

Table 20. Results of studies of genotoxicity with isoxaflutole

Type of study	Organism/cell line	Dose range tested	Purity (%)	Result	Reference
In vitro					
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA1538	50–1 000 µg/plate (in acetone)	98.7	Negative ±S9 mix	Percy (1993)
Chromosomal aberration	Human lymphocytes	0–500 µg/mL (in acetone)	99.4	Negative ±S9 mix	Dance (1993)
Chromosomal aberration	Human lymphocytes	0–300 µg/mL (−S9 mix; in acetone) 0–600 µg/mL (+S9 mix; in acetone)	98.7	Negative ±S9 mix	Dance (1992)
Gene mutation in mammalian cells	Chinese hamster lung cells (V79)	50–100 µg/mL (in acetone)	99.4	Negative ±S9 mix	Lloyd (1992)
Gene mutation in mammalian cells	Mouse lymphoma L5178Y cells	0–600 µg/mL (in acetone)	98.7	Negative ±S9 mix	Strang (1993)
In vivo					
Mouse micronucleus	Male and female CD-1 mice	0, 200, 1 000 and 5 000 mg/kg bw (gavage in corn oil)	98.7	Negative	Edwards (1993)
DNA repair UDS assay (mouse spot test)	Male Sprague-Dawley rats	0, 600 and 2 000 mg/kg bw (gavage in 1% aqueous methyl cellulose)	99.8	Negative	Proudlock, Gant & Dawe (1997)

DNA: deoxyribonucleic acid; S9: 9000 × *g* supernatant fraction from rat liver homogenate; UDS: unscheduled DNA synthesis

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

Rats

In a multigeneration reproductive toxicity study, male and female Crl:CD®BR VAF/Plus® rats (30 of each sex per group) were fed diets containing varying concentrations of isoxaflutole (purity 98.7%) to provide constant doses at all phases of the study of 0, 0.5, 2, 20 and 500 mg/kg bw per day. Adults of the F_0 generation were maintained on control or treatment diets for a 10-week premating period, then throughout mating, gestation and lactation of the F_1 litters. At weaning, the F_1 pups were then fed the same dose level of isoxaflutole as their parents for at least 10 weeks between weaning and mating and throughout mating, gestation and lactation of the F_2 litters. Ophthalmological examinations were conducted prior to the study start, on all adults during the final week of the premating periods, on at least two F_2 pups per litter between lactation days (LDs) 16 and 28 and on all F_2 weanlings receiving 20 or 500 mg/kg bw per day prior to the end of the study. Pairings were on a one-to-one basis within treatment groups. Litters were culled to contain no more than eight pups, wherever possible four males and four females, on LD 4. At necropsy of adults, the reproductive tract, liver and eyes were weighed and/or preserved for histopathological examination, as appropriate. The reproductive tract, liver and eyes were examined by histopathology in pups, but organ weights were not determined.

There were no treatment-related mortalities or clinical signs observed in any dose group or generation. In the F_0 group, at 500 mg/kg bw per day, body weights were significantly decreased relative to controls at all phases of the study. Body weight gain was statistically significantly reduced in 500 mg/kg bw per day males from week 1. In males, the body weight gain was reduced by 14.6% compared with controls during premating (0–10 weeks). Body weight gain was reduced in a statistically significant manner in females at 500 mg/kg bw per day during premating (21.6% compared with controls), gestation days (GDs) 7–14 (27.17% compared with controls) and LDs 0–4 (1.2 g loss of body weight). Females at 500 mg/kg bw per day lost significantly less weight than controls on LDs 14–21 and gained significantly more weight on LDs 4–7. There was no effect on either body weight or body weight gain at 0.5, 2 or 20 mg/kg bw per day in the F_0 group.

F_1 males and females at 500 mg/kg bw per day began the second generation premating period at significantly reduced mean body weights. These differences from control were observed in both sexes throughout the premating period. During the 10-week premating interval, mean body weight values for F_1 males were 18–26% less than those of controls for the 500 mg/kg bw per day dose group; for F_1 females, mean body weight values were 11–20% less than those of controls. For F_1 males, the body weight gain over the 10-week premating interval was significantly lower (15% during weeks 0–10) at 500 mg/kg bw per day than in controls. For F_1 females, the body weight gain was lower (11% during weeks 0–10) at 500 mg/kg bw per day than in controls. Premating body weight and body weight gain values for F_0 and F_1 rats at lower dose levels were not adversely affected by treatment with isoxaflutole. There were no treatment-related ophthalmological findings in the F_0 adults. At 500 mg/kg bw per day, a compound-related increased occurrence of chronic keratitis was noted in F_1 males (both eyes: 13/30; right eye: 5/30; left eye: 5/30) and females (both eyes: 14/30; right eye: 2/30; left eye: 3/30). This finding was not noted in controls or at any lower dose. At 500 mg/kg bw per day, terminal body weights of animals in both generations were lower (9–20%) than those of the controls. There was a compound-related significant increase in mean absolute and relative liver weights in F_0 adults at 20 and 500 mg/kg bw per day and in F_1 adults at 500 mg/kg bw per day when compared with controls. The relative liver weight in F_1 males was increased (12%) at 20 mg/kg bw per day; the absolute liver weight was unaffected. Significant decreases in absolute and/or relative weights of the epididymis, seminal vesicle, testis and ovary in F_0 and F_1 males/females at 20 and/or 500 mg/kg bw per day were attributed to lower body weights. Necropsy observations reported at 500 mg/kg bw per day for F_0 and F_1 adult rats revealed a treatment-related liver effect consisting of mottled liver (F_0: 6/30 and 4/30; F_1: 6/30 and 2/30, in males and females, respectively, versus none in controls). At 20 and 500 mg/kg bw per day, compound-related changes observed in the liver consisted of centrilobular hypertrophy in males and females as well as vacuolation in males from both

generations. These changes were severe and frequent in males at 500 mg/kg bw per day. At 500 mg/kg bw per day, subacute bilateral or unilateral inflammation of cornea was noted in F_1 generation males and females.

Treatment-related clinical signs in the F_1 offspring were limited to some observations of enlarged eyes, eyes opaque and eyes darker than normal. There were no treatment-related clinical signs for F_2 pups. There were no biologically significant differences in mean litter size, mean number of live and dead offspring and sex ratio between treated and control groups in either generation. At 20 and 500 mg/kg bw per day, there were significant decreases in the viability indices (parental and F_1 animals) and mean body weights of male and female offspring of both generations compared with controls. At 500 mg/kg bw per day, there were significant decreases in the viability indices of F_1 and F_2 animals. Significant decreases in live birth indices observed at 0.5, 20 and 500 mg/kg bw per day in the F_0 generation were not considered to be treatment related because this effect was not seen in the F_1 generation. There were no significant differences in gestation index, weaning index or mean number of pups delivered. There were no differences in sex ratio. The viability index, however, was reduced at 20 and 100 mg/kg bw per day in F_1 pups and at 500 mg/kg bw per day in F_2 pups; the decreased viability index was associated with a dose-related increase in pup mortality during LDs 0–4. A compound-related decrease in mean pup body weight of both the F_1 (11–35%) and F_2 (8–29%) pups was noted at 500 mg/kg bw per day from postnatal day (PND) 0 to PND 21. Ophthalmic examination revealed chronic keratitis in F_2 male and female pups (LDs 16–28) and weanlings (days 20–37 of age) at 500 mg/kg bw per day. Additionally, F_2 pups at 500 mg/kg bw per day had a low incidence of inflammation of the iris, as well as retinal and vitreous bleeding. At 500 mg/kg bw per day, there was an increase in the number of F_1 pups and litters, culled on postpartum 4, with no milk in the stomach. At 500 mg/kg bw per day, there was a slight increase in the incidence of underdeveloped renal papilla in pups from both generations. Organ weights were not determined in weanlings of either the F_1 or the F_2 generation. There were no treatment-related findings at any dose in the F_1 or F_2 weanlings.

In conclusion, the NOAEL for parental systemic toxicity and offspring toxicity was 2 mg/kg bw per day. The parental systemic toxicity LOAEL of 20 mg/kg bw per day was based upon increased liver weights, liver hypertrophy and vacuolation. The offspring toxicity LOAEL of 20 mg/kg bw per day was based on decreased pup weights and reduced pup viability. The NOAEL for reproductive toxicity was 500 mg/kg bw per day, the highest dose tested (Henwood, 1995).

(b) Developmental toxicity

Rats

In a developmental toxicity study, isoxaflutole (purity not specified; lot/batch 40 ADM 93) was administered to 25 female Sprague-Dawley rats by gavage in a volume of 10 mL/kg bw at a dose level of 0, 10, 100 or 500 mg/kg bw per day from GDs 6 to 15, inclusive. The test material was suspended in 0.5% aqueous carboxymethyl cellulose. Females were weighed on days 0, 3, 6–16 inclusive, 18 and 20 of gestation. Feed consumption and water consumption were recorded periodically throughout the study. At necropsy on GD 20, the reproductive tracts were dissected and examined. Fetuses were weighed, examined externally and then either dissected immediately for visceral examination followed by fixation and skeletal staining or fixed for serial sectioning.

No mortality was noted. Compound-related increased salivation was observed in 10 dams at 500 mg/kg bw per day, within 1.5 hours of dosing, beginning from GD 7 through GD 15. Similar findings were also noted in one dam at 100 mg/kg bw per day on GD 7. Compound-related decreases in body weight and body weight gain were observed at 500 mg/kg bw per day. At this dose level, significant decreases in body weight gain were observed for GDs 8–12 (19%), GDs 6–15 (19%) and GDs 0–20 (6%). During GDs 16–20, a slight compensatory increase was noted for this dose group. Maternal body weight was slightly decreased on GDs 8 through 15 (1–3%) during the treatment period and remained lower (10%) from GD 16 through GD 20, compared with controls, during the post-treatment period. A compound-related significant decrease (10%, $P < 0.01$) in feed consumption was observed at 500 mg/kg bw per day only during GDs 6–8. A significant increase (9–11%, $P < 0.01$) in feed consumption was noted at 100 and 500 mg/kg bw per day during post-treatment, on GDs

16–17. No compound-related effect on water consumption was observed. No compound-related gross pathological findings were noted. There was no effect on the number of corpora lutea or on the incidence of preimplantation or postimplantation losses or resorptions.

There was no effect of treatment on the number of viable fetuses or sex ratio. At 100 and 500 mg/kg bw per day, fetal weights were decreased significantly (8% and 14%, respectively, $P \leq 0.001$) in a dose-related manner when compared with controls. External examination revealed that there was a dose-related increase in the incidence of small fetus and a decrease in the incidence of large fetus at 100 and 500 mg/kg bw per day. Visceral anomalies were observed at 500 mg/kg bw per day and included subcutaneous oedema and a slight space between body wall and organs. The skeletal anomalies seen at 100 mg/kg bw per day included decreases in sternebral (nos 3, 4 and 5), metacarpal and metatarsal ossification. Additional anomalies noted at 500 mg/kg bw per day consisted of a lack of ossification of the 1st thoracic vertebral centrum and one or more pubic bones, incomplete ossification of caudal vertebrae, an increase in the number of 14th ribs or enlarged 14th ribs and presacral vertebrae, and asymmetrical pelvis.

In conclusion, the NOAEL for maternal toxicity was 100 mg/kg bw per day, based on the decreased body weight gain observed at 500 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 10 mg/kg bw per day, based on decreased fetal weight and delayed ossification observed at 100 mg/kg bw per day (Reader, 1995b).

Rabbits

In a developmental toxicity study, isoxaflutole (purity not specified) was administered to 25 female New Zealand White rabbits by gavage in a dosing volume of 5 mL/kg bw at a dose level of 0, 5, 20 or 100 mg/kg bw per day from GDs 6 to 19, inclusive. The test material was suspended in 1% aqueous carboxymethyl cellulose. Body weight and feed consumption were measured throughout the study. At necropsy on GD 29, the reproductive tracts were dissected and examined. The fetuses were weighed and examined externally and viscerally. The heads of one third of the fetuses were then fixed for serial sectioning, with the torsos and all remaining fetuses fixed for skeletal examination.

No compound-related mortalities were noted. The incidental findings included death of one control and one mid-dose (20 mg/kg bw per day) female on GD 10 and GD 19, respectively. Necropsy of these animals revealed that these deaths were incidental. Compound-related clinical signs observed at 100 mg/kg bw per day included increased number of does with little diet consumed and few faeces in the cages beginning from GD 6 through GD 19. Body weights in the groups receiving 5 or 20 mg/kg bw per day were unaffected by treatment. From GD 10 (after 4 days of treatment), significant body weight losses were recorded in the 100 mg/kg bw per day group. Body weight gain was lower than that of the controls from the start of treatment. A large number of animals in this group continued to lose weight until GD 16, but after cessation of treatment, the rate of body weight gain was higher than that of the controls. The initial deficit was, however, not fully regained by GD 29. A compound-related significant decrease (15–24%, $P < 0.05$) in feed consumption was observed at 100 mg/kg bw per day, during GDs 6–19. A compensatory increase (17–32%, $P < 0.01$) in feed consumption was noted during post-treatment, on GDs 20–28. No adverse effects on feed consumption were noted at lower dose levels. No compound-related gross pathological findings were noted.

The numbers of corpora lutea and implantations were unaffected by treatment (Table 21). At 100 mg/kg bw per day, the mean number of late resorptions was slightly higher than that of concurrent and historical controls (1.5 versus 0.7 resorptions/litter in concurrent control; historical control range: 0.4–1.3). This resulted in an increased postimplantation loss (21% versus 11% in control) and reduced the mean number of viable pups (8.3/litter versus 9.8/litter). In addition, mean fetal weight was slightly but non-significantly lower (3%) than in controls.

Table 21. Caesarean section observations in a rabbit developmental toxicity study with isoxaflutole

Parameter	0 mg/kg bw per day	5 mg/kg bw per day	20 mg/kg bw per day	100 mg/kg bw per day
No. of animals assigned	25	25	25	25
No. of animals mated	25	25	25	25
No. of animals pregnant	20	21	23	20
Pregnancy rate (%)	100	100	100	100
No. died/no. non-pregnant	0	0	0	0
No. died/no. pregnant	1	0	1	0
No. non-pregnant	5	4	1	5
No. aborted	0	0	0	1
No. of premature deliveries	0	0	0	0
Total no. of corpora lutea	247 (19)	280 (21)	291 (23)	236 (19)[a]
No. of corpora lutea/dam	13.0 ± 3.1[b]	13.3 ± 2.2	12.7 ± 2.3	12.4 ± 2.3
Total no. of implantations	210	247	248	201
No. of implantations/dam	11.1 ± 3.2	11.8 ± 2.8	10.8 ± 3.8	10.6 ± 3.1
Total no. of live fetuses	186	210	209	159
No. of live fetuses/dam	9.8 ± 2.7	10.0 ± 2.4	9.0 ± 3.3	8.3 ± 2.7
Total no. of resorptions	24	37	39	42
- No. of early resorptions	10	13	21	13
- No. of late resorptions	14	24	18	29
No. of resorptions/dam	1.3 ± 1.1	1.8 ± 1.3	1.7 ± 1.3	2.2 ± 1.5
Total no. of dead fetuses	0	0	0	0
No. of dead fetuses/dam	0	0	0	0
Fetal weight/litter (g)	38.9 ± 1.6	40.0 ± 1.8	40.8 ± 1.6	37.9 ± 1.5
Preimplantation loss (%)	16.3	12.7	16.2	15.9
Postimplantation loss (%)	11.4	15.0	15.7	20.9
Sex ratio (% male)	54	51	46	48

[a] One dam aborted on GD 26; data excluded from analysis.
[b] Mean ± standard deviation.
Source: Reader (1995a)

The incidental finding noted was occurrence of small fetuses (Table 22) in all dose groups. Single fetuses were noted at necropsy to have anomalies in three litters at 20 mg/kg bw per day and one litter at 100 mg/kg bw per day. As there was no relationship to treatment and only one fetus per litter was affected, these observations were considered to be not related to treatment. There were no visceral findings observed during fetal examination that were considered to be related to treatment. Serial sections of fetal heads revealed an increased incidence of fetuses (litters) with incisors not erupted at 100 mg/kg bw per day, suggesting a delay in development. At examination of the skeletons, there was a dose-related increase in the incidence of 27 presacral vertebrae from 5 mg/kg bw per day. At the low dose, the incidence of this observation was only slightly outside the historical control data, and there was no increase in the number of ribs observed. At 20 mg/kg bw per day, there was an increased incidence of 13 pairs of ribs as well as an increased incidence of 27 presacral vertebrae. Additionally, the ossification of the heads of long limb bones, metacarpals, phalanges and centrales was reduced at 20 mg/kg bw per day. At 100 mg/kg bw per day, the incidence of 13 pairs of ribs, rudimentary first ribs and 27 presacral vertebrae was increased, whereas the incidence of the ossification of the heads of long limb bones, metacarpals, phalanges and pubic bones was decreased.

Table 22. Fetal weight and skeletal findings in the rabbit developmental toxicity study with isoxaflutole

Observation	Fetuses or litters	0 mg/kg bw per day	5 mg/kg bw per day	20 mg/kg bw per day	100 mg/kg bw per day	Control data	
						721 fetuses	11 studies
No. of fetuses	–	186	210	209	159	–	–
No. of litters	–	19	21	23	19	–	–
Small fetus (< 2.0 g)	Fetuses	20.4	13.3	18.2	19.5	11.15	0.0–21.4
	Litters	10	13	11	8	–	–
Ribs 12/12	Fetuses	61.8	49.5	23.4	15.7	53.63	35.3–65.1
	Litters	17	19	15	8	–	–
Ribs 12/13	Fetuses	9.7	12.9	14.8	9.4	12.33	6.8–16.9
	Litters	14	15	16	11	–	–
Ribs 13/13	Fetuses	28.5	37.6	61.2	74.8	33.98	22.7–57.3
	Litters	14	17	22	19	–	–
Rudimentary 1st rib or ribs	Fetuses	0.0	0.0	0.0	1.9	0.10	0.0–1.0
	Litters	0	0	0	2	–	–
26 presacral vertebrae	Fetuses	83.3	65.2	51.7	13.8	76.68	65.8–88.4
	Litters	19	20	22	11	–	–
27 presacral vertebrae	Fetuses	16.7	34.8	47.8	86.2	23.05	11.6–34.2
	Litters	12	19	20	18	–	–
Incomplete ossification, heads, long limb bones	Fetuses	46.2	40.0	45.0	44.0	42.52	31.6–50.0
	Litters	18	20	21	17	–	–
Heads of long limb bones unossified	Fetuses	7.5	11.4	22.0	32.7	8.94	3.9–19.3
	Litters	7	9	12	13	–	–
Incomplete ossification, one or both centrales	Fetuses	0.5	1.4	5.7	3.8	2.10	0.0–4.4
	Litters	1	2	5	5	–	–
Incomplete ossification, metacarpals or phalanges	Fetuses	6.5	6.7	15.3	13.2	5.01	0.8–11.5
	Litters	8	7	11	11	–	–
Metacarpals and/or phalanges unossified	Fetuses	4.3	7.6	16.7	10.7	6.41	2.4–12.0
	Litters	7	6	9	10	–	–
Incomplete ossification of pubic bones	Fetuses	1.6	1.4	2.9	7.5	2.32	0.0–4.7
	Litters	2	3	3	7	–	–
Incisors not erupted	Fetuses	13.1	22.1	18.1	37.0	16.78	0.0–30.0
	Litters	6	9	9	12	–	–

Source: Reader (1995a)

In conclusion, the NOAEL for maternal toxicity was 20 mg/kg bw per day, based on decreased maternal body weight, decreased feed consumption and increased numbers of resorptions seen at 100 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 20 mg/kg bw per day,

based on slightly delayed development of the fetuses, decreased fetal weights and delayed ossification at 100 mg/kg bw per day (Reader, 1995a).

2.6 Special studies

(a) Acute neurotoxicity

Rats

In an acute neurotoxicity study, CD rats (10 of each sex per group) received a single oral gavage administration of isoxaflutole (purity 99.2%) in 0.5% aqueous methyl cellulose at a dose of 0 (vehicle only), 125, 500 or 2000 mg/kg bw. Animals were fasted for about 18 hours prior to administration of the test article. Neurobehavioural tests were conducted on 10 animals of each sex per group and consisted of functional observational battery and evaluation of motor activity. Based on pharmacokinetic studies, these tests were performed on day 1 (within 1–2 and 2–4 hours, respectively, after dosing) and on days 8 and 15 post-dosing. The animals were observed for mortality and clinical signs of toxicity for 14 days post-dosing.

No treatment-related clinical signs were observed in animals receiving doses up to 2000 mg/kg bw. No compound-related effects on body weight, body weight gain or feed consumption were noted. There were no significant differences in group motor activity values among the control and treated animals on days 1, 8 and 15. The decrease in group mean motor activity values for high-dose males on day 8 compared with controls during the 1-hour measurement period was not statistically significant and was considered to be incidental. A significant decrease in mean forelimb grip strength in mid-dose males on day 8 was considered incidental. Additional incidental findings noted during the functional observational battery tests consisted of absence of response to a tail pinch in a few males and females from various dose groups on days 1, 8 and 15, absence of pupillary response in two mid-dose males on day 15 and piloerection in one or two males in the controls and each dose group on day 15. Landing foot splay was statistically significantly decreased relative to controls in males at 2000 mg/kg bw on study days 1 and 15 and in males from 500 mg/kg bw on study day 15 (Table 23). However, this is not considered to be related to treatment, as control values increased on study days 1 and 15, the measurements in treated animals were similar to those in controls during the pretest period and there were no other findings related to hindlimb function in this study.

Table 23. Effects of isoxaflutole on functional observational battery in male rats

Dose level (mg/kg bw)	Trial	Mean forelimb grip strength (g)				Mean hindlimb grip strength (g)				Mean landing foot splay (cm)			
		Pre-test	1 day	8 days	15 days	Pre-test	1 day	8 days	15 days	Pre-test	1 day	8 days	15 days
Control	1	319	409	886	564	191	173	499	534	6.0	7.4	6.2	7.8
	2	702	409	858	501	211	170	588	460	6.5	6.9	6.6	7.8
125	1	375	415	701	516	167	157	495	518	5.8	6.3	5.8	6.4
	2	411	362	769	508	176	154	538	446	5.9	6.5	5.9	6.0
500	1	386	451	725	557	213	123	542	530	4.8	6.2	4.9	5.5**
	2	385	390	566**	469	229	142	502	459	5.5	6.3	5.2	6.0*
2 000	1	407	481	778	542	247	121	538	557	5.5	5.1*	5.2	4.7**
	2	418	422	718	442	201	150	542	433	5.8	5.3	5.5	5.3**

*: $P < 0.05$; **: $P < 0.01$
Source: Mandella (1995a)

There were no compound-related pathological findings in the central or peripheral nervous system in treated animals examined histopathologically. The brain and pituitary weights between control and treated animals were comparable.

The NOAEL for systemic toxicity and neurotoxicity was 2000 mg/kg bw. A LOAEL was not observed in this study (Mandella, 1995a).

(b) Subchronic neurotoxicity

Rats

In a subchronic dietary neurotoxicity study, isoxaflutole (purity 99.2%) was administered to CD rats (10 of each sex per group) at a dietary level of 0, 25, 250 or 750 mg/kg bw per day for 90 days. Stability, homogeneity and concentrations in the diet were confirmed analytically. All animals were euthanized following 13 weeks of treatment. All animals were observed twice daily for mortality and moribundity. Detailed physical examinations were performed weekly. Individual body weights and feed consumption were recorded weekly. Functional observational battery and locomotor activity data were recorded for all animals prior to the initiation of treatment and during study weeks 5, 9 and 13. Ophthalmic examinations were not performed. At terminal sacrifice, all animals were subjected to complete gross pathological examination; abnormal tissues were preserved in 10% neutral buffered formalin. Five animals of each sex from the high-dose group were selected for neuropathological evaluation and were anaesthetized and killed by perfusion fixation. Animals from the control and high-dose groups were subjected to neuropathological examinations of the central and peripheral nervous systems. All abnormal tissues were preserved in 4% paraformaldehyde and glutaraldehyde in phosphate-buffered saline. Brain and pituitary weights from each animal were determined.

The test article was homogeneously distributed in the diets, and diets were stable for 14 days at the ambient temperature. The concentration analyses of all test samples indicated values within ±5% of the target concentrations.

No treatment-related deaths occurred. At 250 mg/kg bw per day, one male was sacrificed moribund on day 46 of the study. This isolated finding was considered to be incidental. There were no treatment-related clinical signs of toxicity observed in treated animals. The mean body weights for high-dose males were consistently lower (6–13%) than those of controls throughout the study period, resulting in an overall decrease in body weight gain of 19% over 13 weeks (Table 24). For high-dose females, the mean body weight and body weight gains were comparable over 13 weeks, with the exception of a slight decrease in week 2 and weeks 11–13, resulting in an overall decrease in body weight gain of 9% over 13 weeks. Increases in body weight and body weight gain in low- and mid-dose females were not considered to be toxicologically significant. No effect on feed consumption was noted.

Table 24. Body weight in male and female rats in the 90-day neurotoxicity study with isoxaflutole

Week	Males				Females			
	0 mg/kg bw per day	25 mg/kg bw per day	250 mg/kg bw per day	750 mg/kg bw per day	0 mg/kg bw per day	25 mg/kg bw per day	250 mg/kg bw per day	750 mg/kg bw per day
0	194.3	195.5	191.0	191.7	146.4	152.7	154.7	153.5
5	412.2	407.4	389.7	367.0**	219.2	241.7*	237.4	222.2
9	504.1	498.2	477.2	445.2**	246.4	274.5*	265.8	245.9
13	558.7	541.6	523.2	486.8**	260.7	299.7**	280.7	258.0
Body weight gain (g), weeks 0–13	364.4	346.1	332.2	295.1	114.3	147.0	126.0	104.5
% change from controls	–	−5	−9	−19	–	+29	+11	−9

*: $P < 0.05$; **: $P < 0.01$
Source: Mandella (1995b)

Motor activity was unaffected by the treatment. Functional observational battery findings observed during the 13-week treatment period are summarized in Table 25. At 25 mg/kg bw per day and above, decreases in both forelimb and hindlimb grip strengths were noted in treated males during week 13. A non-significant decrease in forelimb grip strength was also noted during week 13. The decrease in landing foot splay in females at 25 mg/kg bw per day in week 9 was considered incidental. Additional findings consisted of moderate difficulty in handling, piloerection and absence of a tail pinch response seen prior to treatment and/or with similar frequency in the control group. There were no macroscopic or microscopic findings that were considered to be related to treatment in either males or females at any dietary concentration of isoxaflutole. No treatment-related lesions of the nervous system were noted in high-dose animals.

Table 25. Effect of isoxaflutole on functional observational battery in male rats

Dose level (mg/kg bw)	Trial no.	Mean forelimb grip strength (g)				Mean hindlimb grip strength (g)				Mean landing foot splay (cm)			
		Pre-test	Week 5	Week 9	Week 13	Pre-test	Week 5	Week 9	Week 13	Pre-test	Week 5	Week 9	Week 13
Control	1	396	619	880	993	255	315	727	639	4.6	7.5	6.7	6.3
	2	369	619	787	901	230	270	753	734	4.7	7.2	6.4	6.4
25	1	359	712	821	913	196	325	766	498	5.2	6.9	5.9	5.9
	2	449	625	581	731	232	254	674	504**	5.5	7.1	6.4	5.7
250	1	420	689	704	779	269	283	776	586	5.3	7.4	6.3	5.4
	2	426	643	719	740	244	272	701	562*	5.5	7.2	6.7	5.7
750	1	329	639	818	670**	267	280	797	476*	5.1	6.4	6.3	6.4
	2	414	614	669	765	235	258	695	583	5.1	6.8	6.6	6.4

*: $P < 0.05$; **: $P < 0.01$
Source: Mandella (1995b)

A NOAEL for systemic toxicity was not identified, as only limited parameters were evaluated in this study. The NOAEL for neurotoxicity was 750 mg/kg bw per day, the highest dose tested (Mandella, 1995b).

(c) Developmental neurotoxicity

Rats

In a developmental neurotoxicity study, isoxaflutole (purity 99.15%) in 1% methyl cellulose was administered by gavage in a dosing volume of 5 mL/kg bw to pregnant Crl:CD®(SD)IGS BR (25 per dose) from GD 6 to LD 10 at a dose of 0, 5, 25 or 250 mg/kg bw per day. Parental dams (P) were allowed to deliver naturally. All P females were killed on LD 21. On PND 4, eight pups (four of each sex) per litter were randomly selected in order to reduce variability among the litters; the remaining offspring were weighed and euthanized. The acquisition of balanopreputial separation and vaginal patency was assessed for each pup, and body weight was measured on the day of acquisition. Pups were assessed for acoustic startle response on PND 20 or 60, for locomotor activity on PND 13, 17, 21 or 61 and for swimming ability and learning and memory beginning on PND 22 or 62. Subsequently, 10 pups of each sex per group were selected for neurobehavioural testing and neuropathological examination. Pups not selected for behavioural and neuropathological evaluations were terminated on PND 28 or 29. Morphometric analyses, as required by Organisation for Economic Co-operation and Development and United States Environmental Protection Agency guidelines, were not performed on offspring (PND 11 or 72), as the evaluation of brains by light microscopy did not reveal any structural abnormalities, nor were there any clear functional differences between the control and treated groups.

No unscheduled parental deaths occurred during the study. Clinical or functional observational battery signs, gross pathology, pregnancy rate, number of implantations per dam,

gestation length and sex ratio were unaffected by treatment. No treatment-related differences in live litter size, postnatal survival, sex ratios, clinical signs or sexual maturation were observed in any treated group. Pup swimming ability, learning, memory, motor activity, auditory startle response, brain weights and dimensions, and neuropathology were unaffected by the test substance.

Body weights and body weight gains for the P females are presented in Table 26. At 5 and 25 mg/kg bw per day, there were no effects on maternal body weight, body weight gain or feed consumption. Body weights were slightly decreased ($P \leq 0.05$) in the 250 mg/kg bw per day P females during GDs 9–15 (decrease 4–6%) and LDs 1–10 (decrease 7–10%). Body weight gains were decreased ($P \leq 0.0l$) in the 250 mg/kg bw per day P females during GDs 6–9 (decrease 91%) and 9–12 (decrease 39%). Body weight gains were also decreased for the entire gestation treatment interval (GDs 6–20; decrease 14%, $P \leq 0.01$) and overall gestation (GDs 0–20; decrease 11%, $P \leq 0.05$). During the lactation treatment interval (LDs 1–10), body weight gains were comparable with those of controls in all treated groups. Feed consumption was unaffected by the treatment at 5 and 25 mg/kg bw per day. At 250 mg/kg bw per day, feed consumption was statistically significantly reduced compared with that of controls throughout gestation and the first 4 days of lactation.

Table 26. Selected mean body weights for P females (n = 23–25) administered isoxaflutole from GD 6 to LD 10

Treatment interval (days)	Mean body weights ± SD (g)			
	0 mg/kg bw per day	5 mg/kg bw per day	25 mg/kg bw per day	250 mg/kg bw per day
Gestation				
0	261 ± 14.7	258 ± 11.9	256 ± 131	258 ± 14.1
6	294 ± 19.8	292 ± 11.9	286 ± 15.4	291 ± 15.1
9	305 ± 18.7	304 ± 13.3	297 ± 17.9	292* ± 12.2 (↓4)
15	341 ± 28.4	334 ± 21.7	332 ± 21.3	320** ± 15.4 (↓6)
20	411 ± 35.3	408 ± 20.1	403 ± 26.6	392 ± 19.3
Treatment (6–20)	117 ± 25.0	116 ± 13.9	116 ± 17.4	101** ± 16.7 (↓14)
Overall (0–20)	150 ± 27.6	149 ± 18.8	146 ± 19.4	133* ± 19.1 (↓11)
Lactation				
1	309 ± 30.6	305 ± 22.7	297 ± 19.4	279** ± 18.5 (↓0)
4	326 ± 32.3	321 ± 20.3	315 ± 19.5	300** ± 17.1 (↓8)
10	351 ± 26.7	344 ± 20.0	337 ± 20.4	325** ± 23.6 (↓7)
21	345 ± 23.0	341 ± 19.3	336 ± 21.2	333 ± 17.6
Treatment (1–10)	43 ± 12.0	40 ± 17.6	40 ± 11.9	46 ± 18.2
Post-treatment (10–21)	−6 ± 15.2	−3 ± 14.7	−1 ± 14.0	8** ± 16.9 (↑133)
Overall (1–21)	37 ± 21.2	36 ± 17.6	39 ± 15.6	54** ± 20.8 (↑46)

*: $P \leq 0.05$; **: $P \leq 0.01$
Source: Nemec (1999)

Decreased ($P \leq 0.05$) survival was observed in the 250 mg/kg bw per day pups during PNDs 0–1 only (93.2% treated versus 98.3% controls). No treatment-related clinical signs were observed. Body weights of offspring in the 5 and 25 mg/kg bw per day groups were comparable with those of the controls. In the 250 mg/kg bw per day pups, body weights were decreased ($P \leq 0.05$) in the males from PND 1 to PND 28 (decreased 7–12%) and from PND 49 to PND 63 (decreased 4–5%). In the females, body weights were decreased ($P \leq 0.05$) sporadically between PND 1 and PND 35 (decreased 5–12%). Decreased ($P \leq 0.05$) body weight gains were observed sporadically in the males between PND 1 and PND 28 (decreased 6–21%) and only during PNDs 4–7 in the females (decreased

15%). Absolute brain weights were also reduced (11–12%) in males and females on PND 11 at 250 mg/kg bw per day, an effect likely related to the decreased body weights and body weight gains observed in these animals. No morphometric measurements of the brain were performed in this study.

In conclusion, the maternal NOAEL was 25 mg/kg bw per day, based on decreased maternal body weight, body weight gain and feed consumption at 250 mg/kg bw per day. The offspring toxicity NOAEL was 25 mg/kg bw per day, based on decreased pup survival, body weight and body weight gain at 250 mg/kg bw per day. In the absence of any neurotoxic findings, the NOAEL for neurotoxicity in the rat was 250 mg/kg bw per day, the highest dose tested (Nemec, 1999).

(d) Microsomal enzymes

A special study was conducted to establish the dose–response relationship and to investigate the role of the mixed-function oxidase system with respect to liver enlargement in isoxaflutole-treated mice. Groups of 25 male CD-1 mice received isoxaflutole (purity 99.6%) in their diet at a dose level of 0, 175, 700, 2800 or 7000 ppm (equivalent to 0, 26.3, 105, 420 and 1050 mg/kg bw per day) for 14 days.

There were no mortalities, no treatment-related clinical observations and no effects on body weight or feed consumption. Isoxaflutole administration caused an increase (≥ 11%) in absolute and relative liver weights in mice at and above 700 ppm. This increase was attributed to the induction of mixed-function oxidase enzymes in the liver. The total cytochrome P450 levels were increased in a dose-dependent manner; this was statistically significant at and above 700 ppm. Analysis of P450 isoenzymes indicated that the elevated P450 levels were mainly due to a significant increase in absolute and relative pentoxyresorufin *O*-depentylase (PROD) activity (P450 2 family, B1 isoenzymes) at 175 ppm and above and at 700 ppm and above, respectively, and to a significant increase in absolute and relative benzoxyresorufin *O*-debenzylase (BROD) activity (P450 2B family, B1 and B2 isoforms) at 175 ppm and above. Absolute ethoxyresorufin *O*-deethylase (EROD) activity (P450 1 family, A1 isoenzymes) was significantly increased at 2800 and 7000 ppm. Absolute methoxyresorufin *O*-demethylase (MROD) activity (P450 1 family, A2 isoenzymes) was significantly increased at 700 ppm and above, but there was no dose–response relationship. Absolute lauric acid 11- and 12-hydroxylase activities (peroxisome proliferation) were significantly increased at 7000 ppm. The results suggest that isoxaflutole caused a dose-related increase in liver weight in male mice, owing to marked elevation in cytochrome P450 enzymes of the P450 2B family, similar to phenobarbital. It does not appear to induce other P450 isoenzymes significantly or cause peroxisome proliferation. There was no NOAEL. The LOAEL was 175 ppm (equivalent to 26.3 mg/kg bw per day) on the basis of elevated absolute and relative BROD activity and absolute PROD activity (Price, 1994a).

In another study conducted to establish the dose–response relationship and to investigate the role of the mixed-function oxidase system with respect to liver enlargement in isoxaflutole-treated rats, groups of five male Sprague-Dawley CD1 rats received isoxaflutole (purity 99.6%) in the diet at a dose level of 0, 10, 100 or 400 mg/kg bw per day for 14 days.

There were no mortalities, no treatment-related clinical observations and no effects on body weight or feed consumption. Isoxaflutole administration caused an increase (≥ 33%) in absolute and relative liver weights in rats at 100 and 400 mg/kg bw per day. This increase was attributed to induction of mixed-function oxidase enzymes in the microsomal fraction of the homogenized liver. The total cytochrome P450 levels were increased in a dose-dependent manner; this was statistically significant at all dose levels. The specific forms of isoenzymes responsible for this increase were PROD and BROD enzymes, the induction of which may be attributed to the P450 2B family (i.e. phenobarbital type). Therefore, isoxaflutole appears to function as a phenobarbital-type inducer of the P450 2B family. There was no increase in other P450 isoenzyme levels, including MROD and EROD, nor did the test compound induce lauric acid hydroxylases, which are associated with peroxisome proliferation. Analysis of P450 isoenzymes revealed a statistically significant increase in absolute and relative (to total liver P450) PROD (P450 2 family, B1 isoenzymes) and BROD (P450 2B family, B1

and B2 isoforms) activities at all dose levels. Absolute EROD activity (P450 1 family, A1 isoenzymes) was significantly, but not dose-relatedly, increased in all dose groups and was decreased in relation to total liver P450 at 100 and 400 mg/kg bw per day (statistically significant at 400 mg/kg bw per day). MROD activity (P450 1 family, A2 isoenzymes) was not markedly altered in comparison with other P450 isoenzymes. Absolute lauric acid 11- and 12-hydroxylase activities (peroxisome proliferation) were significantly increased at 400 mg/kg bw per day.

The results suggest that isoxaflutole caused a dose-related increase in liver weight in male rats that may be due to marked elevation in cytochrome P450 enzymes of the P450 2B family (PROD and BROD), similar to phenobarbital. It does not appear to induce other P450 isoenzymes significantly or cause peroxisome proliferation. There was no NOAEL. The LOAEL was 10 mg/kg bw per day on the basis of elevated absolute and relative PROD and BROD activities (Price, 1994b).

(e) Tyrosine levels

The plasma samples taken from the 2-week dietary study in mice (Price, 1994a) were analysed for tyrosine concentrations. Plasma tyrosine levels increased at all doses (175–7000 ppm) studied relative to the untreated control mouse plasma. No dose–response relationship was observed. The results of this study suggest that the supplementation of the mouse diet with isoxaflutole for 14 days resulted in an elevation of tyrosine concentrations in the plasma. A no-effect level was not observed and is therefore assumed to be less than 175 ppm (Little, 1993b).

The plasma samples taken from the 2-week dietary study in Sprague-Dawley CD1 rats (Price, 1994b) were analysed for tyrosine concentrations. Plasma tyrosine levels increased at all doses (10–400 mg/kg bw per day) studied compared with that of the untreated control rat plasma. Plasma tyrosine levels increased 3-fold compared with control levels at the lowest dose applied (10 mg/kg bw per day), increasing to 3.6- and 3.5-fold at 100 and 400 mg/kg bw per day, respectively. No dose–response relationship was observed. The results of this study suggest that the supplementation of the rat diet with isoxaflutole for 14 days resulted in an elevation of tyrosine concentrations in the plasma. A no-effect level was not observed and is therefore assumed to be less than 10 mg/kg bw per day (Little, 1993c).

(f) Thyroid mechanism

The mechanism of action of isoxaflutole on the thyroid was investigated in male Sprague-Dawley rats. In this study, isoxaflutole (purity 99.6%) was administered in the diet to male Sprague-Dawley rats (12 per dose) at a dose level of 0 or 500 mg/kg bw per day for 14 days. A third group (positive control) of rats received sodium phenobarbital by gavage at 80 mg/kg bw per day and an untreated diet. Following the treatment period, the liver enzyme activities, including cytochrome P450 and *p*-nitrophenol uridine diphosphate-glucuronosyltransferase (UDPGT), as well as thyroxine (T_4) and triiodothyronine (T_3) levels were monitored, and thyroid weights were determined. The rate of T_4 disappearance from blood was measured in rats (six animals from each group) after intravenous administration of sodium [^{125}I]thyroxine. The effects on blood concentration half-life, thyroid gland iodine uptake and thyroid weights were measured.

There were no mortalities, no treatment-related clinical observations and no treatment-related effects on body weight, body weight gain, feed consumption or feed efficiency. Isoxaflutole administration caused more than a 2-fold increase in Phase I (cytochrome P450–dependent mixed-function oxidase system, as indicated by increased PROD activity) and Phase II (as indicated by increased UDPGT activity) enzymes, which resulted in increased clearance of ^{125}I-labelled T_4 from the blood, as indicated by a shorter half-life and decreases in plasma T_4 levels. In addition, there were increases in liver and thyroid weights. The plasma T_3 level was unaffected. The significant reduction in the level of circulating T_4 was possibly the result of enhanced glucuronidation by hepatic UDPGT and a rapid systemic clearance of total ^{125}I-labelled T_4 in the isoxaflutole-treated group. Following intravenous administration of ^{125}I-labelled T_4, the thyroid iodine uptake was slightly higher and thyroid weights were significantly higher in isoxaflutole-treated rats compared with controls. The results of the study (Table 27) appear to support the hypothesis that the increased incidence of thyroid

tumours in male rats at 500 mg/kg bw per day in the carcinogenicity study may be due to an imbalance of thyroid hormones created by an induction of UDPGT followed by decreased plasma T_4 levels, increased clearance of T_4 and a decreased half-life for T_4.

Table 27. Comparison of isoxaflutole and phenobarbital in rats

Parameters measured	Dose groups[a]		
	Control	Isoxaflutole	Phenobarbital
Absolute liver weight (g) (% of control)	13.9	21.1** (152%)	19.8** (143%)
Relative liver weight (g) (% of control)	5.07	7.5 ** (149%)	7.3** (145%)
Microsomal protein (mg/g liver) (% of control)	18.1	31.6** (175%)	24.6** (136%)
P450 (nmol/g liver)	16.1	57.9**	41.3**
Absolute thyroid weight (mg) (% of control)	18.7	20.1 (107%)	23.6* (126%)
UDPGT (mmol/g liver per hour)	57	216*	169**
PROD (nmol/g liver per hour)	5.1	137**	104**
T_4 (ng/dL)	5.7	3.2**	4.9**
T_3 (µg/dL)	74	68	69
$[^{125}I]T_4$ K_{el} (/h)	0.040 7	0.052 0***	0.042 8
$[^{125}I]T_4$ $t_{½}$ (h)	17.0	13.3	16.2
$[^{125}I]T_4$ clearance (mL/min)	0.038	0.065***	0.049*

K_{el}: mean terminal rate constant; PROD: pentoxyresorufin *O*-depentylase; $t_{½}$, half-life; T_3: triiodothyronine; T_4: thyroxine; UDPGT: uridine diphosphate-glucuronosyltransferase; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$
[a] Doses: isoxaflutole = 500 mg/kg bw per day; phenobarbital = 80 mg/kg bw per day.
Source: Chambers (1995)

Isoxaflutole appears to act like a phenobarbital-type inducer of hepatic Phase I and Phase II drug-metabolizing enzymes. The development of thyroid tumours in male rats treated with isoxaflutole at 500 mg/kg bw per day may be secondary to the treatment-related effects on the liver, which, in turn, produced alterations in thyroid–pituitary hormonal feedback mechanisms and a concomitant hormonal imbalance (Chambers, 1995).

To examine the potential for isoxaflutole to cause hepatic cell proliferation in the rat and to determine the reversibility of any effects observed, groups of 10 female rats were administered isoxaflutole (purity 99.43%) at varying concentrations to provide constant doses of 0, 2, 20, 50, 200 and 500 mg/kg bw per day for either 2 or 13 weeks. Reversibility groups of 10 animals were fed diets that provided 0 or 500 mg/kg bw per day for either 2 or 13 weeks, then transferred to control diets for a further 2 weeks. For the week prior to termination, animals were administered bromodeoxyuridine in the drinking-water to assess cell proliferation.

There were no deaths or clinical signs of toxicity during the study. Body weights and/or body weight gains at the terminal sacrifice were statistically significantly reduced for animals in the 500 mg/kg bw per day dose group for 2 weeks as well as for those given 200 or 500 mg/kg bw per day for 13 weeks. Corresponding decreases in feed consumption were seen for animals in these groups. Liver weights (absolute and relative to body weight) were increased in rats exposed to 200 and 500 mg/kg bw per day for 2 weeks as well as those exposed for 13 weeks. The hepatocyte labelling index (LI; indicator of hepatic cell proliferation) was increased at 2 and 13 weeks for animals given 200 and 500 mg/kg bw per day.

In the recovery group of rats treated with 500 mg/kg bw per day for 2 weeks and then placed on control (isoxaflutole-free) diet for 2 weeks, body weight gain, liver weights (absolute and relative) and LI were similar to those of controls. These results indicate that isoxaflutole-induced responses are

rapidly reversible. Reversibility of hepatic changes was also seen in rats treated with 500 mg/kg bw per day for 13 weeks followed by 2 weeks on control diet.

In conclusion, isoxaflutole demonstrated dose- and time-dependent effects on hepatocyte proliferation similar to those produced by other non-genotoxic hepatocarcinogens, such as phenobarbital. Hepatocyte proliferative effects were observed only at the highest doses of isoxaflutole, were correlated with changes in liver weight and were shown to be reversible upon cessation of isoxaflutole exposure. These data support the hypothesis that cell proliferation is the non-genotoxic mode of action for isoxaflutole tumorigenicity and that this response is dose dependent and reversible (Moser, 2001).

In a comparative tyrosine tolerance study, isoxaflutole (purity 98.7%) or 2-(2-nitro-4-trifluoromethylbenzoyl)-cyclohexane-1,3-dione (NTBC; purity 99.8%), a therapeutic agent, was administered via gavage to male Sprague-Dawley rats (five per dose) at a dose level of 0 or 10 mg/kg bw per day for 1 week. The animals then received tyrosine at 500 mg/kg bw per day on the day of treatment and on days 2, 3 and 8 after the test substance administration. Urine collected at 4, 8 and 24 hours was analysed for tyrosine metabolites.

Administration of tyrosine to rats pretreated with isoxaflutole or NTBC increased the urinary excretion of tyrosine metabolites, *N*-acetyl tyrosine, 4-hydroxyphenyl acetate (HPAA) and 4-hydroxyphenyl lactate (HPLA). The effect of isoxaflutole was reversible within 48 hours after administration, whereas that of NTBC was not completely reversed by 1 week after administration. The results of this functional assay suggest that both isoxaflutole and NTBC affect the main catabolic pathway for tyrosine by inhibiting the liver enzyme HPPD (Esdaile, 1995).

In a comparative metabolism study, isoxaflutole (purity 98.3%) was administered to groups (five per species) of male Sprague-Dawley (CD) rats and CD-1 mice by gavage at a single dose (10 mg/kg bw) followed 1 hour later with a single oral dose of [^{14}C]tyrosine (500 mg/kg bw). The total radioactivity in the urine and expired carbon dioxide was estimated at intervals of 0–5, 5–12, 12–24 and 12–48 hours. Metabolite analysis was performed on the urine of the rat and the mouse to analyse the quantitative differences in their ability to utilize a bypass metabolic route for the blocked tyrosine pathway via HPLA and HPAA.

For both species, a major portion of the administered dose of [^{14}C]tyrosine was eliminated via urine and expired air. Urinary elimination (mice: 19.90%; rats: 8.42%) was predominant in the mouse, whereas a significant portion of radiolabel was predominantly excreted via the expired air as carbon dioxide in the rat (mice: 13.23%; rat: 20.41%) during the first 48 hours following administration of [^{14}C]tyrosine. High-performance liquid chromatographic analysis of [^{14}C]tyrosine metabolites found in the urine of both species revealed higher amounts of two major metabolies, HPLA and HPAA, in the mouse than in the rat. The enzymatic hydrolysis of conjugates indicated that some metabolites were excreted as glucuronides and/or sulfates in urine; these did not include HPLA and HPAA.

This study demonstrated species-related qualitative and quantitative differences in the excretion of tyrosine following single simultaneous administrations of isoxaflutole and [^{14}C]tyrosine to male mice and rats. The results suggest that the elimination of tyrosine as HPLA and HPAA is more efficient in the mouse than in the rat, with twice as much of the administered dose of [^{14}C]tyrosine observed in the mouse urine as in the rat urine (Filaquier, 1995).

It was hypothesized, following the development of several herbicides sharing an HPPD-inhibiting mode of action, that a number of effects (organ weight and histopathology in liver, kidney, pancreas, thyroid; histopathology in eye; fetal skeletal development) were not primary toxic effects of the compounds, but were secondary to the increased plasma tyrosine concentrations observed in the rat.

The objective of this study was to measure blood tyrosine levels in the rat following daily oral gavage administration of NTBC between days 1 and 18, together with administration of diet containing 2% weight per weight (w/w) L-tyrosine between days 15 and 19. NTBC (purity 99.7%), an HPPD inhibitor, was administered at a dose level of 5, 10, 20 or 40 µg/kg bw per day. Each group consisted of three female Sprague-Dawley rats. A control group received the vehicle alone (demineralized water). Body weights were recorded for all females on days 1, 5, 8, 12, 15 and 19. Feed consumption was measured for all females during the intervals days 1–5, 5–8, 8–12, 12–15 and 15–19. Clinical observations were recorded daily. Blood samples were collected on days 15 and 19 and subsequently analysed for tyrosine levels. At scheduled sacrifice, the pancreas and thyroid gland were preserved for possible histological examination.

There were no mortalities during the study. At NTBC doses from 10 µg/kg bw per day, one or more animals in each group were noted with white areas on the eye (bilateral) on study day 19. There were no clinical findings at an NTBC dose of 5 µg/kg bw per day. At an NTBC dose of 40 µg/kg bw per day, there was an overall mean body weight loss of 4 g between study days 1 and 19, compared with a mean body weight gain of 21 g in the controls over the corresponding period. At an NTBC dose of 40 µg/kg bw per day, mean feed consumption was reduced by 22% between study days 15 and 19, when compared with the controls. The effect was statistically significant ($P < 0.05$). On study day 15, prior to the addition of 2% tyrosine to the diet, plasma tyrosine concentrations at an NTBC dose of 5 µg/kg bw per day were similar to those measured in the control group, whereas plasma tyrosine was increased from an NTBC dose of 10 µg/kg bw per day (Table 28). On study day 19, after the animals were fed diets containing 2% tyrosine from study days 15 to 19 while also being treated daily with NTBC by oral gavage on study days 15 through 18, plasma tyrosine concentrations were increased in all groups including controls. The increase was dose related in the groups administered NTBC.

Table 28. Plasma tyrosine concentration after administration of NTBC and dietary tyrosine in rats

Study day	Plasma tyrosine concentration (mg/L)				
	Control	5 µg/kg bw per day NTBC	10 µg/kg bw per day NTBC	20 µg/kg bw per day NTBC	40 µg/kg bw per day NTBC
15	8.09	9.7	73.77	25.93	167.7
19	20.7	33.13	305.7	388.7	419.7

NTBC: 2-(2-nitro-4-trifluoromethylbenzoyl)-cyclohexane-1,3-dione
Source: Blanck (2005)

Minimal to slight mottled kidney (bilateral) was observed in 2/3 animals at an NTBC dose of 40 µg/kg bw per day and in 1/3 animals at NTBC doses of 20 and 5 µg/kg bw per day. No other macroscopic findings were observed.

In conclusion, oral gavage administration of NTBC together with administration of diet containing 2% w/w L-tyrosine provoked tyrosinaemia in a dose-related manner and was associated with an increase in the incidence of rats with white area on both eyes compared with the control group (Blanck, 2005).

In a study conducted to assess the potential effects of increased plasma tyrosine concentration on selected organs (eye, liver, pancreas and thyroid gland) of rats, four groups were used, each consisting of 10 Wistar rats of each sex and treated for 28 days as follows: *Group 1*: Animals received the vehicle alone (demineralized water) by gavage and untreated diet throughout the study. This group acted as a control. *Group 2*: Animals received the vehicle alone (demineralized water) by gavage and L-tyrosine at 2% w/w in the diet throughout the study. *Group 3*: Animals received NTBC, an inhibitor of HPPD, a key enzyme of tyrosine catabolism, by gavage at a volume of 10 mL/kg bw and untreated diet throughout the study. *Group 4*: Animals received NTBC by gavage at a volume of 10 mL/kg bw

and L-tyrosine at 2% w/w in the diet throughout the study. Plasma tyrosine concentrations were measured, organ weights were determined and selected organs were examined by histopathology.

One female in the NTBC only group died prematurely due to an accidental trauma. In the NTBC + 2% L-tyrosine group, treatment-related clinical signs consisted of white area on the eye in 9/10 males between study days 23 and 26 on one or more occasions. Body weight, body weight gain and feed consumption were not affected by the treatment. In the NTBC + tyrosine group, at the end of the study, 9/10 male rats were noted with corneal oedema, all 10 males were noted with snowflake corneal opacity and 3/10 females were noted to have snowflake corneal opacity. These are considered to be treatment related, as they are linked with increased plasma tyrosine concentration. There were no treatment-related ophthalmological findings in either the NTBC only or the L-tyrosine only group. When measured on study day 29 or 30, after overnight fasting, plasma tyrosine concentrations were similar to those of controls in the tyrosine group and markedly increased relative to controls in both the NTBC and the NTBC + tyrosine groups (Table 29).

Table 29. Plasma tyrosine concentration after administration of NTBC or tyrosine or co-administration of NTBC + tyrosine in rats

Sex	Plasma tyrosine concentration (nmol/mL)			
	Control	Tyrosine	NTBC	NTBC + tyrosine
Males	70.17	77.71	1 302.05	1 477.45
Females	66.73	63.06	1 531.85	1 474.14

NTBC: 2-(2-nitro-4-trifluoromethylbenzoyl)-cyclohexane-1,3-dione
Source: Blanck (2006a)

These results were not those that had been expected in advance of the study. Rather, it had been expected that the plasma tyrosine concentrations in the group administered NTBC at 10 μg/kg bw per day would be similar to those observed in the groups receiving either control diet or diet supplemented with 2% tyrosine. In the NTBC + tyrosine group, relative liver weight was significantly increased in both males and females relative to controls. At necropsy, in the NTBC + tyrosine group, 3/10 males were noted with eye opacity. There were no other treatment-related macroscopic findings. Findings that are considered to be treatment related were observed in the pancreas, thyroid and eye in the male and female rats in the NTBC + tyrosine group (Table 30). Findings in the pancreas were acinar atrophy/fibrosis and/or acinar degeneration/apoptosis of the exocrine pancreas and interstitial inflammation. In the thyroid gland, treatment-related findings were colloid alteration and potentially follicular cell hypertrophy based on increased severity in one animal in the NTBC + tyrosine group. In the eye, the treatment-related change was unilateral or bilateral keratitis.

In conclusion, administration of NTBC at 10 μg/kg bw per day plus 2% dietary tyrosine markedly increased plasma tyrosine concentrations and produced findings in the eye, pancreas and thyroid. These findings were not observed after administration of either tyrosine or NTBC alone, despite an increase in plasma tyrosine levels after gavage administration of NTBC alone at 10 μg/kg bw per day (Blanck, 2006a).

In a study conducted to assess the potential effects of increased plasma tyrosine concentration on selected organs (eye, kidney, liver, pancreas and thyroid gland) of rats, four groups were used, each consisting of five Wistar rats of each sex and treated for 28 days as follows: *Group 1*: Animals received the vehicle alone (demineralized water) by gavage and untreated diet throughout the study. This group acted as a control. *Group 2*: Animals received the vehicle alone (demineralized water) by gavage and L-tyrosine at 2% w/w in the diet throughout the study. *Group 3*: Animals received NTBC, an inhibitor of HPPD, a key enzyme of tyrosine catabolism, by gavage at a volume of 10 mL/kg bw and untreated diet throughout the study. *Group 4*: Animals received NTBC by gavage at a

Table 30. Histopathology of selected organs after administration of NTBC or tyrosine or co-administration of NTBC + tyrosine in rats

	Males				Females			
	Control	TYR	NTBC	NTBC + TYR	Control	TYR	NTBC	NTBC + TYR
No. examined	10	10	10	10	10	10	9	10
Pancreas								
Acinar atrophy/fibrosis: focal/multifocal	1	2	0	3	0	0	0	5
Acinar degeneration/ apoptosis: focal/ multifocal	0	1	0	2	0	3	0	5
Interstitial inflammation: focal/multifocal	0	1	0	3	0	0	0	5
Interstitial inflammation: diffuse	0	0	0	0	0	0	0	1
Thyroid								
Follicular cell hypertrophy: diffuse	2	3	1	3	0	1	0	0
Colloid alteration	0	1	1	6	0	0	0	0
Eye								
Keratitis: diffuse: unilateral and bilateral	1	0	0	9	0	0	0	1

NTBC: 2-(2-nitro-4-trifluoromethylbenzoyl)-cyclohexane-1,3-dione; TYR: L-tyrosine
Source: Blanck (2006a)

volume of 10 mL/kg bw and L-tyrosine at 2% w/w in the diet throughout the study. During the acclimatization phase, all animals were subjected to an ophthalmological examination; all animals were re-examined during week 4. Blood samples were collected for measurement of blood tyrosine levels at selected intervals. All animals were necropsied, and selected organs were weighed, collected, fixed and examined microscopically.

There were no mortalities during the study. Treatment-related clinical signs were limited to the NTBC + tyrosine group. These signs were white area on the eye in all males and one female between study days 24 and 30 and half-closed eyes in 4/5 males between study days 22 and 30 on one or more occasions. At NTBC at 10 µg/kg bw per day + 2% L-tyrosine, in females, mean body weight was reduced by between 2% and 5% during the second half of the study. Mean body weight gain per day was 2.1 g compared with 3.6 g in the controls between study days 8 and 15, resulting in an overall cumulative body weight gain reduction of 11% compared with the controls by study day 29. These effects were not statistically significant, but were considered to be biologically relevant. At an NTBC dose of 10 µg/kg bw per day and at 2% L-tyrosine, body weight evolution was unaffected by treatment in either sex. In females of the NTBC + tyrosine group, feed consumption was decreased throughout the study, with the greatest effect observed during week 2. There was no effect on feed consumption in any other group. Treatment-related ophthalmological findings were limited to the NTBC + tyrosine group and consisted of corneal oedema and snowflake corneal opacities in all five males, neovascularization of the cornea in one male, snowflake corneal opacity in one female and anterior synechia of the iris in another female.

In the NTBC + tyrosine group, a time-dependent increase in blood tyrosine was observed from the first measurement at study day 2, with a plateau reached at measurement on study day 21 (Table 31). In the NTBC group, blood tyrosine was increased relative to controls only on study day 29 or 30, prior to fasting. In the L-tyrosine group, blood tyrosine was increased relative to controls at all

time points. Blood tyrosine concentration was similar to that of controls in the L-tyrosine group and markedly elevated in both the NTBC and the NTBC + tyrosine groups.

Table 31. Plasma tyrosine concentration after administration of NTBC or L-tyrosine or co-administration of NTBC + tyrosine in rats

Day	Plasma tyrosine concentration (nmol/mL)							
	Males				Females			
	Control	TYR	NTBC	NTBC + TYR	Control	TYR	NTBC	NTBC + TYR
2	73.78	341.08	81.79	367.57	50.65	208.84	56.12	152.99
7	73.29	310.83	84.66	389.98	46.28	148.13	55.18	104.75
14	73.96	314.26	83.12	1 166.95	44.40	141.84	66.37	235.88
21	76.94	279.71	98.57	1 852.20	50.15	152.11	66.37	921.57
29/30	75.39	229.26	249.02	1 981.35	52.92	138.64	306.31	892.32
30/31	67.66	73.85	1 231.86	1 846.68	54.63	61.66	1 451.51	1 421.71

NTBC: 2-(2-nitro-4-trifluoromethylbenzoyl)-cyclohexane-1,3-dione; TYR: L-tyrosine
Source: Blanck (2006b)

In females in the NTBC + tyrosine group, relative liver weight was increased compared with control animals. There were no other differences in organ weight. Macroscopic examination revealed opacity of the eye in all males and in 1/5 females in the group treated with NTBC + tyrosine. All other gross pathologicdal changes were considered to be incidental and not treatment related.

Findings that are considered to be treatment related were observed in the pancreas, thyroid and eye in the male and female rats in the NTBC + tyrosine group (Table 32). Findings in the pancreas were diffuse interstitial mixed inflammation, along with acinar degeneration/apoptosis of the exocrine pancreas. In the thyroid gland, the only treatment-related finding was colloid alteration, which was observed only in the males. In the eye, the treatment-related change was bilateral keratitis. No pathological treatment-related effect was seen in the liver.

Table 32. Treatment-related histopathological findings after administration of NTBC or L-tyrosine or co-administration of NTBC + tyrosine in rats

	Males				Females			
	Control	TYR	NTBC	NTBC + TYR	Control	TYR	NTBC	NTBC + TYR
No. examined	5	5	5	5	5	5	5	5
Pancreas								
Acinar degeneration/ apoptosis: focal/ multifocal	1	1	1	3	0	0	1	5
Interstitial mixed cell inflammation: diffuse	0	0	0	2	0	0	1	1
Thyroid								
Follicular cell hypertrophy: diffuse	0	0	0	1	0	0	0	0
Colloid alteration	0	0	0	3	0	0	0	0
Eye								
Keratitis: diffuse: bilateral	0	0	0	5	0	0	0	1

NTBC: 2-(2-nitro-4-trifluoromethylbenzoyl)-cyclohexane-1,3-dione; TYR: L-tyrosine
Source: Blank (2006b)

The results of this study clearly show that sustained systemic tyrosinaemia provokes bilateral keratitis of the eye, focal or multifocal acinar degeneration or apoptosis of the exocrine pancreas, and minimal to slight colloid alteration of the thyroid follicles. These data taken together support the hypothesis that a threshold plasma tyrosine concentration exists below which tyrosine-related findings will not be observed (Blank, 2006b).

In another study, the objectives of which were to assess the relationship between the increase in blood tyrosine levels and potential effects on pregnancy and embryo/fetal development, pregnant Sprague-Dawley rats were administered NTBC by gavage at 10 µg/kg bw per day from GD 6 to GD 20 and L-tyrosine at 2% w/w in the diet from GD 6 to GD 21. Similar groups were co-administered vehicle alone (demineralized water) by gavage plus untreated diet (control group), NTBC by gavage plus untreated diet, or vehicle alone by gavage plus diet supplemented with L-tyrosine at the same dosage. Twenty-three pregnant rats were used per dose group.

There were no treatment-related mortalities or clinical signs during the course of the study. The pregnancy rate was unaffected by treatment with L-tyrosine at 2% in the diet, oral administration of NTBC at 10 µg/kg bw per day or co-administration of L-tyrosine at 2% in the diet plus NTBC at 10 µg/kg bw per day by gavage. Mean maternal body weight gains and mean maternal body weights in the group treated with L-tyrosine alone were similar to those of the control group (Table 33). There was a reduction of 58% (not statistically significant) in mean maternal body weight gain between GD 6 and GD 8 in the group treated with NTBC alone compared with the controls. Thereafter, mean maternal body weight gains were comparable to the control values, resulting in an overall similar body weight gain between GD 6 and GD 21. There was a reduction of 55% (not statistically significant) in mean maternal body weight gain between GD 6 and GD 8 in the group co-treated with L-tyrosine plus NTBC compared with the control group. Thereafter, mean maternal body weight gains in this treated group were similar to the control values, but the overall body weight gain between GD 6 and GD 21 was still slightly reduced by 6% (not statistically significant) in comparison with the control group. Mean maternal corrected body weight change in all treated groups was comparable to the controls. There was no treatment-related effect on feed consumption in any group.

Table 33. Maternal body weight change, maternal plasma L-tyrosine level and fetal weight after administration of NTBC or L-tyrosine or co-administration of NTBC + tyrosine in rats

Parameter	Days	Control	TYR	NTBC	NTBC + TYR
Maternal body weight gain (g)	0–6	29.0	30.7	29.5	30.9
	6–8	6.6	4.7	2.8	3.0
	6–10	15.9	12.6	11.4	12.4
	6–14	33.9	32.4	30.9	30.7
	6–18	75.2	74.3	70.9	73.0
	6–21	123.7	124.1	120.4	116.1
Maternal corrected body weight change (g)	–	45.7	50.7	48.7	45.9
Maternal plasma tyrosine (nmol/mL)	–	46.04	216.4	388.6	2 888
Fetal weight, both sexes (g)	–	5.43	5.39	5.30	5.04**
Fetal weight, males (g)	–	5.56	5.54	5.43	5.17**
Fetal weight, females (g)	–	5.31	5.25	5.16	4.93**

NTBC: 2-(2-nitro-4-trifluoromethylbenzoyl)-cyclohexane-1,3-dione; TYR: L-tyrosine; **: $P < 0.01$
Source: Kennel (2006)

Maternal plasma tyrosine concentrations were increased in all groups, with the greatest increase observed in the NTBC + tyrosine group (Table 33). Mottled kidney of minimal to slight severity was observed in all treatment groups, but not in the control group. Four animals showed minimal unilateral or bilateral corneal opacity in the NTBC + tyrosine group; no corneal opacities were observed in other groups. There was no effect of treatment on liver weight in any group.

There were no effects of treatment on the numbers of live or dead fetuses, corpora lutea, early or late resorptions, or preimplantation or postimplantation losses.

Fetal weight was statistically significantly decreased in the NTBC + tyrosine group. There was also a slight, statistically non-significant decrease in fetal weight in the NTBC group (Table 33). There were no external findings observed that were considered to be treatment related. There was no effect of treatment on the number of runt fetuses observed. A limited number of skeletal structures was examined – namely, those that were observed to differ from controls in other studies conducted with HPPD inhibitors. In the NTBC + tyrosine group, there was a decrease in ossification of specific bones and an increase in the incidence of short 14th rib and extra ossification points on the 14th thoracic vertebrae (Table 34). All of these findings are classified as variants.

Table 34. Fetal skeletal observations after administration to dams of NTBC or L-tyrosine or co-administration of NTBC + tyrosine in rats

	Fetuses				Litters			
	Control	TYR	NTBC	NTBC + TYR	Control	TYR	NTBC	NTBC + TYR
No. examined	175	162	146	170	23	22	20	23
7th cervical centrum, unossified/normal cartilage	1	6	9	72	1	5	6	19
5th sternebra, incomplete ossification/normal cartilage	49	36	57	71	20	12	17	21
6th sternebra, incomplete ossification/normal cartilage	0	1	0	10	0	1	0	2
5th sternebra, unossified/ normal cartilage	1	2	1	12	1	2	1	6
14th thoracic rib (unilateral/bilateral), short	2	2	0	4	1	2	0	4
Extra ossification point (unilateral/bilateral) on 14th thoracic vertebra	2	8	5	20	2	5	4	11
Forepaw, 3rd and/or 4th phalanges, unossified/ normal cartilage	0	1	0	8	0	1	0	3
5th metacarpal, incomplete ossification/normal cartilage or unossified/ normal cartilage	7	7	6	21	5	4	5	8
1st metatarsal, unossified/ normal cartilage	1	4	2	6	1	3	2	4
Fewer than 9 sacrocaudal vertebrae ossified/normal cartilage	1	0	0	7	1	0	0	4

NTBC: 2-(2-nitro-4-trifluoromethylbenzoyl)-cyclohexane-1,3-dione; TYR: L-tyrosine
Source: Kennel (2006)

In conclusion, co-administration of L-tyrosine at 2% w/w in the diet between GD 6 and GD 21 plus NTBC by oral gavage at 10 µg/kg bw per day between GD 6 and GD 20 provoked a marked maternal tyrosinaemia, which caused a general delay of ossification in fetuses. Furthermore, the results observed with animals treated with only tyrosine or only NTBC, where maternal plasma tyrosine was not markedly increased and where there was little or no effect on fetal ossification, show that the effects of increased maternal plasma tyrosine on fetal ossification are threshold based. Marked increases in maternal plasma tyrosine concentration are required for fetal effects to be observed (Kennel, 2006).

To compare the metabolism of tyrosine in untreated and HPPD-inhibited conditions across species, hepatocyte preparations (Liverbeads™) from rat, mouse, rabbit, dog and human were incubated with tyrosine, in the absence or the presence of NTBC, a potent inhibitor of the HPPD enzyme. Incubation times ranged from 0 to 4 hours; at the end of the incubation, the concentrations of tyrosine and HPLA were measured.

Basal tyrosine levels were similar across the species and did not change during the incubation period, with the exception of the rabbit, which presented slightly lower basal levels. After addition of NTBC, tyrosine levels were similar across the species at all incubation times, with the rabbit displaying the lowest levels. After addition of enriched tyrosine medium, tyrosine levels were similar across the species and did not change during the incubation period, with the exception of the mouse. After addition of enriched tyrosine medium and NTBC, tyrosine levels were similar across the species at all incubation times (Table 35).

Table 35. Tyrosine concentration in incubation medium after incubation of hepatocytes with or without excess tyrosine and with or without NTBC

Condition	Time (h)	Tyrosine concentration (mg/L)				
		Rat	Dog	Rabbit	Mouse	Human
Basal	0	23.69	26.22	15.25	29.12	24.33
	2	25.93	26.47	17.95	20.75	23.03
	4	25.18	26.22	16.27	21.13	24.40
Basal + NTBC	0	23.82	26.25	15.35	28.48	24.42
	2	26.87	27.60	17.30	24.63	24.48
	4	27.60	27.48	16.85	29.25	27.33
Basal + TYR	0	82.17	77.18	74.45	69.45	76.07
	2	74.42	81.18	74.55	60.63	74.03
	4	74.78	82.60	78.13	54.02	74.47
Basal + TYR + NTBC	0	84.92	78.77	75.60	70.12	74.62
	2	79.07	82.77	76.05	68.52	76.73
	4	79.30	81.38	79.77	73.17	77.70

NTBC: 2-(2-nitro-4-trifluoromethylbenzoyl)-cyclohexane-1,3-dione; TYR: L-tyrosine
Source: Totis (2006)

Basal HPLA was not detected in any species, with the exception of the mouse, at any time point. In the mouse Liverbeads™, basal levels of HPLA were barely detectable and did not increase during incubation times. After addition of NTBC to basal medium, HPLA was not observed in the rat, dog or rabbit Liverbeads™ incubations at any time point. In mouse and human incubations, however, HPLA concentration increased with time.

In basal medium supplemented with tyrosine, HPLA was similarly observed only in mice. After the addition of NTBC to basal + L-tyrosine medium, HPLA was observed in the rat, rabbit,

mouse and human incubations. The concentration of HPLA increased with time in the mouse and human incubations, whereas in the rat, there was little or no increase in HPLA with time. In the rabbit, HPLA was observed only at the 4-hour time point. HPLA was not detected in dog Liverbeads™ incubations under any conditions (Table 36).

Table 36. HPLA concentration in incubation medium after incubation of hepatocytes with or without excess tyrosine and with or without NTBC

Condition	Time (h)	HPLA concentration (µg/mg protein)				
		Rat	Dog	Rabbit	Mouse	Human
Basal	0	< LOQ	< LOQ	< LOQ	0.15	< LOQ
	2	< LOQ	< LOQ	< LOQ	0.24	< LOQ
	4	< LOQ	< LOQ	< LOQ	0.25	< LOQ
Basal + NTBC	0	< LOQ	< LOQ	< LOQ	0.18	< LOQ
	2	< LOQ	< LOQ	< LOQ	0.42	0.33
	4	< LOQ	< LOQ	< LOQ	0.69	0.54
Basal + TYR	0	< LOQ	< LOQ	< LOQ	0.17	< LOQ
	2	< LOQ	< LOQ	< LOQ	0.20	< LOQ
	4	< LOQ	< LOQ	< LOQ	0.26	< LOQ
Basal + TYR + NTBC	0	< LOQ	< LOQ	< LOQ	0.12	< LOQ
	2	0.19	< LOQ	< LOQ	0.73	0.54
	4	0.23	< LOQ	0.36	1.31	1.08

LOQ: limit of quantification; NTBC: 2-(2-nitro-4-trifluoromethylbenzoyl)-cyclohexane-1,3-dione; TYR: L-tyrosine
Source: Totis (2006)

In conclusion, the results of this study allowed the classification of species into two main groups: 1) human and mouse, which are able to produce HPLA and to use an alternative pathway for tyrosine catabolism when HPPD is inhibited; and 2) rabbit, dog and rat, for which this alternative pathway is much less efficient under "normal" and "stressed" conditions (Totis, 2006).

2.7 Studies on metabolites

(a) Metabolite RPA 202248: 2-Cyano-3-cyclopropyl-4-(2-methylsulfonyl-4-trifluoromethylphenyl)propan-1,3-dione

Acute toxicity

In an acute oral toxicity study, groups of five male and five female Sprague-Dawley rats were orally administered RPA 202248 (purity > 99.0%) in 0.5% methyl cellulose and distilled water (20 mL/kg bw) at a dose level of 2000 or 5000 mg/kg bw. The animals were observed for mortality and clinical signs of toxicity for 15 days post-dosing. Two males and two females at 5000 mg/kg bw died by day 2; the clinical signs of toxicity observed in both sexes on day 1 included palpebral ptosis, piloerection, reduced motor activity, tremors (females) and coldness to touch (females).

Based on the results of this study, the acute oral LD_{50} for RPA 202248 was greater than 5000 mg/kg bw for both sexes (Bigot, 1995a).

In a second acute oral toxicity study, groups of five male and five female Sprague-Dawley rats received a single oral administration of RPA 202248 (purity 99.9%) in 0.5% aqueous methyl cellulose at a dose level of 2000, 2710, 3690 or 5000 mg/kg bw. Mortalities occurred by study day 3 at 5000 mg/kg bw (one male) and on study day 2 (two males, one female) at 3690 mg/kg bw. There

were no mortalities at either 2000 or 2710 mg/kg bw. The clinical signs of toxicity observed in both sexes, within a few hours of dosing, included piloerection at all dose levels and hunched posture in males at 2710 mg/kg bw as well as in both sexes at 3690 and 5000 mg/kg bw. Reduced motor activity was noted in both sexes at 3690 and 5000 mg/kg bw.

Based on the results of this study, the acute oral LD_{50} for RPA 202248 was greater than 5000 mg/kg bw in both sexes (Katchadourian, 1996).

Mutagenicity

In two independent microbial gene mutation assays, *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 were exposed to RPA 202248 (purity 99.9%) at 250, 500, 1000, 2500 or 5000 µg/plate in the absence or presence of S9 activation. The confirmatory assay was conducted using the preincubation modification to the standard plate incorporation test. The S9 fraction was derived from Aroclor 1254–induced rat livers, and the test material was delivered to the test system in dimethyl sulfoxide (DMSO).

Minimal cytotoxicity was observed at 5000 µg/plate +S9 (plate incorporation method) or 5000 µg/plate −S9 (preincubation method). All strains responded to the mutagenic action of the appropriate positive control. There was, however, no evidence that RPA 202248 induced a mutagenic response in either trial (Percy, 1995).

(b) Metabolite RPA 203328: IFT-BA (2-mesyl-4-trifluoromethylbenzoic acid)

Acute toxicity

In an acute oral toxicity study, a group of five male and five female Sprague-Dawley rats was orally administered RPA 203328 (purity 99.7%) in 0.5% methyl cellulose and distilled water (20 mL/kg bw) at a dose level of 5000 mg/kg bw. The animals were observed for 15 days post-dosing. No mortalities were noted; the clinical signs of toxicity observed in two males and one female included dyspnoea, piloerection, soiled fur, mucoid faeces and increased salivation. The female also exhibited reduced motor activity, hunched posture and noisy breathing.

Based on the results of this study, the acute oral LD_{50} for RPA 203328 was greater than 5000 mg/kg bw for both sexes (Bigot, 1995b).

Short-term studies of toxicity

In a 14-day toxicity study, RPA 203328 (purity 100%) was administered by oral gavage to male and female Sprague-Dawley rats (five of each sex per dose) at a dose of 0, 30, 100, 300 or 1000 mg/kg bw per day. The control group received the vehicle alone (0.5% methyl cellulose) at the same dosing volume as the treated groups (10 mL/kg bw per day). Animals were observed daily for mortality and clinical signs for a period of 14 days. Body weight, feed consumption, haematology and clinical chemistry were monitored during the study, organs were weighed at necropsy and a gross pathological examination was carried out.

There were no treatment-related mortalities in the study. The only treatment-related clinical sign was increased salivation from 300 mg/kg bw per day. Body weight and body weight gain were slightly decreased in males from 300 mg/kg bw per day, but with no effect in females. Feed consumption was unaffected by treatment. No treatment-related changes were observed at the ophthalmological examination. Increased red blood cell count, haemoglobin and haematocrit were noted in males at the top two doses (300 and 1000 mg/kg bw per day), but not in females. Cholesterol concentration was decreased in females at 1000 mg/kg bw per day, but not in males. There was no effect on organ weights in any dose group. Slight to moderate pale abnormal colour of the liver was the only gross finding noted and was seen in females of all treatment groups and in some males at 300 mg/kg bw per day.

The NOAEL for RPA 203328 in this 14-day gavage study was 30 mg/kg bw per day, based on increased salivation, slightly decreased body weight gains and changes in the haematology and clinical chemistry parameters seen at 300 mg/kg bw per day (Dange, 1994).

In a 28-day toxicity study, RPA 203328 (purity 99.7%) was administered in the diet to male and female Sprague-Dawley rats (10 of each sex per dose) at a concentration of 0, 150, 500, 5000 or 15 000 ppm (equal to 0, 11.14, 37.57, 377.0 and 1118 mg/kg bw per day for males and 0, 12.68, 42.70, 421.5 and 1268.7 mg/kg bw per day for females, respectively). Clinical signs, body weights and feed consumption were monitored. Blood and urine were collected near or at the end of the study for haematological, clinical chemical and urine analysis determinations. Ophthalmological examinations were conducted near the end of the study in the control and 15 000 ppm animals. At the end of the study, selected organs were weighed, and histopathological examinations were conducted.

Among males, a slightly lower urinary pH at 15 000 ppm and minimally higher urinary refractive index at 500 and 15 000 ppm were noted. In the absence of other adverse effects on other parameters, these changes were considered to be a normal physiological response to ingestion of an acidic compound. There were no mortalities, clinical signs or changes in body weight or body weight gain in either males or females. No treatment-related ophthalmological abnormalities were noted. No effects were observed on haematological or clinical chemistry parameters. There were no effects of administration of RPA 203328 over 28 days on organ weights or histopathological observations.

The NOAEL of this 28-day dietary study with RPA 203328 was 15 000 ppm (equal to 1118 mg/kg bw per day), the highest dose tested (Dange, 1995).

In a 90-day toxicity study, male and female Sprague-Dawley rats (10 of each sex per dose) were fed diets containing RPA 203328 (purity 99.0%) at a concentration of 0, 1200, 4800 or 12 000 ppm (equal to 0, 73.21, 306.1 and 768.9 mg/kg bw per day for males and 0, 93.10, 371.4 and 952.4 mg/kg bw per day for females, respectively). Clinical signs, body weights and feed consumption were monitored. Blood and urine were collected near or at the end of the study for haematological, clinical chemical and urine analysis determinations. In addition, grasping, righting, corneal, pupillary, auditory startle and head shaking reflexes were examined once during the acclimatization phase and during week 12. Ophthalmological examinations were conducted near the end of the study in the control and 12 000 ppm animals. At the end of the study, selected organs were weighed, and histopathological examinations were conducted.

There were no mortalities, clinical signs or changes in body weight or body weight gain in either males or females. No effects were observed on haematological or clinical chemistry parameters. From 4800 ppm, urine pH was increased in females, but in the absence of any other findings, the toxicological significance of this finding is unclear. No clearly treatment-related macroscopic organ changes were found at necropsy. At gross necropsy, dark or yellowish liver, marked lobular liver and/or dark kidneys were noted in some animals. In the absence of histological changes, these were not considered to be related to treatment. No treatment-related changes were noted at microscopic examinations.

The NOAEL for RPA 203328 in this 90-day dietary study was 12 000 ppm (equal to 768.9 mg/kg bw per day), the highest dose tested (Bigot, 1998).

Mutagenicity

The genotoxicity studies conducted on RPA 203328 (a metabolite of isoxaflutole) are summarized in Table 37.

Developmental toxicity

In a developmental toxicity study, RPA 203328 (purity 99.0%) was administered to 25 female CD rats by gavage in a volume of 10 mL/kg bw at a dose level of 0, 75, 250 or 750 mg/kg bw per day from GD 6 to GD 20, inclusive. The test material was suspended in 0.5% aqueous methyl cellulose. Females were weighed on days 0, 3, 6, 8, 10, 12, 14, 16, 18 and 21 of gestation. Feed consumption was recorded periodically throughout the study. At necropsy on GD 21, the gravid uterine weight was recorded, and the dams were evaluated for number of corpora lutea and number and status of

Table 37. Results of studies of genotoxicity with RPA 203328

Type of study	Organism/cell line	Dose range tested	Purity (%)	Result	Reference
In vitro					
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537	100–5 000 µg/plate (in DMSO)	97.7	Negative ±S9 mix	Percy (1994)
Chromosomal aberration	Chinese hamster ovary cells	18.3–2 710 µg/mL (in DMSO)	99.0	Negative ±S9 mix	Murli (1998)
Gene mutation in mammalian cells	Chinese hamster ovary cells (HPRT)	5.3–2 700 µg/mL (in DMSO)	99.0	Negative ±S9 mix	Cifone (1998)
In vivo					
Mouse micronucleus	Male CD-1 mice	0, 500, 1 000 and 2 000 mg/kg bw (gavage in 0.5% methyl cellulose)	99.0	Negative	Curry (1998)

DMSO: dimethyl sulfoxide; HPRT: hypoxanthine–guanine phosphoribosyltransferase; S9: 9000 × *g* supernatant fraction from rat liver homogenate

implantations (resorptions, dead and live fetuses). Live fetuses were removed from the uteri, counted, weighed, sexed and examined externally. Approximately half of the live fetuses from each litter were fixed in Bouin's solution and subsequently dissected for internal examination. The remaining half were eviscerated, fixed in alcohol and stained with alizarin red S for skeletal examination.

At 250 and 750 mg/kg bw per day, 6 and 18 females (24% and 72%), respectively, had at least one occurrence of transient salivation upon treatment. In some animals dosed at 750 mg/kg bw per day, this was associated with red nasal discharge within a few minutes following administration. These observations disappeared approximately 1 hour after treatment and were probably linked with the acidic nature of the test substance. Statistically significantly reduced body weight changes occurred in the 750 mg/kg bw per day group during the interval GDs 8–10 and in the 250 mg/kg bw per day group during the interval GDs 10–14. Body weight parameters at 75 mg/kg bw per day were comparable to those of the control (Table 38). Mean feed consumption was significantly reduced for the entire treatment period at 750 mg/kg bw per day and from GDs 8 to 21 at 250 mg/kg bw per day. Feed consumption at 75 mg/kg bw per day was comparable to that of the control.

Table 38. Maternal body weight and body weight change in a rat teratology study with RPA 203328

	Day	0 mg/kg bw per day	75 mg/kg bw per day	250 mg/kg bw per day	750 mg/kg bw per day
Maternal body weight (g)	0	270.5	270.1	267.2	266.6
	6	306.8	305.7	300.4	301.0
	8	314.9	311.4	306.1	305.6
	10	325.3	321.5	313.4	309.2
	14	346.8	342.0	329.4	325.2
	18	390.7	385.1	372.0	367.3
	21	445.8	439.9	423.7	415.7
Corrected body weight change (g)		68.2	63.9	46.8**	43.1**

**: $P < 0.01$
Source: Repetto-Larsay (1999)

There was no effect of administration of RPA 203328 on gestation rate, implantation rate, the number of viable young, sex ratio or fetal weight. On examination of the fetuses, there was no effect of treatment on external, visceral or skeletal observations.

Based on the decreased body weight gain and feed consumption from 250 mg/kg bw per day, the maternal NOAEL was 75 mg/kg bw per day. The fetal NOAEL was 750 mg/kg bw per day, the highest dose tested (Repetto-Larsay, 1999).

Based on the available data, the Meeting concluded that there are no toxicological concerns for RPA 203328; therefore, the Meeting concluded that it should not be included in the residue definition. Isoxaflutole benzamide (2-mesyl-4-trifluoromethyl benzamide), a metabolite found in glyphosate-tolerant soya bean, is not likely to be of toxicological concern based on its structural similarity to RPA 203328; therefore, the Meeting concluded that it should not be included in the residue definition. For another metabolite in soya bean, 4-trifluoromethylbenzoic acid, the Meeting concluded that it should not be included in the residue definition due to its anticipated low toxicity based on the toxicity of benzoic acid.

The diketonitrile metabolite RPA 202248, RPA 205834 (2-aminomethylene-1-cyclopropyl-3-(2-mesyl-4-trifluoromethylphenyl)-propane-1,3-dione) and RPA 207048 (1-cyclopropyl-2-hydroxymethylene-3-(2-mesyl-4-trifluoromethylphenyl)-propane-1,3-dione) are structurally similar to the parent compound, and their toxicity is expected to be similar to that of the parent compound. Therefore, in the absence of limited to no data, the Meeting concluded that these three metabolites should be included in the residue definition.

3. Observations in humans

Employees working in isoxaflutole manufacturing plants have been monitored since 1997. Regular medical examinations are done every 1–5 years, depending on age and job tasks. The examinations include blood count, fasting blood sugar, liver enzymes, blood fat, blood pressure, audiometry and lung function testing and also address lifestyle factors such as nutrition. Biomonitoring is also included. No adverse effects were reported in about 58–63 employees working in the isoxaflutole manufacturing plant (Shipp, 2012).

Comments

Biochemical aspects

Following oral gavage dosing of rats, isoxaflutole was rapidly absorbed: about 70% after low dose (1 mg/kg bw) administration and about 40% after high dose (100 mg/kg bw) administration. The maximum concentrations in blood (C_{max}) were achieved between 0.5 and 1 hour post-dosing. Only about 1.4–4.3% of the dose was recovered in the tissues (e.g. kidney, liver, blood and plasma) 7 days after dosing. The elimination of the radioactivity associated with [^{14}C]isoxaflutole following oral administration was rapid, with the majority (80%) of the radioactivity being eliminated within 48 hours at the high dose level and within 24 hours at the low dose level. The urine was the major route of elimination for the low-dose groups (about 69–74% of the dose), whereas faeces was the major route of elimination for the high-dose group (about 55–63% of the dose). Isoxaflutole and/or its metabolites have a mean β-phase elimination half-life of about 60 hours, irrespective of the dose level. Up to nine radiolabelled components were found in the urine, and up to 11 in the faeces. The major component identified in urine, faeces and liver was a diketonitrile (RPA 202248, or 3-cyclopropyl-2-[2-mesyl-4-trifluoromethylbenzoyl]-3-oxopropane nitrile), followed by RPA 203328 (2-mesyl-4-trifluoromethylbenzoic acid). Unchanged isoxaflutole was detected primarily in faeces in the high-dose animals. There were no sex differences in absorption, distribution or metabolism.

Toxicological data

The acute oral LD_{50} in rats was greater than 5000 mg/kg bw, and the acute dermal LD_{50} in both rats and rabbits was greater than 2000 mg/kg bw. The acute inhalation LC_{50} in rats was greater than the maximum achievable concentration of 5.23 mg/L air. Isoxaflutole was non-irritating to rabbit skin and minimally irritating to rabbit eyes. It was not a skin sensitizer in guinea-pigs, as determined by the Buehler method and the Magnusson and Kligman test.

The liver was the primary target organ in mice, rats and dogs in repeated-dose toxicity studies. Thyroid, kidney and the haematopoietic system were also target organs in dogs and rats. Corneal opacity was observed in repeated-dose toxicity studies in rats, but not in mice or dogs.

In a 28-day toxicity study in mice using dietary concentrations of 0, 175, 700, 2800 and 7000 ppm (equal to 0, 29.4, 120.7, 474.6 and 1140.1 mg/kg bw per day for males and 0, 34.7, 142.9, 534.4 and 1347.4 mg/kg bw per day for females, respectively), the NOAEL was 175 ppm (equal to 29.4 mg/kg bw per day), based on increases in liver enzymes (ALAT and ASAT), clinical chemistry changes (decreased bilirubin and creatinine levels) and increased liver weight at 700 ppm (equal to 120.7 mg/kg bw per day). In the absence of any other significant findings at 175 ppm, the increased liver weights observed at this dose were considered a minor adaptive change.

In a 90-day toxicity study in mice using dietary concentrations of 0, 50, 1000 and 2000 ppm (equal to 0, 7.6, 170.0 and 324.1 mg/kg bw per day for males and 0, 8.7, 181.2 and 376.2 mg/kg bw per day for females, respectively), the NOAEL of 50 ppm (equal to 7.6 mg/kg bw per day) was based on increased ALAT and ASAT activities, increased absolute and relative liver weights and increased incidence of periacinar hepatocytic hypertrophy at 1000 ppm (equal to 170.0 mg/kg bw per day).

In a 6-week toxicity study in rats given diets providing doses of 0, 25, 100, 400 and 1000 mg/kg bw per day, the LOAEL was 25 mg/kg bw per day, based on corneal opacities and effects on the liver observed at all doses. Most of the corneal opacities were resolved by the 2nd week of the reversibility period. In a 90-day dietary toxicity study in rats at doses of 0, 1.0, 3.0, 10 and 100 mg/kg bw per day, the NOAEL was 3.0 mg/kg bw per day, based on haematological changes, corneal opacity and liver toxicity observed at 10 mg/kg bw per day.

In a 1-year toxicity study in dogs using dietary concentrations of 0, 240, 1200, 12 000 and 30 000 ppm (equal to 0, 8.56, 44.81, 453 and 1265 mg/kg bw per day for males and 0, 8.41, 45.33, 498 and 1254 mg/kg bw per day for females, respectively), the NOAEL was 1200 ppm (equal to 44.81 mg/kg bw per day), based on reduced weight gains, increased liver weight, histopathological findings in the liver and changes in haematological and clinical chemistry parameters at 12 000 ppm (equal to 453 mg/kg bw per day).

In a 78-week study of toxicity and carcinogenicity in mice using dietary concentrations of 0, 25, 500 and 7000 ppm (equal to 0, 3.2, 64.4 and 977.3 mg/kg bw per day for males and 0, 4.0, 77.9 and 1161.1 mg/kg bw per day for females, respectively), the NOAEL was 25 ppm (equal to 3.2 mg/kg bw per day), based on liver effects seen at 500 ppm (equal to 64.4 mg/kg bw per day). The NOAEL for carcinogenicity was 500 ppm (equal to 64.4 mg/kg bw per day), based on an increased incidence of hepatocellular adenomas and carcinomas in both sexes at 7000 ppm (equal to 977.3 mg/kg bw per day).

In a 2-year chronic toxicity and carcinogenicity study in rats given diets providing doses of 0, 0.5, 2, 20 and 500 mg/kg bw per day, the NOAEL was 2 mg/kg bw per day, based on liver, thyroid, ocular and nervous system toxicity in males and liver toxicity in females seen at 20 mg/kg bw per day. An increased incidence of adenomas and carcinomas of the liver was found in male and female rats at 500 mg/kg bw per day. In male rats, an increase of thyroid follicular cell adenomas was also observed at 500 mg/kg bw per day.

A 14-day dietary study in mice and rats indicated a marked increase in microsomal enzyme induction (increased PROD and BROD activities) and increased liver weights. There was no peroxisome proliferation. The data were inadequate to elucidate the precursor events leading to tumour formation and dose concordance for hepatocellular adenomas and carcinomas in mice and

rats. In a 14-day oral gavage study in rats, isoxaflutole was found to decrease T_4 levels, with little or no change in T_3 levels, and an increased systemic clearance of ^{125}I-labelled T_4 was observed. The results of these mechanistic studies were suggestive of the induction of microsomal enzymes and tumour formation, but failed to establish the mode of action.

The Meeting concluded that isoxaflutole is carcinogenic in mice and rats.

Special studies conducted to evaluate the corneal opacity seen in rats suggest that the lesion may be linked to the inhibition of the enzyme HPPD in the catabolic pathway of tyrosine. The studies have shown that if HPPD is inhibited, alternative pathways may be utilized to remove excess tyrosine, and species specificity may be linked to the differences in activity of these alternative pathways. The results of the comparative metabolism study in mice and rats suggest that the elimination of tyrosine as HPLA and HPAA is more efficient in the mouse than in the rat, with twice as much of the administered dose of [^{14}C]tyrosine observed in mouse urine as in rat urine. The results of special studies indicate that rats are more sensitive than mice, dogs and humans to tyrosinaemia.

Isoxaflutole was tested for genotoxicity in vitro and in vivo in an adequate range of assays. No genotoxicity was observed.

The Meeting concluded that isoxaflutole is unlikely to be genotoxic.

On the basis of the absence of genotoxicity and other available toxicological information, the Meeting concluded that the mode of action for the increased incidences of hepatocellular adenomas and carcinomas in both male and female mice and rats and the increased incidence of thyroid follicular cell adenomas in male rats, while not completely understood, is likely to involve a threshold. Therefore, the Meeting concluded that isoxaflutole is unlikely to pose a carcinogenic risk to humans from the diet.

In a two-generation reproductive toxicity study in rats given diets providing doses of 0, 0.5, 2, 20 and 500 mg/kg bw per day, the NOAEL for parental systemic toxicity and offspring toxicity was 2 mg/kg bw per day. The parental systemic toxicity LOAEL of 20 mg/kg bw per day was based on increased liver weights, liver hypertrophy and vacuolation. The offspring toxicity LOAEL of 20 mg/kg bw per day was based on decreased pup weights and reduced pup viability. The NOAEL for reproductive toxicity was 500 mg/kg bw per day, the highest dose tested.

In a developmental toxicity study in rats that tested doses of 0, 10, 100 and 500 mg/kg bw per day, the maternal NOAEL was 100 mg/kg bw per day, based on decreased body weight gain observed at 500 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 10 mg/kg bw per day, based on decreased fetal weight and delayed ossification observed at 100 mg/kg bw per day.

In a developmental toxicity study in rabbits that tested doses of 0, 5, 20 and 100 mg/kg bw per day, the NOAEL for maternal toxicity was 20 mg/kg bw per day, based on decreased maternal body weight, decreased feed consumption and increased numbers of resorptions seen at 100 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 20 mg/kg bw per day, based on slightly delayed development of the fetuses, decreased fetal weights and delayed ossification at 100 mg/kg bw per day.

The Meeting concluded that isoxaflutole is not teratogenic in rats or rabbits.

In an oral acute neurotoxicity study in rats that tested doses of 0, 125, 500 and 2000 mg/kg bw, no evidence of neurotoxicity or systemic toxicity was observed at doses up to 2000 mg/kg bw. In a 90-day neurotoxicity study in rats given diets providing doses of 0, 25, 250 and 750 mg/kg bw per day, no neurotoxicity was observed at doses up to 750 mg/kg bw per day. A NOAEL for systemic toxicity was not identified, as only limited parameters were evaluated in this study.

In a developmental neurotoxicity study in rats that tested gavage doses of 0, 5, 25 and 250 mg/kg bw per day, the maternal NOAEL was 25 mg/kg bw per day, based on decreased maternal body weight, body weight gain and feed consumption at 250 mg/kg bw per day. The offspring toxicity NOAEL was 25 mg/kg bw per day, based on decreased pup survival, body weight and body weight

gain at 250 mg/kg bw per day. In the absence of any neurotoxic findings, the NOAEL for neurotoxicity in the rat was 250 mg/kg bw per day, the highest dose tested.

The Meeting concluded that isoxaflutole is not neurotoxic.

Toxicological data on metabolites and/or degradates

The acute oral LD_{50} of metabolite RPA 202248, a major metabolite of urine, faeces and liver, was greater than 5000 mg/kg bw. The metabolite was not genotoxic in the Ames test.

Metabolite RPA 203328, detected in urine and faeces, was extensively studied. The acute oral LD_{50} in rats was greater than 5000 mg/kg bw. RPA 203328 was not genotoxic in a range of in vivo and in vitro genotoxicity assays. In a 14-day gavage toxicity study in rats, the NOAEL for RPA 203328 was 30 mg/kg bw per day, based on increased salivation, slightly decreased body weight gains and changes in the haematology and clinical chemistry parameters seen at 300 mg/kg bw per day. Dietary 28-day and 90-day toxicity studies in rats were conducted for RPA 203328 at doses up to 15 000 ppm (equal to 1118 mg/kg bw per day) and 12 000 ppm (equal to 769 mg/kg bw per day), respectively. No evidence of systemic toxicity was observed in these studies. No evidence of teratogenicity or developmental toxicity in rats was observed in a developmental toxicity study for RPA 203328 at doses up to 750 mg/kg bw per day.

Human data

In reports on employees working in isoxaflutole manufacturing plants, no adverse health effects were reported.

The Meeting concluded that the existing database on isoxaflutole was adequate to characterize the potential hazards to 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 2 mg/kg bw per day in a 2-year dietary study of toxicity and carcinogenicity in rats, on the basis of liver, thyroid, ocular and nervous system toxicity in males and liver toxicity in females at 20 mg/kg bw per day. A safety factor of 100 was applied. This ADI is supported by a NOAEL for parental systemic toxicity and offspring toxicity of 2 mg/kg bw per day in a dietary two-generation reproductive toxicity study in rats, based on increased liver weights, liver hypertrophy, vacuolation, decreased pup weights and reduced pup viability observed at 20 mg/kg bw per day. The ADI provides a margin of exposure of at least 25 000 relative to the LOAEL for liver and thyroid tumours in rats and at least 48 000 relative to the LOAEL for the liver tumour response in mice. Thus, the Meeting considered that isoxaflutole is not likely to pose a carcinogenic risk to humans from the diet.

The Meeting concluded that it was not necessary to establish an acute reference dose (ARfD) for isoxaflutole 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 isoxaflutole

Species	Study	Effect	NOAEL	LOAEL
Mouse	Ninety-day study of toxicity[a]	Toxicity	50 ppm, equal to 7.6 mg/kg bw per day	1 000 ppm, equal to 170 mg/kg bw per day
	Eighteen-month study of toxicity and carcinogenicity[a]	Toxicity	25 ppm, equal to 3.2 mg/kg bw per day	500 ppm, equal to 64.4 mg/kg bw per day
		Carcinogenicity	500 ppm, equal to 64.4 mg/kg bw per day	7 000 ppm, equal to 977 mg/kg bw per day

Species	Study	Effect	NOAEL	LOAEL
Rat	Acute neurotoxicity study[b]	Toxicity	2 000 mg/kg bw[c]	–
	Ninety-day study of toxicity[a]	Toxicity	3 mg/kg bw per day	10 mg/kg bw per day
	Two-year study of toxicity and carcinogenicity[a]	Toxicity	2 mg/kg bw per day	20 mg/kg bw per day
		Carcinogenicity	20 mg/kg bw per day	500 mg/kg bw per day
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	500 mg/kg bw per day[c]	–
		Parental toxicity	2 mg/kg bw per day	20 mg/kg bw per day
		Offspring toxicity	2 mg/kg bw per day	20 mg/kg bw per day
	Developmental toxicity study[b]	Maternal toxicity	100 mg/kg bw per day	500 mg/kg bw per day
		Embryo and fetal toxicity	10 mg/kg bw per day	100 mg/kg bw per day
Rabbit	Developmental toxicity study[b]	Maternal toxicity	20 mg/kg bw per day	100 mg/kg bw per day
		Embryo and fetal toxicity	20 mg/kg bw per day	100 mg/kg bw per day
Dog	One-year study of toxicity[a]	Toxicity	1 200 ppm, equal to 44.8 mg/kg bw per day	12 000 ppm, equal to 453 mg/kg bw per day

LOAEL: lowest-observed-adverse-effect level; NOAEL: no-observed-adverse-effect level
[a] Dietary administration.
[b] Gavage administration.
[c] Highest dose tested.

Estimate of acceptable daily intake

0–0.02 mg/kg bw

Estimate of acute reference dose

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 isoxaflutole

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid, 40–70%, depending on dose
Dermal absorption	Low, < 4.5%
Distribution	Widely distributed (highest levels in kidney and liver)
Potential for accumulation	None
Rate and extent of excretion	Rapid and complete, about 80% in urine and faeces in 24 h in rats
Metabolism in animals	Extensive; saturated at high doses
Toxicologically significant compounds in animals, plants and the environment	Isoxaflutole, RPA 202248[a], RPA 205834[a], RPA 207048[a]

Acute toxicity	
Rat, LD_{50}, oral	> 5 000 mg/kg bw
Rat, LD_{50}, dermal	> 2 000 mg/kg bw
Rat, LC_{50}, inhalation	> 5.23 mg/L (whole-body exposure)
Rabbit, dermal irritation	Non-irritating
Rabbit, ocular irritation	Minimally irritating
Guinea-pig, dermal sensitization	Non-sensitizing (Buehler method and Magnusson-Kligman test)
Short-term studies of toxicity	
Target/critical effect	Eye, liver and red blood cells
Lowest relevant oral NOAEL	3 mg/kg bw per day (rat)
Lowest relevant dermal NOAEL	1 000 mg/kg bw per day (rat)
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Liver and thyroid
Lowest relevant oral NOAEL	2 mg/kg bw per day (rat)
Carcinogenicity	Unlikely to pose a carcinogenic risk to humans from the diet
Genotoxicity	
	Not genotoxic
Reproductive toxicity	
Target/critical effect	Pup viability and pup weights
Lowest relevant parental NOAEL	2 mg/kg bw per day
Lowest relevant offspring NOAEL	2 mg/kg bw per day
Lowest relevant reproductive NOAEL	500 mg/kg bw per day, the highest dose tested
Developmental toxicity	
Developmental target/critical effect	Delayed ossification, decreased fetal weights
Lowest maternal NOAEL	20 mg/kg bw per day (rabbit)
Lowest embryo/fetal NOAEL	10 mg/kg bw per day (rat)
Neurotoxicity	
Acute and subchronic neurotoxicity	Not neurotoxic
Other toxicological studies	
Studies on metabolites	Rat, LD_{50}, oral: > 5 000 mg/kg bw (RPA 203348 and RPA 203328)
	Lowest relevant short-term NOAEL: 769 mg/kg bw per day (RPA 203328)
	Not genotoxic (RPA 202248 and RPA 203328)
	No developmental toxicity (RPA 203328) at doses up to 750 mg/kg bw per day, highest dose tested
Medical data	
	No adverse effects

LC_{50}: median lethal concentration; LD_{50}: median lethal dose; NOAEC: no-observed-adverse-effect concentration; NOAEL: no-observed-adverse-effect level

[a] Based on structural similarity to the parent compound.

Summary

	Value	Study	Safety factor
ADI	0–0.02 mg/kg bw	Two-year study of toxicity and carcinogenicity in rats	100
ARfD	Unnecessary	–	–

ADI: acceptable daily intake; ARfD: acute reference dose

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TOLFENPYRAD

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Explanation

Tolfenpyrad, a pyrazole insecticide, is the International Organization for Standardization–approved name for 4-chloro-3-ethyl-1-methyl-*N*-[4-(*p*-tolyloxy)benzyl]pyrazole-5-carboxamide (International Union of Pure and Applied Chemistry), which has the Chemical Abstracts Service number 129558-76-5. Tolfenpyrad has broad insecticidal activity against a variety of pests on egg, larval, nymphal and adult stages and is used on a variety of crops. The pesticidal mode of action is thought to be the inhibition of complex I of the respiratory electron transport chain in the mitochondria.

Tolfenpyrad has not previously been reviewed by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) and is being evaluated 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) unless otherwise specified.

Evaluation for acceptable daily intake

1. Biochemical aspects

The structure of tolfenpyrad and the positions of the labels used in the study of Okada (1998) are shown in Fig. 1.

Fig. 1. Structure of tolfenpyrad

*: position of ^{14}C label in the tolyl ring ([TO-^{14}C]tolfenpyrad) in study of Okada (1998)

+: position of ^{14}C label in the pyrazole group ([PY-^{14}C]tolfenpyrad) in study of Okada (1998)

1.1 Absorption, distribution and excretion

Rats

The absorption, distribution and excretion of [^{14}C]tolfenpyrad, labelled at either the tolyl ring ([TO-^{14}C]tolfenpyrad, radiochemical purity 99.5%) or the 3 position of the pyrazole group ([PY-^{14}C]tolfenpyrad, radiochemical purity 99.7%), were studied in groups of male and female F344 rats dosed orally by gavage at a single dose of 1 or 20 mg/kg body weight (bw). In addition, rats were administered either [PY-^{14}C]tolfenpyrad (five males and five females) or [TO-^{14}C]tolfenpyrad (five males) at a dose of 1 mg/kg bw per day for 14 consecutive days (14 radiolabelled doses). These materials were dissolved in 0.5% weight per volume (w/v) sodium carboxymethyl cellulose and 0.5% w/v Tween 80. The identification of tolfenpyrad and its metabolites in tissues and excreta is described in section 1.2 (Ogawa, 1999a). The experimental designs are presented in Table 1.

After the administration of single doses, similar toxicokinetic parameters for radioactivity in blood were observed with both labels at the two dose levels, indicating that no difference in the disposition of tolfenpyrad was attributable to radiolabel positions (Table 2). The time to reach the maximum concentration in plasma (T_{max}) was higher after a high dose than after a low dose. At least 58% of the 1 and 20 mg/kg bw doses were absorbed. Seven days after administration, 88–93% of the radioactivity was excreted in faeces, and 2–3% of the radioactivity was excreted in urine, independent of dose or sex. No radioactivity was found in expired air. Forty-eight hours after dosing, biliary excretion was 64–70% in males and 51–55% in females. At 48 hours, up to 3% of radioactivity was excreted in urine, 5–11% remained in the carcass and 13–37% remained in the gastrointestinal tract. Radioactivity was widely distributed to the tissues, the highest levels being found in liver, kidney and brown fat 4–12 hours after dosing (Table 3). Tissue levels were similar between males and females, except for brown fat, in which concentrations were about 2 times higher in females than in males. In kidney and brown fat, highest levels were observed 12 hours after dosing. Concentrations of radioactivity decreased rapidly, and by 168 hours, only very low levels of radioactivity were observed in tissues (Okada, 1998).

After the administration of 14 daily doses, the absorption, distribution and excretion of the labelled materials were investigated for up to 168 hours. Different groups of rats were used for determination of radioactivity levels in blood, urine, faeces and tissues. Urine and faeces were collected over 24-hour intervals after the 1st to 13th administrations and at 24, 48, 72, 120 and 168 hours after the 14th administration. Blood was collected at 24 hours after each daily dose, then at the following time points after the 14th dose: 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72, 120 and 168 hours. Samples of an extensive range of tissues were taken from groups of rats at 4, 12 and 168 hours after the 14th

dose. The identification of parent compound and metabolites in tissues and excreta is described in section 1.2 (Ogawa, 1999b).

The concentration of radioactivity in plasma samples tended to reach a maximum after two or three administrations, at 1.5–3 times the plasma concentration after the first dose. Plasma concentrations in females were about 2 times higher than those in males at all time points. The plasma concentrations reached the maximum level at 8–12 hours after the final administration and thereafter demonstrated biphasic elimination. Calculations based on the second phase showed a relatively slow decrease in plasma concentration, with half-lives of 20–46 hours. Tissue concentrations were highest in liver, kidney, bone marrow and brown fat (Table 4, levels in other tissues are not presented). The decline in radioactivity level in bone marrow at 168 hours appeared to be slower than in other tissues. The tissue distribution after repeated administration tended to be similar to that after a single administration. Urinary and faecal excretions of radioactivity were comparable and almost as complete as with a single administration. Total excretion exceeded 95% 168 hours after the final administration in all treatment groups, suggesting that tolfenpyrad exhibits no residuality in rats.

Table 1. Study designs for absorption, distribution and excretion in rats

Test group	Labelled compound	Dose (mg/kg bw)	Sex	Number of rats	Time post-dosing (h)
Group 1 Measurement of blood concentrations	[PY-^{14}C]Tolfenpyrad	1	Male	5	0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72, 120 and 168
			Female	5	
		20	Male	5	
			Female	5	
	[TO-^{14}C]Tolfenpyrad	1	Male	5	
			Female	5	
		20	Male	5	
			Female	5	
Group 2 Measurement of excretion rates into urine, faeces and expired air	[PY-^{14}C]Tolfenpyrad	1	Male	5	0–24, 24–48, 48–72, 72–120 and 120–168
			Female	5	
		20	Male	5	
			Female	5	
	[TO-^{14}C]Tolfenpyrad	1	Male	5	
			Female	5	
Group 3 Measurement of excretion rates into bile, urine and faeces	[PY-^{14}C]Tolfenpyrad	1	Male	4	Bile: 3, 6, 12, 24 and 48 Urine and faeces: 0–24 and 24–48
			Female	4	
		20	Male	4	
			Female	4	
Group 4 Measurement of tissue concentrations and distribution rates	[PY-^{14}C]Tolfenpyrad	1	Male	5	4, 12 and 168
			Female	5	4, 12 and 168
		20	Male	5	6, 24 and 168
			Female	5	6, 24 and 168
	[TO-^{14}C]Tolfenpyrad	1	Male	5	4, 12 and 168
			Female	5	4, 12 and 168

Source: Okada (1998)

Table 2. Toxicokinetic parameters after a single oral administration of radiolabelled tolfenpyrad to rats

	1 mg/kg bw		20 mg/kg bw	
	Males	Females	Males	Females
[PY-^{14}C]Tolfenpyrad				
C_{max} (µg eq/mL)	0.304	0.253	1.93	2.23
T_{max} (h)	2–4	6–8	8	12
$AUC_{0-\infty}$ (µg·h/mL)	3.1	2.8	44.5	52.4
$t_{½}$ (h)	16.4	27.6	16.3	14.2
[TO-^{14}C]Tolfenpyrad				
C_{max} (µg eq/mL)	0.268	0.284	2.22	2.37
T_{max} (h)	2–4	4	6–8	4
$AUC_{0-\infty}$ (µg·h/mL)	3.0	3.4	62.7	70.8
$t_{½}$ (h)	12.1	11.0	12.6	11.5

AUC: area under the plasma–concentration time curve; C_{max}: maximum concentration; eq: equivalents; $t_{½}$: half-life; T_{max}: time to reach C_{max}
Source: Okada (1998)

Table 3. Tissue concentrations after a single oral dose of tolfenpyrad administered to rats

Organ or tissue	Dose; ^{14}C label	Tissue concentration (mg eq/kg)			
		Males		Females	
		4 h (1 mg/kg bw); 6 h (20 mg/kg bw)	12 h (1 and 20 mg/kg bw)	4 h (1 mg/kg bw); 6 h (20 mg/kg bw)	12 h (1 and 20 mg/kg bw)
Liver	1 mg/kg bw; PY	5.40 ± 0.633	4.72 ± 0.465	5.70 ± 0.656	6.24 ± 0.564
	1 mg/kg bw; TO	5.56 ± 0.314	5.11 ± 0.477	5.74 ± 0.555	6.23 ± 0.835
	20 mg/kg bw; PY	18.6 ± 0.650	19.8 ± 4.95	20.0 ± 1.11	24.1 ± 3.99
Kidney	1 mg/kg bw; PY	1.35 ± 0.192	2.06 ± 0.209	1.38 ± 0.063	2.08 ± 0.486
	1 mg/kg bw; TO	1.65 ± 0.144	2.25 ± 0.163	1.41 ± 0.058	1.83 ± 0.261
	20 mg/kg bw; PY	4.88 ± 0.185	4.26 ± 0.490	4.95 ± 0.207	4.27 ± 0.741
Brown fat	1 mg/kg bw; PY	1.01 ± 0.085	1.73 ± 0.486	1.11 ± 0.089	4.29 ± 0.375
	1 mg/kg bw; TO	0.928 ± 0.099	2.14 ± 0.552	1.39 ± 0.244	5.44 ± 1.10
	20 mg/kg bw; PY	3.12 ± 0.398	20.6 ± 6.46	5.17 ± 1.46	34.6 ± 2.80

Source: Okada (1998)

The toxicokinetics of tolfenpyrad in rats after repeated administration was similar to that after a single administration. Although the declines in organ/tissue concentrations of radioactivity were slightly slower compared with those after a single administration, tolfenpyrad exhibited neither accumulation nor residuality in rats (Okada, 1999).

1.2 Biotransformation

(a) In vivo

Rats

The metabolism of tolfenpyrad ^{14}C-radiolabelled in either the pyrazole or tolyl ring was studied in male and female F344 rats after a single oral gavage administration of 1 or 20 mg/kg bw and after 14 daily oral gavage administrations of 1 mg/kg bw. The levels of tolfenpyrad and its

Table 4. Tissue concentrations after 14 daily oral doses of tolfenpyrad administered to rats

Organ or tissue	Dose; position of label	Tissue concentration (mg eq/kg)					
		Males			Females		
		4 h	12 h	168 h	4 h	12 h	168 h
Liver	1 mg/kg bw; PY	7.75 ± 0.45	7.77 ± 0.61	0.263 ± 0.027	10.6 ± 1.2	11.3 ± 0.3	0.336 ± 0.161
	1 mg/kg bw; TO	10.1 ± 1.3	8.88 ± 0.55	0.275 ± 0.079	–	–	–
Kidney	1 mg/kg bw; PY	2.54 ± 0.13	2.98 ± 0.24	0.187 ± 0.015	2.24 ± 0.19	2.88 ± 0.33	0.162 ± 0.038
	1 mg/kg bw; TO	3.43 ± 0.25	3.55 ± 0.3	0.210 ± 0.059	–	–	–
Brown fat	1 mg/kg bw; PY	3.62 ± 0.94	3.01 ± 0.42	0.278 ± 0.52	6.80 ± 1.81	7.27 ± 1.25	0.178 ± 0.069
	1 mg/kg bw; TO	3.24 ± 0.53	3.02 ± 0.07	0.273 ± 0.067	–	–	–
Bone marrow	1 mg/kg bw; PY	1.18 ± 0.32	1.48 ± 0.24	0.76 ± 0.21	2.94 ± 0.47	3.06 ± 0.45	1.20 ± 0.41
	1 mg/kg bw; TO	1.49 ± 0.23	1.28 ± 0.16	0.63 ± 0.12	–	–	–

Source: Okada (1998)

metabolites were determined in plasma, urine, faeces and bile excreted over 48 hours after dosing. The rats were dosed as part of the study by Okada (1998) described in section 1.1.

Faeces and bile were extracted with methanol, after which samples were digested with glucuronidase and sulfatase preliminary to conjugate analysis. Radioactivity in samples was determined by liquid scintillation counting and radioisotope high-performance liquid chromatography (HPLC), and metabolites were identified by co-chromatography against authentic reference standards. The full chemical names of the metabolites described below can be found in the legend of Fig. 2 below.

Within 48 hours after single administration, 4–15% of the dose was eliminated in faeces as tolfenpyrad, but the largest proportion (24–49%) of the administered dose found in faecal extracts was PT-CA (see Table 5). Smaller fractions found in faecal extracts were Sul-OH-PT-CA (5–12%) and OH-PT-CA (6–13%). Minor amounts of metabolites were not identified. In plasma, liver and kidney, 91–100% of radioactivity represented PT-CA, whereas maximally 3% of radioactivity represented other, unidentified metabolites These data suggest a rapid metabolism of tolfenpyrad in the liver. In bile, 50–67% of the administered dose was excreted within 48 hours (see Table 6), the major part as PT-CA-TA, PT-CA-Gluc and PT-CA (31–43%; these three metabolites could not be further separated). Low levels of Sul-OH-PT-CA (5–8%) and CO-PT (4–6%) and several unidentified metabolites were detected. These data indicate a rapid conjugation of PT-CA in the liver and subsequent excretion into the bile. Less than 0.7% of the administered dose was present as unchanged tolfenpyrad in bile. In bile duct–cannulated rats, only 3–8% of the administered dose was excreted into faeces, predominantly as tolfenpyrad (up to 6%) and PT-CA (up to 1%). The data from the studies in unoperated rats and the bile duct–cannulated rats indicate that following biliary excretion into the gastrointestinal tract, PT-CA is deconjugated, probably by enterobacteria, and excreted in faeces. In urine, no intact tolfenpyrad was detected. Various metabolites (among others, OH-PAM and CA-T-CA) were present at levels less than 0.5% of the administered dose, with the exception of PT-CA and PT-CA-TA (these metabolites could not be further separated), which were present at up to 1.9% of the administered dose. The urinary metabolite profiles were different between the groups of

rats administered [PY-^{14}C]tolfenpyrad and [TO-^{14}C]tolfenpyrad. The complicated metabolite profile in the urine indicates that metabolism of tolfenpyrad includes cleavage of the C–N bond of the benzylamine moiety and oxidation of the side-chains of both of the ^{14}C-labelled rings. In summary, the data indicate that after absorption in the gastrointestinal tract, tolfenpyrad is rapidly metabolized to PT-CA in the liver, which is subsequently polarized by conjugation and then excreted into bile. Once back in the gastrointestinal tract, it is deconjugated and thus appears in faeces as PT-CA. Observed differences in metabolite levels between sexes, doses and position of radiolabel were minor.

Table 5. Amount of tolfenpyrad and its metabolites in faeces of rats

Identification	% of administered dose											
	1 mg/kg bw [PY-^{14}C]				20 mg/kg bw [PY-^{14}C]				1 mg/kg bw [TO-^{14}C]			
	0–24 h		0–48 h		0–24 h		0–48 h		0–24 h		0–48 h	
	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females
Extracted	61.5	55.5	77.3	75.3	27.9	24.2	62.7	50.6	56.5	50.0	75	73.3
Sul-OH-PT-CA	7.4	5.0	9.0	8.2	2.4	1.5	10.5	5.3	9.2	8.0	11.5	8.1
OH-PT-CA	6.4	4.8	8.8	8.9	1.5	1.8	7.9	6.4	6.2	7.4	9.0	12.9
PT-CA	36.2	30.0	47.7	41.6	11.1	9.0	29.0	23.9	35.6	28.0	48.9	45.2
Tolfenpyrad	10.6	14.8	10.9	15.1	12.5	11.7	14.3	14.7	4.1	5.6	–	0.3
Total of unidentified	0.8	0.9	0.8	1.4	0.4	0.1	0.6	0.3	1.5	1.0	1.5	1.2
Unextracted	3.2	4.1	2.3	3.5	1.3	1.1	5.1	2.8	6.1	5.8	2.0	3.2

Source: Ogawa (1999a)

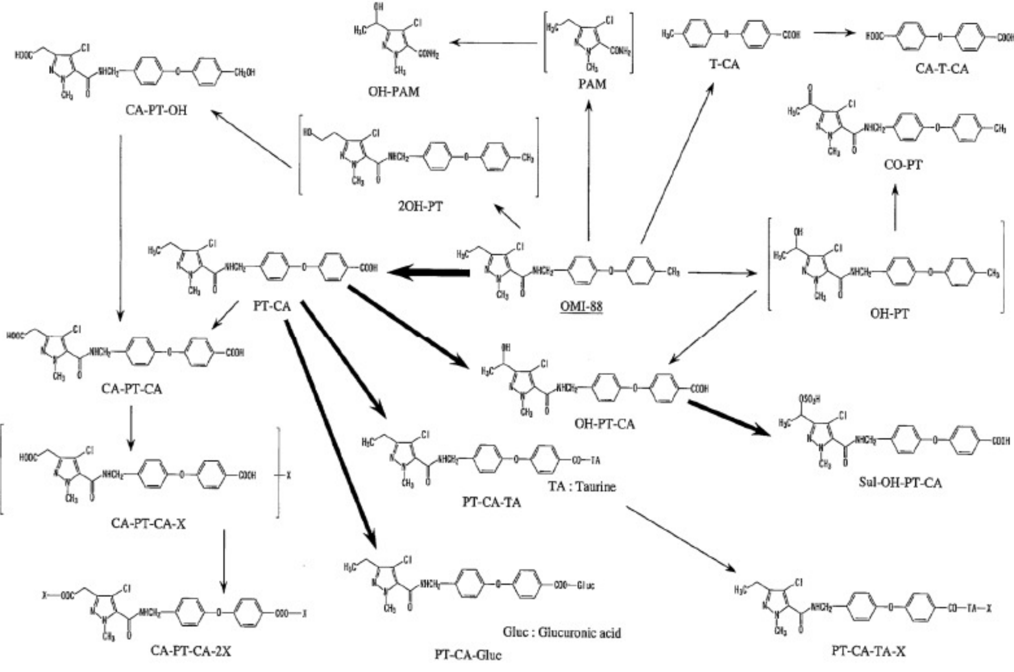
Table 6. Amount of tolfenpyrad and its metabolites in bile of rats

Identification	% of administered dose							
	1 mg/kg [PY-^{14}C]				20 mg/kg [PY-^{14}C]			
	0–24 h		0–48 h		0–24 h		0–48 h	
	Males	Females	Males	Females	Males	Females	Males	Females
Extracted	29.9	26.4	61.6	52.8	35.7	22.6	67.4	50.0
Sul-OH-PT-CA	3.0	2.8	5.9	4.7	4.4	2.6	7.7	5.4
OH-PT-CA	0.2	0.8	0.8	0.8	1.1	0.7	2.0	1.5
PT-CA-TA PT-CA-Gluc PT-CA	20.6	14.3	42.9	34.1	19.0	13.1	41.2	31.3
CO-PT	2.0	4.2	3.7	5.0	5.6	3.1	6.4	5.9
Tolfenpyrad	–	0.3	–	0.3	0.5	0.5	0.5	0.7
Total of unidentified	4.9	3.9	9.0	7.0	5.1	2.1	8.5	5.3
Unextracted	0.8	1.0	2.0	1.9	1.2	0.6	2.1	1.3

Source: Ogawa (1999a)

The proposed metabolic pathway for tolfenpyrad is presented in Fig. 2 (Ogawa, 1999a).

Fig. 2. Proposed metabolic pathway in rats



OMI-88 Tolfenpyrad
OH-PT 4-Chloro-3-(1-hydroxyethyl)-1-methyl-*N*-[4-(*p*-tolyloxy)benzyl]pyrazole-5-carboxamide
CO-PT 3-Acetyl-4-chloro-1-methyl-*N*-[4-(*p*-tolyloxy)benzyl]pyrazole-5-carboxamide
PT-CA 4-[4-[(4-Chloro-3-ethyl-1-methylpyrazol-5-yl)carbonylaminomethyl]phenoxy]benzoic acid
CA-PT-OH [4-Chloro-5-[*N*-[4-(4-hydroxymethyl)phenoxy]benzylcarbamoyl]-1-methylpyrazol-3-yl]acetic acid
OH-PT-CA 4-[4-[(4-Chloro-3-(1-hydroxyethyl)-1-methylpyrazol-5-yl)carbonylaminomethyl]phenoxy]benzoic acid
CA-PT-CA 4-[4-[(3-Carboxymethyl-4-chloro-1-methylpyrazol-5-yl)carbonylaminomethyl]phenoxy]benzoic acid
2OH-PT 4-Chloro-3-(2-hydroxyethyl)-1-methyl-*N*-[4-(*p*-tolyloxy)benzyl]pyrazole-5-carboxamide
T-CA 4-(*p*-Tolyloxy)benzoic acid
CA-T-CA 4,4′-Oxydibenzoic acid
PAM 4-Chloro-3-ethyl-1-methylpyrazole-5-carboxamide
OH-PAM 4-Chloro-3-(1-hydroxyethyl)-1-methylpyrazole-5-carboxamide
Sul-OH-PT-CA 4-[4-[(4-Chloro-1-methyl-3-(1-sulfoxyethyl)pyrazol-5-yl)carbonylarninomethyl]phenoxy]benzoic acid
PT-CA-TA 2-[4-[4-[(4-Chloro-3-ethyl-1-methylpyrazol-5-yl)carbonylaminomethyl]phenoxy]phenylcarbonylamino]-ethane-1-sulfonic acid
PT-CA-Gluc Glucuronide conjugate of 4-[4-[(4-chloro-3-ethyl-1-methylpyrazol-5-yl)carbonylaminomethyl]phenoxy]-benzoic acid
X Biological component

(b) In vitro

In an in vitro metabolism study, unlabelled tolfenpyrad (1.0 mg, purity 99.8%), [TO-^{14}C]tolfenpyrad (0.1 mg, radiochemical purity 99.8%) or [PY-^{14}C]tolfenpyrad (0.1 or 1.0 mg, radiochemical purity 99.3%) was incubated with a 9000 × *g* supernatant fraction from a rat liver homogenate (S9). Metabolites of tolfenpyrad were identified by gas chromatography–mass spectrometry (GC-MS), liquid chromatography–mass spectrometry (LC-MS), hydrogen nuclear magnetic resonance (^{1}H-NMR) and HPLC.

Without S9, no significant degradation of tolfenpyrad was observed. After incubation with S9, 5–6% of tolfenpyrad was cleaved between the amide of the pyrazole ring and the methylene bond of the tolyl ring. There was little difference in the metabolite profiles between the labels or substrate amounts (0.1 and l mg). From the metabolite investigation by GC-MS, LC-MS, ^{1}H-NMR and HPLC, unchanged tolfenpyrad made up about 10% of the total substrate; identified or tentatively identified

metabolites made up about 88%, with 2% remaining unidentified. The major metabolic pathways of tolfenpyrad in rat liver S9 were attributed to the ω-1 oxidation (hydroxylation and carbonylation) of the ethyl group of the pyrazole ring and the oxidation of the methyl group of the tolyloxybenzyl group (hydroxylation and carbonylation). Other metabolic pathways included cleavage between the amide and methylene moiety, the demethylation of N-CH_3 and the conversion of the ethyl group of the pyrazole ring to a vinyl group (Ogawa, 1998).

2. Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

The results of studies of acute toxicity with tolfenpyrad are summarized in Table 7.

Table 7. Results of studies of acute toxicity with tolfenpyrad

Species	Strain	Sex	Route	Vehicle	Purity (%)	LD_{50} (mg/kg bw) / LC_{50} (mg/L)	Reference
Mouse	Crl:CD-1(ICR)BR	M/F	Oral	Aqueous carboxymethyl cellulose	99.33	114 (M) 117 (F)	Glaza (1997a)[c]
Rat	Crl:CD(SD)BR	M/F	Oral	Aqueous carboxymethyl cellulose	99.33	386 (M) 150 (F)	Glaza (1997b)[b]
Rat	Crj:CD(SD) IGS	M/F	Oral	Aqueous carboxymethyl cellulose	99.83	260 (M) 113 (F)	Ishii (2000a)[c]
Rat	Crj:CD(SD) IGS	M/F	Oral	Olive oil	99.83	86 (M) 75 (F)	Ishii (2000b)[d]
Rat	Crl:CD®(SD)BR	M/F	Dermal	Distilled water	99.33	> 2 000 (M) > 3 000 (F)	Glaza (1997c)[e]
Rat	Crl:CD®(SD)BR	M/F	Inhalation	–	99.8	2.21 mg/L (M) 1.50 mg/L (F)	Wesson (2000)[f]

F: female; LC_{50}: median lethal concentration; LD_{50}: median lethal dose; M: male

[a] Lot no. 6D-01-2. Performed according to Organisation for Economic Co-operation and Development (OECD) Test Guideline 401. The mice were given doses of 25–250 mg/kg bw. Mortality was observed at doses of 100 mg/kg bw and higher. No clinical signs were observed at 25 mg/kg bw. Two males and two females treated at 50 mg/kg bw showed hypoactivity and/or staggered gait. In addition, at higher doses, prostration, clonic convulsions, dyspnoea, thin appearance, hunched posture, hypothermic to touch and tremors were observed.

[b] Lot no. 6D-01-2. Performed according to OECD Test Guideline 401. Males were given doses of 100–750 mg/kg bw, and females were given doses of 50–250 mg/kg bw. Mortality was observed at doses of 75 mg/kg bw and higher. Clinical signs of toxicity were observed at all dose levels and included thin appearance, hypoactivity, staggered gait, hunched posture, rough haircoat, red-stained face, soft stool, few faeces and wet and/or yellow- or dark-stained urogenital area. At dose levels of 150 mg/kg bw and higher, weight losses during the 1st week were noted in the majority of the animals. At necropsy, gastric lesions were observed in three males given 500 mg/kg bw and in one female given 250 mg/kg bw.

[c] Lot no. 9L-03. Performed according to OECD Test Guideline 401. The rats were given doses of 40–640 mg/kg bw. Mortality and clinical signs of toxicity were observed at dose levels of 80 mg/kg bw and higher. Clinical signs included decrease in locomotor activity, diarrhoea, soiled perineal region, emaciation, hunchback position, lateral position, prone position or supine position and irregular respiration or bradypnoea. Decreases in body weights were observed in one male in the 40 mg/kg bw group and in all males and females in the 80 mg/kg bw group and higher on day 4. No abnormalities were observed in necropsy of animals that

survived. In animals that died, necropsy revealed haemorrhage in the glandular stomach, white raised patches in the forestomach, small thymus and small spleen.

[d] Lot no. 9L-03. Performed according to OECD Test Guideline 401. The rats were given doses of 20–320 mg/kg bw. Mortality was observed at doses of 80 mg/kg bw and higher. Diarrhoea and soiled perineal region were observed at all dose levels. At higher doses, decrease in locomotor activity, prone position, hunchback position, lateral position, ataxic gait, irregular respiration or dyspnoea, tonic or clonic convulsion and salivation were observed. Necropsy revealed no abnormalities.

[e] Lot no. 6D-01-2. Performed according to OECD Test Guideline 402. Male rats were given a dose of 2000 mg/kg bw, and female rats were given doses of 1000–3000 mg/kg bw. Two females at 2000 mg/kg bw were killed in moribund condition, one on day 8 and one on day 11. At all three dose levels, clinical signs of toxicity were observed, primarily between days 4 and 10, and included hypoactivity, staggered gait, hunched posture, red-stained face, thin appearance, decreased feed consumption, yellow-stained urogenital area, few faeces, soft stool and hypothermia. Necropsy revealed no visible lesions. No dermal irritation was observed.

[f] Lot no. 9L-03. Performed according to OECD Test Guideline 403. The rats were exposed to concentrations of 0.95–2.07 mg/L. In males, mortality was observed at the highest concentration. In females, mortality was observed at all concentrations. Clinical signs were observed at all concentrations and included increased or decreased respiratory rate, laboured and/or noisy respiration, wet fur, hunched posture, piloerection, pallor of the extremities, ataxia, lethargy, tiptoe gait, ptosis, red/brown staining around the snout or eyes, cyanosis, hypothermia and coma. The mass median aerodynamic diameter was 4.1–4.9 µm.

(b) Dermal irritation

In an acute dermal irritation study, performed in accordance with Organisation for Economic Co-operation and Development (OECD) Test Guideline 404, the intact skin of six female Hra:(NZW)SPF rabbits was exposed for 4 hours under semi-occlusion to 0.5 g tolfenpyrad (purity 99.33%; lot no. 6D-01-2) moistened with 0.5 mL of distilled water. Dermal irritation was scored according to the Draize system at 0.5, 24, 48 and 72 hours after patch removal.

Very slight erythema was observed in two rabbits after patch removal. No other signs of dermal irritation were observed. All signs of irritation had cleared at 72 hours after patch removal. Tolfenpyrad was considered not irritating to rabbit skin (Glaza, 1996a).

(c) Ocular irritation

In an acute eye irritation study, performed according to OECD Test Guideline 405, 0.041 g (0.1 mL weight equivalent) of tolfenpyrad (purity 99.33%; lot no. 6D-01-2) was instilled into the conjunctival sac of the right eye of nine male Hra:(NZW)SPF rabbits. The untreated left eye served as a control. In three rabbits, the treated eyes were washed 30 seconds after instillation; in the remaining six rabbits, the treated eyes were not washed. The eyes were macroscopically examined for signs of irritation according to the Draize system at 1, 24, 48, 72 and 96 hours and at days 7 and 14 post-instillation.

In all six animals with unwashed eyes, mild irritation of the conjunctivae (redness, chemosis) was observed, which cleared in all animals by day 14. In one of these rabbits, iritis was observed 1 hour after instillation. In the three rabbits with treated eyes receiving a washout, the test material produced slight to moderate conjunctival irritation (redness, chemosis), which cleared by 96 hours after treatment. Tolfenpyrad was slightly irritating to the eye of rabbits (Glaza, 1996b).

(d) Dermal sensitization

In a dermal sensitization study using the Magnusson and Kligman maximization test, performed in accordance with OECD Test Guideline 406, tolfenpyrad (purity 99.33%; lot no. 6D-01-2) was tested in 20 female Hartley strain SPF guinea-pigs. A preliminary study established 1% and 5% test substance concentrations in olive oil as suitable for the intradermal induction and topical dermal induction phases, respectively. A 1% topical dermal application was used for the challenge phase. The control group consisted of 10 animals. 2,4-Dinitrochlorobenzene was used as a positive control.

After the dermal challenge treatment on day 22, no signs of irritation were observed in the control and the treated groups 24 and 48 hours after the removal of the occlusive bandage. None of the 20 guinea-pigs in the treated group showed a positive skin response after the challenge procedure. Under the conditions of this study, tolfenpyrad was not a skin sensitizer (Shibata, 1997).

2.2 *Short-term studies of toxicity*

(a) Oral administration

Mice

In a 4-week dietary range-finding study, tolfenpyrad (purity 99.33%; lot no. 6D-01-2) was administered to groups of 10 male and 10 female Crl:CD-1 mice at 0, 30, 100, 300 or 600 parts per million (ppm) (equal to 0, 5.3, 17.5, 51 and 104 mg/kg bw per day for males and 0, 6.5, 21.8, 67 and 126 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. All mice underwent complete necropsy. Liver and gallbladder were weighed.

In mice treated at 600 ppm, one female was found dead and one male and one female were killed in a moribund condition on day 6. At 600 ppm, rough haircoat, hunched posture, ataxia, hypoactivity, animal cold to the touch, few faeces and/or a thin appearance were observed. No mortality or clinical signs were observed at lower doses. At 600 ppm, males and females showed a body weight loss of 10–12%, whereas control mice gained 4–5% during this period. During the remaining 3 weeks of the study, the high-dose animals did gain body weight. However, terminal body weights of both sexes at 600 ppm were statistically significantly lower (16–17%) than those of controls. Total feed consumption in high-dose mice was reduced by 22–27% compared with controls. Body weight gains and feed consumption of the other dose groups were not affected by treatment. Relative liver weights were statistically significantly higher at 300 ppm (12–16%) and 600 ppm (33–35%) than those in controls. Necropsy revealed no toxicologically relevant gross findings (Trutter, 1999a).

Tolfenpyrad (purity 99.33%; lot no. 6D-01-2) was administered via the diet to groups of 10 male and 10 female CD-1 mice for 13 weeks at a dietary level of 0, 15, 100 or 300 ppm (equal to 0, 2.4, 15.9 and 46.2 mg/kg bw per day for males and 0, 3.0, 20.2 and 57.9 mg/kg bw per day for females, respectively). The mice were examined daily for mortality and clinical signs. A detailed clinical examination was performed weekly. Feed consumption and body weights were recorded weekly. Ophthalmological examinations were performed prior to and during week 13. Haematology, clinical chemistry and urine analysis were performed at the end of the treatment period. All mice were necropsied, and weights of brain with brainstem, heart, liver with gallbladder, kidneys, adrenals and testes with epididymides were recorded. A wide range of tissues of mice of the control and 300 ppm groups and the lung, liver, kidneys and gross lesions from mice of the 15 and 100 ppm groups were examined microscopically.

No mortalities or treatment-related clinical signs were observed. At the end of the study, there was a 7% reduction in body weight gain in males of the 300 ppm group, which was not statistically significant and probably the result of an 8% lower feed consumption. No toxicologically significant effects were observed in ophthalmology or haematology. Aspartate aminotransferase (ASAT) activity in the 300 ppm male mice was statistically significantly increased (86%). In males at 300 ppm, an increase in relative heart weight (16%) was found. Relative liver weight was increased (10–18%) in both sexes at 300 ppm. Necropsy and histological examination showed no treatment-related changes.

The NOAEL was 100 ppm (equal to 15.9 mg/kg bw per day), based on elevated ASAT activity and increased relative heart weight in males and increased relative liver weight in both sexes at 300 ppm (equal to 46.2 mg/kg bw per day) (Trutter, 1999b).

Rats

In a 13-week dietary toxicity study, tolfenpyrad (purity 99.33%; lot no. 6D-01-2) was administered to groups of 10 male and 10 female F344/DuCrj rats at 0, 15, 80 or 160 ppm (equal to 0, 0.906, 4.78 and 9.33 mg/kg bw per day for males and 0, 1.01, 5.17 and 9.32 mg/kg bw per day for females, respectively). In addition, six male and six female F344/DuCrj rats were fed tolfenpyrad at a dietary concentration of 0 or 160 ppm followed by a 4-week recovery period. Animals were checked daily for clinical signs of toxicity. Body weights and feed consumption were measured weekly. Ophthalmological examinations were carried out on all rats before dosing and in control and 160 ppm rats at week 13. Blood was sampled for haematology and clinical biochemistry at termination. Urine was sampled during week 13 for analysis. All rats were necropsied, and a wide range of tissues of rats of the control and 160 ppm groups and the lung, liver and kidneys from rats of the 15 and 80 ppm groups were examined microscopically.

No deaths, clinical signs or ophthalomological changes were observed. Body weight gain was slightly reduced (6–7%) at 80 ppm and markedly reduced (22–24%) at 160 ppm. At 160 ppm, feed consumption was reduced by 12–16%. During the recovery period, feed consumption at 160 ppm was similar to that of controls, whereas body weights of the 160 ppm rats were 11–16% lower. Low white blood cell counts were observed at 80 ppm (−29%) and 160 ppm (−27%) in females. A lower result in males of the 160 ppm group of 14.4% was not statistically significant. After the 4-week recovery period, there was no reduction in white blood cell count in females of the 160 ppm group (+1.9%), whereas in males, the reduction of 14.9%, although essentially unchanged during this period, was statistically significant. Blood chemistry analysis revealed high glucose levels (+31% and +66%, respectively) and a slight reduction in total protein (−8% and −12%, respectively) in females treated at 80 and 160 ppm. In females at 160 ppm, an increase in gamma-glutamyltransferase (GGT, 0 International Units [IU]/L in controls, 0.3 IU/L at 160 ppm) was found. Blood urea nitrogen levels were increased in males at 80 ppm (25%) and in males and females at 160 ppm (39% and 13%, respectively). A reduced triglyceride level (48%) was found in males treated at 160 ppm. Triglyceride levels were slightly reduced (15%) in females at 160 ppm. Furthermore, increased inorganic phosphorus levels (14–19%) were observed in both sexes at 160 ppm, and increased potassium levels were observed in females treated at 80 ppm (26%) and in males and females treated at 160 ppm (27–29%). At necropsy, dark brown change of the liver and brown change of the Harderian gland were observed in males treated at 160 ppm and in females treated at 80 ppm and above. Furthermore, small seminal vesicles and prostate were observed in males treated at 160 ppm, and small ovaries, uteri and vaginas were seen in females treated at 160 ppm. Reduced absolute (−38%) and relative ovary weights (−20% compared with body weight, −35% compared with brain weight) were found in females treated at 160 ppm. At 80 and 160 ppm, compared with body weight, increased relative liver weights (14–15% and 24–25%, respectively) and kidney weights (9–10% and 18–24%, respectively) were observed in both sexes. Compared with brain weight, no changes in liver or kidney weights were observed. Slight, but statistically significant, increases in relative liver weight in males (5%) and relative kidney weight in females (5%) observed at 15 ppm were considered not toxicologically relevant. Histopathological findings included an increase in mast cells in the mesenteric lymph nodes, diffuse hypertrophy of the hepatocytes, hypertrophy of the pancreatic acinar cells and hypersecretion of the Harderian glands in both sexes at doses of 80 ppm and above. Hyaline droplets were observed in the proximal renal tubular epithelium of males treated at 160 ppm; a slightly increased incidence observed in males at 80 ppm was not considered toxicologically relevant. Hypertrophy of the proximal renal tubular epithelium was observed in females treated at 80 ppm and above. Hypertrophy of the acinar cells in the mandibular glands was observed in males treated at 160 ppm and in females treated at 80 ppm and above. Furthermore, a decrease in haematopoietic cells was observed in the marrow of the femur and sternum, as well as atrophy of the ovaries and uteri in females treated at 160 ppm. The observed effects on urea nitrogen, total protein, inorganic phosphorus, potassium and relative liver and kidney weights in females and changes in triglyceride levels and the mesenteric lymph nodes in males and females were still present at the end of the recovery period. The other observed changes partly or completely recovered after discontinuation of treatment.

The NOAEL was 15 ppm (equal to 0.906 mg/kg bw per day), based on increased blood urea nitrogen levels, a reduced white blood cell count, high blood glucose and potassium levels, dark brown change of the liver and brown change of the Harderian gland, hypertrophy of the proximal renal tubular epithelium, hypertrophy of the acinar cells in the mandibular glands in females and increase in mast cells in the mesenteric lymph nodes, diffuse hypertrophy of hepatocytes, hypertrophy of pancreatic acinar cells and hypersecretion of the Harderian glands in both sexes at 80 ppm (equal to 4.78 mg/kg bw per day) (Chida, 1999a).

Dogs

In a 28-day oral (capsule) toxicity study, tolfenpyrad (purity 99.33%; lot no. 6D-01-2) was administered to groups of two male and two female Beagle dogs at 0, 1, 5 or 10 mg/kg bw per day. The dogs were checked daily for clinical signs of toxicity. Feed consumption was measured daily. Body weights were measured weekly. Blood and urine were sampled for haematology, clinical biochemistry and urine analysis 2 weeks before and 2 and 4 weeks after the start of treatment. All animals were necropsied, selected organs were weighed and a wide range of tissues was examined microscopically.

No mortality or morbidity occurred. Vomiting was repeatedly observed in two males and one female treated at 5 mg/kg bw per day and one male and two females treated at 10 mg/kg bw per day, but only once in one control male. Vomiting was already observed on the 1st day of dosing in these dogs, except for the control male and one female at 10 mg/kg bw per day. Increased incidences of mucoid, soft and watery faeces were observed in males and females at 10 mg/kg bw per day. One high-dose male displayed a decrease in body weight of 2.1 kg at the end of the treatment period. This dog showed a reduced feed consumption from week 3 onward, and at week 4, blood urea nitrogen and creatinine and protein levels in urine were increased. Histopathological examination of this dog revealed an increase in cytoplasmic eosinophilia in the hepatocytes, swelling of distal tubular epithelium and vacuolation in collecting tubular epithelium of the kidney. In the other animals, no changes in haematological, clinical chemistry and urine analysis parameters or gross pathology and histopathology were observed.

The NOAEL was 1 mg/kg bw per day, based on the increased incidence of vomiting at 5 mg/kg bw per day in both sexes (Nagashima, 1997a).

In a 90-day toxicity study, four male and four female Beagle dogs per dose group received capsules containing tolfenpyrad (purity 99.33%; lot no. 6D-01-2) at 0, 1, 5 or 10 mg/kg bw per day. Animals were checked daily for clinical signs. Feed consumption and body weights were measured weekly. Ophthalmological examinations were performed pretreatment and in weeks 6 and 12. Haematology, clinical chemistry and urine analysis were performed pretest and in weeks 7 and 13. All dogs were necropsied, and weights of brain, thyroid glands, heart, lungs, liver, adrenals, kidneys, spleen, testes and ovaries were recorded. Histology was performed on a large selection of organs from all dogs.

The incidence of vomiting was increased at 5 and 10 mg/kg bw per day. Soft or mucoid faeces were observed in one male treated at 5 mg/kg bw per day and one female treated at 10 mg/kg bw per day. These signs were already observed on the 1st or 2nd day of treatment. A decrease in urine volume was recorded in females of the 5 and 10 mg/kg bw per day groups in weeks 7 and 13. However, the urine volumes were within the range of background variation and not accompanied by histological or other changes. In addition, no changes in urine volume in males were observed. Therefore, the reduction in urine volume in females was not considered to be toxicologically significant. There were no treatment-related effects on body weight gain, feed consumption, haematological, biochemical, ophthalmological or pathological examinations or organ weights.

The NOAEL was 1 mg/kg bw per day, based on the increased incidence of vomiting at 5 mg/kg bw per day in both sexes (Nagashima, 1997b).

In a 90-day toxicity study, four male and four female Beagle dogs per dose group received capsules containing tolfenpyrad (purity 99.33%; lot no. 6D-01-2) at 0, 10, 30 or 100 mg/kg bw per day. Animals were checked daily for clinical signs. Feed consumption and body weights were measured weekly. Ophthalmological examinations were performed pretreatment and in weeks 6 and 12. Haematology and clinical chemistry were performed pretest and in weeks 4, 7 and 13. Urine analysis was performed pretest and in weeks 7 and 13. All dogs were necropsied, and weights of brain, thyroid glands, heart, lungs, liver, adrenals, kidneys, spleen, testes and ovaries were recorded. Histology was performed on a large selection of organs from all dogs.

Dose-dependent increased incidences of vomiting, soft faeces and mucoid faeces were observed in all treatment groups. One male at 30 mg/kg bw per day and all the dogs in the 100 mg/kg bw per day group died or were killed in extremis. In dogs that died, clinical signs included no faeces, emaciation, a decrease in spontaneous movement, staggering gait, hypothermia, prone position, lateral position and mydriasis. The dogs that were killed in extremis showed decreases in body weight and feed consumption as well as emaciation. In the surviving male dogs at 30 mg/kg bw per day, one dog showed a reduced body weight at the end of the study, but no other dogs at 30 or 10 mg/kg bw per day showed any significant changes in body weight gain. The feed consumption in the dogs reflected the body weight gains. No ophthalmological changes were observed. At 100 mg/kg bw per day, increases in erythrocyte count, haemoglobin levels and haematocrit were observed in one male and one female, and a decrease in leukocytes and an increase in segmented neutrophils were observed in one high-dose male dog. Clinical chemistry showed an increase in alanine aminotransferase (ALAT) in one male in the 30 mg/kg bw per day group and in one female in the 100 mg/kg bw per day group. Blood urea nitrogen was high in males in the 30 mg/kg bw per day group and in both sexes in the 100 mg/kg bw per day group. In the animals that were sacrificed in extremis in the 100 mg/kg bw per day group, an increase in creatinine was observed. Observed decreases in blood cholesterol, triglycerides and phospholipids were attributed to malnutrition.

Microscopic examinations revealed a dose-dependent increase in cytoplasmic eosinophilia and centrilobular vacuolation in hepatocytes at 30 mg/kg bw per day and above. These changes were considered to be treatment related. In the cerebrum and cerebellum, focal haemorrhage with degeneration was observed in one male and one female in the 100 mg/kg bw per day group that died or was killed in extremis. There were no reactive changes in the area of degeneration. Therefore, it was assumed that the lesions were relatively new and similar to focal changes induced by cardiovascular injury. Other changes observed in the 30 and 100 mg/kg bw per day groups included atrophy of seminiferous tubules in the testis, a decrease in sperm numbers and the presence of cell debris in the epididymis, atrophy of the prostate and ileal, mesenteric and submandibular lymph nodes, hypocellularity in the bone marrow (sternum, femur) and atrophy of glandular cells in the parotid and sublingual glands.

A NOAEL could not be established. Mild toxicity (i.e. vomiting, soft and mucoid faeces) was observed at 10 mg/kg bw per day. Severe toxicity, including mortality, was observed at doses of 30 mg/kg bw per day and above (Nagashima, 1999a).

In a 1-year toxicity study, four male and four female Beagle dogs per dose group received capsules containing tolfenpyrad (purity 99.3%; lot no. 6D-01-2) at 0, 1, 5 or 20/10 mg/kg bw per day. The initial top dose of 20 mg/kg bw per day was reduced to 10 mg/kg bw per day at week 5 due to a death on day 26 and decreased feed consumption or body weight loss in the other dogs of this dose group. Animals were examined daily for clinical signs of toxicity. Feed consumption was measured weekly. Body weights were recorded weekly for the first 14 weeks and once every 2 weeks thereafter. Ophthalmological examinations were performed pretreatment and in months 6 and 12. Haematology, clinical chemistry and urine analysis were performed pretest and in months 3, 6, 9 and 12. All dogs were necropsied, and weights of brain, pituitary, thyroid glands, heart, lungs, liver, adrenals, kidneys, spleen, testes, prostate, uterus and ovaries were recorded. Histology was performed on a large selection of organs from all dogs.

One female in the 20 mg/kg bw per day group died on day 26, and one male in the 20/10 mg/kg bw per day group died on day 83. These dogs exhibited body weight loss, low feed intake, salivation, no defecation, decrease in spontaneous movement, emaciation, hypothermia, lateral position, staggering gait and paleness of the mucosa (oral and conjunctival mucosa) prior to death. Dose-dependent increased incidences of vomiting, soft or mucoid stool and salivation were observed at 5 mg/kg bw per day and above. These signs were already observed after the 1st or 2nd day of treatment. Test article–like material was identified in the vomitus of some individuals. Body weight of one male treated with 20/10 mg/kg bw per day decreased from week 38 up to week 42 and was unchanged thereafter. In one female treated with 20/10 mg/kg bw per day, body weight decreased from week 4 up to week 7 and repeatedly increased and decreased from week 24. In another female of the same group, body weight exhibited repeated increases and decreases from week 24. Low feed intake values were recorded simultaneously with variations in body weight. Blood chemistry examination revealed an elevation of ALAT activity in one male treated at 5 mg/kg bw per day in months 9 (850%) and 12 (240%) and in one male treated with 20/10 mg/kg bw per day in month 12 (340%). Microscopic examination revealed increases of cytoplasmic eosinophilia in hepatocytes of two males and three females treated with 20/10 mg/kg bw per day. An increase in pigmentation in hepatocytes and Kupffer cells was observed in females treated at 20/10 mg/kg bw per day. There were no treatment-related effects observed in urine analysis, haematology, ophthalmology, gross pathology or organ weight.

The NOAEL was 1 mg/kg bw per day, based on increased incidences of vomiting, soft or mucoid stool and salivation and increased ALAT levels at 5 mg/kg bw per day (Nagashima, 1999b).

(b) Dermal application

Groups of 10 male and 10 female Sprague-Dawley rats were dermally exposed 6 hours/day for 21 days to tolfenpyrad (purity 99.5%; lot no. 365-65A) at a dose of 0, 10, 50 or 200 mg/kg bw per day. The rats were checked daily for clinical signs. A detailed examination was performed on days 8, 15 and 22. Body weights and feed consumption were recorded weekly. Ophthalmoscopy was performed pretreatment and at termination. 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, adrenals, kidneys, testes, epididymides, ovaries, thymus and uterus were recorded. Histology was performed on a wide range of organs and tissues of the control and high-dose rats.

Minimal skin flaking was observed in all treatment groups, and slight erythema was observed at 50 and 200 mg/kg bw per day. No other treatment-related clinical signs were noted. Body weight gain was slightly (−7%), but statistically significantly, reduced in females at 50 and 200 mg/kg bw per day. Body weight gain in males was not affected. No effect on feed consumption or ophthalmological examination was observed. Slight reductions in total white blood cell count were observed in males at 50 (−20%) and 200 mg/kg bw per day (−21%) and in females at 200 mg/kg bw per day (−31%). However, the white blood cell counts were at the low end of the historical control range and were considered not toxicologically relevant. Occasional other changes in haematology and clinical chemistry in the treatment groups were not dose related and/or were within the range of normal variation and were considered not to be treatment related. Macroscopic, histopathological and organ weight examinations showed no toxicologically significant effects of tolfenpyrad treatment.

The NOAEL for systemic effects was 200 mg/kg bw per day, the highest dose tested (Barnett, 2008a).

(c) Exposure by inhalation

Groups of 10 male and 10 female Crl:CD(SD) rats were exposed nose only to dust particulate aerosol atmospheres of tolfenpyrad (purity 99.5%; lot no. 61202) at an actual concentration of 0, 0.5, 2.0 or 10 mg/m^3 air (mass median aerodynamic diameter of 2.8–3.2 µm) 6 hours/day, 5 days/week, over a 4-week period (a total of 20 exposures). The control animals received air only. The behaviour and the general health and condition of the animals were observed daily. Body weights and feed consumption were recorded weekly. Ophthalmoscopy examination was performed pretreatment and

before termination. Haematology, clinical chemistry and urine analysis were performed at the end of the study. One day after the last exposure, the rats were killed and necropsied. Weights of liver, kidneys, heart, lungs, spleen, thymus, adrenals, brain, testes, epididymides, ovaries, oviducts, uterus and cervix were recorded. Histology was performed on a large selection of organs and tissues of the control and high-dose rats and on liver and kidneys of the low- and mid-dose rats.

No treatment-related mortality or clinical signs were observed. Body weights and feed consumption were not affected. Haematology showed a reduction (76% of controls) in white blood cell count in female rats at 10 mg/m^3. Although the decrease was statistically significant, it was within the range of concurrent historical controls. A statistically significant decrease in ALAT levels (74% of control value) was considered not toxicologically relevant. No other effects on haematology, clinical chemistry or urine analysis were observed. Increases in absolute (17%) and relative liver weights (15%) were observed in female rats at 10 mg/m^3. No macroscopic changes were observed. Histological examination revealed hepatocellular hypertrophy in some male and female rats at 10 mg/m^3. In the absence of histopathological damage and relevant blood chemistry changes, the observed hepatocellular hypertrophy is not considered to be adverse. A slight increase in hyaline droplets within the epithelium of the proximal convoluted tubule of the kidneys, observed in two male rats at 10 mg/m^3, was not considered toxicologically relevant.

The no-observed-adverse-effect concentration (NOAEC) was 10 mg/m^3, the highest concentration tested (Kelly, 2008).

2.3 Long-term studies of toxicity and carcinogenicity

Mice

In a 78-week dietary carcinogenicity study, tolfenpyrad (purity 99.33%; lot no. 6D-01-2) was administered to groups of 50 male and 50 female CD-1 mice at 0, 15, 150 or 500 ppm (500 ppm was reduced to 400 ppm at week 13 and from 400 ppm to 300 ppm at week 20 due to suppression in body weight gain, decreased feed consumption and severe clinical signs). Over the entire test period, these doses were equal to 0, 2.2, 20.8 and 60.9 mg/kg bw per day for males and 0, 2.8, 27.1 and 75.9 mg/kg bw per day for females, respectively. The mice were checked daily for mortality and clinical signs. A detailed clinical examination and palpation for nodules and masses were performed weekly. Feed consumption and body weight were recorded weekly during weeks 1–13 and weeks 20–25, at week 17 or 18 and every 4th week from week 25 onward. Blood samples collected at weeks 52 and 79 were examined for erythrocyte, leukocyte, platelet and differential leukocyte counts. Cell morphology was investigated in all mice at week 52 and in control and high-dose mice at week 79. In addition, blood smears of moribund mice were evaluated. All animals were necropsied, and in 10 mice of each sex per dose, the weights of brain, lung, liver, kidneys, adrenals, spleen, testes with epididymides and ovaries were recorded. A wide range of tissues was examined microscopically from all control and high-dose animals and dead or moribund animals during the treatment period in the 15 and 150 ppm groups. In addition, lung, liver, kidney and gross lesions of all mice in the 15 and 150 ppm groups were examined histologically.

After 4 weeks of treatment, survival in males and females at 500 ppm was 90% compared with 100% in control males and females. No further effects on survival were observed. Hunched and/or thin appearance, hypoactivity, few faeces and pale bodies were observed in the high-dose animals during weeks 0–19. A statistically significant decrease in body weight was observed in males at 150 ppm (−7%) and in high-dose males (−17%) and females (−16%) at termination. The reduction of the dietary concentration to 300 ppm resulted in a significant increase in mean body weight gain during weeks 20–23. Feed consumption was statistically significantly reduced in both sexes at 150 ppm (8–9%) and at the high dose (14–16%). No treatment-related effects on haematology were observed.

At the high dose, a statistically significant reduction in absolute brain weight was observed in females (−13%). Relative brain weight was statistically significantly increased in high-dose males

(+17%) and females (+11%). Statistically significant reductions in absolute (50–64% of control) and relative spleen weights (60–70% of control) were observed in mid- and high-dose males. In high-dose females, absolute spleen weight was significantly reduced (62% of control). Absolute testes/epididymides weight was reduced in high-dose males (80% of control). Relative liver weights were significantly increased in high-dose males (124% of control) and females (120% of control). Macroscopic examination showed atrophy of ovaries (absolute weight 18% of control, relative weight 23% of control), uterus and cervix, and a decreased incidence/severity of cystic endometrial hyperplasia was observed in the high-dose females. No other treatment-related histological changes were reported. Treatment with tolfenpyrad did not result in significantly increased incidences of neoplastic lesions.

The NOAEL was 15 ppm (equal to 2.2 mg/kg bw per day), based on decreased body weight gain and feed consumption and changes in organ weights observed in males and females at 150 ppm (equal to 20.8 mg/kg bw per day). Tolfenpyrad was not carcinogenic in CD-1 mice (Ivett, 1999).

Rats

In a 2-year dietary carcinogenicity study, tolfenpyrad (purity 99.33%; lot no. 6D-01-2) was administered to groups of 60 male and 60 female Fischer F344/DuCrj (SPF) rats at 0, 15, 40 or 80 ppm (equal to 0, 0.56, 1.5 and 3.1 mg/kg bw per day for males and 0, 0.69, 1.9 and 3.8 mg/kg bw per day for females, respectively). Ten rats of each sex per group were designated for interim sacrifice after 52 weeks of treatment. The rats were checked daily for mortality and clinical signs. A detailed clinical examination and palpation for nodules and masses were performed weekly. Feed consumption and body weight were recorded weekly during months 1–3 and once every 3 or 4 weeks thereafter. Ophthalmoscopy was performed pretest and at scheduled necropsy at week 105. Haematology, clinical chemistry and urine analysis were performed in 10–14 rats of each sex per dose at weeks 14, 27 and 79 and in all animals at scheduled necropsy at weeks 53 and 105. At termination at 52 or 105 weeks of treatment, all animals were necropsied, and weights of brain, lung, heart, liver, kidneys, adrenal gland, spleen, testes and ovaries were recorded. Histological examinations were performed on a wide range of organs and tissues of all rats in the control and 80 ppm groups and of dead or moribund animals during the treatment period in the 15 and 40 ppm groups. In addition, lung, liver, kidney, mesenteric lymph nodes and Harderian glands of all rats of the 15 and 40 ppm groups were examined histologically.

There were no effects of treatment on mortality or clinical signs. At 80 ppm, body weight gain was statistically significantly reduced in males (−7%) and females (−13%). In males at 80 ppm, feed consumption was statistically significantly reduced (7–12%) during the first 5 weeks of treatment. After that, slight reductions in feed consumption (about 5%) were observed in these rats. In males at 40 ppm, slight reductions in feed consumption (3–5%) were observed during the first 5 weeks of treatment. In females at 80 ppm, feed consumption was consistently reduced (5–15%) throughout the treatment period. Statistically significant reductions in feed consumption (up to 9%) were also consistently observed in females at 40 ppm. Occasionally, reductions in reticulocyte counts (6–9%) and blood platelet counts (9%) and prolonged activated partial thromboplastin time (7%) were observed in males at 80 ppm, and reduced white blood cell counts were observed in females at 40 ppm (28%) and in males (14%) and females (29%) at 80 ppm. These changes were relatively small, were within the historical control range, were not consistently found and were therefore not considered to be toxicologically significant. Furthermore, if evaluations are made on the serial time measurements of white blood cell counts, it is clear that in males and females of all dose levels, including controls, the counts were consistently lower in week 53 than in week 79 (in particular) and week 105. This suggests that involvement of factors other than dietary tolfenpyrad may have been responsible for reductions, particularly after about 1 year. Clinical chemistry revealed occasional changes in triglyceride, potassium and magnesium levels. These changes were generally within the historical control range and are not considered toxicologically relevant. At 80 ppm, at the interim and terminal kills, slight to moderate increases in relative liver weight (7–8% in males, 9–16% in females) and relative kidney weight (10–11% in males, 10–18% in females) were observed. Relevant data from the necropsy and histological examination are presented in Table 8. Necropsy at 53 weeks

Table 8. Macroscopic and histological effects of chronic treatment of rats with tolfenpyrad.

	Incidence of finding (n = 60)							
	Males				Females			
	0 ppm	15 ppm	40 ppm	80 ppm	0 ppm	15 ppm	40 ppm	80 ppm
Necropsy (week 105)								
Liver: White patched	2	1	1	6	7	8	9	20**
Kidney: Dark brown change	8	6	12	30**	3	7	7	37**
Harderian gland: Brown change	0	2	6*	29**	2	1	7	24**
Histopathology (week 53)								
Liver: Basophilic focus of altered hepatocytes	0	0	0	0	1	2	2	8**
Mesenteric lymph nodes: Mast cells	0	–	–	2	0	–	–	0
Kidney: Hypertrophy of the proximal tubular epithelial cells	0	0	0	0	0	0	0	0
Kidney: Hyaline droplets in the proximal tubule	0	1	4*	9**	0	0	0	0
Harderian glands: Hypersecretion	4	–	–	6	1	–	–	2
Histopathology (week 105)								
Liver: Basophilic focus of altered hepatocytes								
- Slight	36	38	37	42	32	26	22**	15**
- Moderate	5	0	7	2	11	15	22	25
- Severe	0	0	1	0	0	1	2	8
Mesenteric lymph nodes: Mast cells	0	0	0	8**	2	6	7	4
Mesenteric lymph nodes: Sinus histiocytosis	2	1	7	19**	3	1	17**	33**
Kidney: Hypertrophy of the proximal tubular epithelial cells	0	0	0	18**	0	0	4*	19**
Kidney: Hyaline droplets in the proximal tubule	0	1	0	0	1	2	0	0
Harderian glands: Hypersecretion	6	0*	10	30**	0	2	2	6*

*: $P < 0.05$; **: $P < 0.01$ (chi-squared test)
Source: Chida (1999b)

revealed no treatment-related changes. At necropsy at 105 weeks, an increase in the incidence of white patch on the liver in females treated at 80 ppm, an increase in dark brown changes to the kidney in both sexes treated at 80 ppm and an increase in brown changes to the Harderian gland in males at 40 and 80 ppm and in females at 80 ppm were observed. Histological examination revealed an increase in mast cells in the mesenteric lymph nodes in males treated at 80 ppm, an increase in sinus histiocytosis in the mesenteric lymph nodes in males treated at 80 ppm and in females at 40 and 80 ppm, hypertrophy of the renal proximal tubular epithelial cells in males treated at 80 ppm and in females treated at 40 and 80 ppm and an increase in hypersecretion of the Harderian glands in both sexes treated at 80 ppm. In addition, there was an increase in severity of basophilic foci of altered

hepatocytes of the liver in females at doses of 40 and 80 ppm. There was also an increase in hyaline droplets in the renal proximal tubular epithelial cells in males at doses of 40 and 80 ppm at the interim kill, but not at the end of the study. Treatment with tolfenpyrad did not result in significantly increased incidences of neoplastic lesions in any of the organs and tissues examined.

The NOAEL was 15 ppm (equal to 0.56 mg/kg bw per day), based on reduced feed intake in males and females and increased severity of basophilic foci of altered hepatocytes, sinus histiocytosis of the mesenteric lymph nodes and hypertrophy of the proximal renal tubular epithelia in females at 40 ppm (equal to 1.5 mg/kg bw per day). Tolfenpyrad was not carcinogenic in Fischer F344/DuCrj (SPF) rats (Chida, 1999b).

2.4 Genotoxicity

Tolfenpyrad was tested for genotoxicity in a range of guideline-compliant assays. Tolfenpyrad did not induce any significant responses in mutagenicity tests in bacteria and mouse lymphoma cells in vitro or in a chromosomal aberration test in vivo. In two studies, polyploidy was observed. In an in vitro chromosomal aberration study with Chinese hamster lung (CHL) cells, two experiments were performed. In one experiment, cell cultures were treated with tolfenpyrad for 24 or 48 hours in the absence of S9 mix, resulting in a marked increase in the frequency of cells exhibiting numerical aberrations (predominantly polyploidy), which exceeded the current historical vehicle control range. Such chromosomal aberrations were also noted at the low dose in the other experiment (in one replicate only). The study author noted that the significance of polyploidy in vitro was not completely understood at the time of this experiment. Furthermore, inconsistency of the effect, even within an experiment in this study, does not permit a reasoned interpretation. In all other cultures in the study, the frequencies of numerical aberrations fell within the contemporary historical vehicle control ranges (Riley, 1997).

Subsequently, a study was performed by Murli (2007) to assess the ability of tolfenpyrad to affect the cell cycle kinetics in cultured CHL cells in the absence of S9, which might explain the polyploidy of chromosomes observed in the Riley (1997) CHL chromosomal aberration study. Tolfenpyrad at a concentration of 5.00, 8.50 or 13.0 µg/mL induced severe cell cycle delay in CHL cells in the absence of S9 at all dose levels when cells were treated for 3, 6 and 24 hours, washed and the culture continued in the presence of 5-bromo-2′-deoxyuridine for 24 hours (anticipated to be 1.5 cell cycles), when they were harvested and assessed. Dose-related increases in polyploidy were observed following 6 and 24 hours of treatment. There was also a correlation between the number of cells delayed in M1 phase (a single cell cycle when both chromatids stain darkly) and M1+ phase and the induction of polyploidy and endoreduplicated chromosomes. Cells that had progressed through two cell cycles (M2) had chromatids clearly differentiated between light and dark throughout their length. In M1+ cells, only parts of the chromosomes were differentially stained. It is concluded that the increased polyploidy and endoreduplication observed in the CHL cell chromosomal aberration study of Riley (1997) was a result of a severe cell cycle delay caused by tolfenpyrad. Endoreduplication is normal in many cell types and may involve nothing more than a loss of M-phase cyclin-dependent kinase activity and oscillations in the activity of S-phase cyclin-dependent kinase, resulting in switching from mitotic cell cycles to endocycles (Lee, Davidson & Duronio, 2009; Zielke, Edgar & DePamphilis, 2013). However, normal, programmed endoreduplication should probably be considered as different from this chemically induced change. Consequences may be a general increase in gene expression, but in the absence of any in vivo evidence for a similar response, this in vitro result appears to have little significance.

It is concluded that tolfenpyrad is unlikely to be genotoxic or mutagenic. The results of the genotoxicity tests are summarized in Table 9.

***Table 9. Overview of genotoxicity tests with tolfenpyrad*[a]**

End-point	Test object	Concentration	Purity (%)	Results	Reference
In vitro					
Gene mutation	*Salmonella typhimurium* strains TA98, TA100, TA102, TA1535, TA1537; *Escherichia coli* WP2 *uvrA*	62.5–1 000 µg/plate (±S9)	99.33	Negative	Ballantyne (1997)[b,c]
Gene mutation	*S. typhimurium* strains TA98, TA100, TA102, TA1535, TA1537; *E. coli* WP2 *uvrA*	156–5 000 µg/plate; precipitation at all doses; 20 min preincubation (±S9)	99.33	Negative	Ozaki (2000)[b,d]
Structural chromosomal aberrations	CHL cells	8.6–32.8 µg/mL (−S9) 42–85.8 µg/mL (+S9)	99.33	Negative	Riley (1997)[e]
Cell cycle kinetics aberrations	CHL cells	5–15 µg/mL (−S9)	99.5	Polyploidy induction	Murli (2007)[f]
Gene mutation	Mouse lymphoma L5178Y *TK* locus	0.005–75 µg/mL (−S9), 0.01–25 µg/mL (+S9)	99.5	Negative	Cifone (2007)[g]
In vivo					
Micronucleus formation	CD-1 mouse bone marrow	Two gavage doses of 5, 10 or 20 mg/kg bw, separated by 24 h	99.5	Negative	Yong (2007)[h]

CHL: Chinese hamster lung; S9: 9000 × *g* supernatant fraction of rat liver homogenate; TK: thymidine kinase

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

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

[c] Batch 6D-01-2. Performed in accordance with OECD Test Guideline 471. In a range-finding assay (8–5000 µg/plate), no cytotoxicity was observed, but precipitation was observed at and above 1000 µg/plate. In the main test (62.5–1000 µg/plate), the assays in the presence of S9 were performed with a 20-minute preincubation step. In the presence of S9, cytotoxicity was observed at 500 and 1000 µg/plate in strains TA102 and TA1535. Treatment of strain TA102 with 2-aminoanthracene in the presence of S9 failed to provide any increase in revertant numbers. This strain is not very sensitive to this treatment, and 2-aminoanthracene is not a diagnostic agent with which the strain can be identified. The latter function is more closely provided by glutaraldehyde in the absence of S9. However, this did not result in a significant response either; therefore, the use of TA102 in this experiment failed to give valid results. The data showing a lack of mutagenic response with TA102 in the Ozaki (2000) study, however, are valid.

[d] Batch 6D-01-2. Performed in accordance with OECD Test Guideline 471. In a range-finding assay (0.305–5000 µg/plate), no cytotoxicity was observed, but precipitation was observed at and above 19.5 µg/plate. In the main test (156–5000 µg/plate), the assays in the presence of S9 were performed with a 20-minute preincubation step. At all test concentrations, deposition of crystals was observed.

[e] Batch 6D-01-2. Performed in accordance with OECD Test Guideline 473. Two experiments were carried out. Precipitation was observed at concentrations of 85.8 µg/mL and higher. In the absence of S9, 32% cell survival was observed at 13.4 µg/mL. In the presence of S9, 35% cell survival was observed at 80 µg/mL.

[f] Batch 365-65A.

[g] Batch 365-65A. Performed in accordance with OECD Test Guideline 476.

[h] Batch 365-65A. Performed in accordance with OECD Test Guideline 474. Mortality was observed in 6/8 animals at 50 mg/kg bw per day and in 1/5 animals at 20 mg/kg bw per day after the first treatment. After the second treatment, 1/4 animals in the 20 mg/kg bw per day dose group died. The two surviving animals from the 50 mg/kg bw per day dose group were used as replacement animals and were dosed on the 2nd day with tolfenpyrad at 20 mg/kg bw per day. At 20 mg/kg bw per day, hypoactivity, hunched posture, sternal recumbancy, laboured respiration, ataxia and/or irregular respiration were observed. At 20 mg/kg bw per day, a slightly lower polychromatic erythrocyte : normochromatic erythrocyte ratio was observed.

2.5 *Reproductive and developmental toxicity*

(a) *Multigeneration studies*

In a two-generation dietary reproduction study performed according to OECD Test Guideline 416, SD (Crj:CD, SPF) rats (30 of each sex per group for the F_0 generation, 22–24 of each sex per group for the F_1 generation) were fed tolfenpyrad (purity 99.33%; lot no. 6D-01-2). Dietary concentrations were adjusted to maintain the desired dose levels of 0, 0.75, 1.5 and 3 mg/kg bw per day. The mean test substance intake for each treatment group during the premating, gestation and nursing periods was within ±20% of the target values. Parental (F_0) rats were exposed from 6 weeks of age until termination, and F_1 rats were exposed from postnatal week 3 until termination. Clinical examination was performed daily, and body weight and feed consumption were recorded weekly (males, females during premating period) and on gestation days (GDs) 0, 7, 14 and 20 and postnatal days (PNDs) 0, 4, 7, 14, 17 and 21 (females). Rats were mated after 10 weeks of treatment. From GD 21 until completion of delivery, each female was observed for parturition on two occasions per day. Pups were weighed on PNDs 0, 4, 7, 14 and 21. On PND 4, litters were culled to four pups of each sex per litter, and at PND 21, one male and one female were selected per litter from 22–24 litters per dose group. All litters were examined (for number of pups, sex of pups, number of stillbirths, number of live births and gross anomalies). In all F_1 and F_2 pups, the days of pinna unfolding (before culling) and eye opening (after culling) were recorded. In all F_1 pups, the days of cleavage of balanopreputial gland (males) and vaginal opening (females) were recorded. All F_1 animals were examined for the surface righting reflex, midair righting reflex and pupillary reflex. At termination, necropsy was performed on F_0 and F_1 parental rats, and weights of brain, pituitary, thymus, liver, kidney, adrenal gland, spleen, testis, epididymis (whole and caudal), seminal vesicle (including coagulating gland and content), prostate (ventral lobe), ovary and uterus (including cervical region) of all animals were recorded. Histological examination was performed on pituitary, testis, epididymis, seminal vesicle, coagulating gland, prostate (ventral lobe), ovary, fallopian tube, uterus (including cervical region) and vagina of the control and 3 mg/kg bw per day groups and any gross lesions. Pups not selected for breeding were necropsied at PND 21. As treatment-related changes in spleen and thymus weights were observed in these pups, these tissues of the control and 3 mg/kg bw per day groups were examined histologically.

At 3 mg/kg bw per day, three F_0 females died or were killed moribund due to dystocia (difficult parturition). Body weight gain was reduced in high-dose F_0 and F_1 females during gestation (up to 13%) and lactation (up to 17%). Small, but statistically significant, reductions in body weight gain were observed at 3 mg/kg bw per day in F_0 males during the mating period (−5%) and in F_0 females during the premating period (−9%). At 3 mg/kg bw per day, feed consumption was statistically significantly reduced in F_0 and F_1 females during gestation (up to 19%) and lactation (up to 18%). Small, but statistically significant, reductions in body weight gain were observed at 1.5 mg/kg bw per day in females during gestation (up to 6%) and lactation (up to 8%). Feed consumption was only slightly (up to 7%, not statistically significant) reduced in F_0 females at 1.5 mg/kg bw per day. At 3 mg/kg bw per day, birth weights were reduced in male and female F_1 pups (8–9%, $P < 0.01$) and F_2 pups (5–6%, not significant). During the lactation period, body weight gain in high-dose pups was statistically significantly decreased, with a 24% reduction at PND 7 in F_1 pups and a 19–20% reduction at PND 7 in F_2 pups. From PND 7 onward, the differences in body weight between control and high-dose rats became less pronounced. About half of the females of the 3 mg/kg bw per day group in F_0 animals had a prolonged gestation (23–24 days, compared with about 22 days in controls). In four of these dams, a prolonged parturition was observed. One of these dams delivered only dead pups, while the other three proceeded to dystocia and died or were killed in a moribund state. In the F_0 females at 3 mg/kg bw per day, an increased incidence of total litter loss, a low gestation index (number of females delivering live pups/number of pregnant rats) and a low birth index (number of live pups/number of implantations) were noted. At the high dose, the birth index in the F_1 generation was decreased (not statistically significant). In F_1 and F_2 high-dose pups, a slightly increased incidence of postnatal death was observed. No treatment-related changes in estrous cycles, sperm parameters, ovarian follicle counts, copulation index (number of animals that mated/number of animals used for mating), fertility index (number of pregnant females/number of females that mated)

or nursing behaviour were found. Blackish abdominal cavity was observed from PND 0 to PND 7 in a small number of F_1 and F_2 pups at 3 mg/kg bw per day. After PND 4, the incidence of this observation decreased. A significant delay in the completion of surface righting reflex was observed in F_1 male pups at 3 mg/kg bw per day. The incidence of pinna unfolding was significantly lower in both sexes on PND 3 in the high-dose F_1 and F_2 pups and also on PND 2 in F_1 females. At the high dose, there was a significant prolongation of the day when eye opening occurred in F_1 females and in F_2 males. No treatment-related effects were noted in vaginal opening or preputial separation of F_1 animals. Thymus weights at weaning were statistically significantly lower in F_1 males (absolute −30%; relative −19%) and females (absolute −26%; relative −15%) at 3 mg/kg bw per day and in F_2 males of all treated groups (absolute −12%, −13% and −33% at 0.75, 1.5 and 3 mg/kg bw per day, respectively; relative −13%, −15% and −24% at 0.75, 1.5 and 3 mg/kg bw per day, respectively) and in F_2 females at 1.5 (absolute −13%; relative −11%) and 3 mg/kg bw per day (absolute −27%; relative −18%). Similar reductions in thymus weight were observed in a modified two-generation reproductive toxicity study, with a focus on the effects of tolfenpyrad on immune function (Atai, 1999; see below). In the absence of changes in other immunological parameters at 0.75 mg/kg bw per day in the study of Atai (1999), the Meeting considered the small reductions in thymus weight observed at this dose as initial, non-adverse events of a process that results in immunotoxic effects at higher doses. Therefore, these effects were considered not relevant as a basis for a lowest-observed-adverse-effect level (LOAEL). Similar considerations cannot be applied to the same findings at 1.5 mg/kg bw per day, because this dose was not tested in the study of Atai (1999), and relevant immunological parameters were not measured in the present study.

In F_1 adults, slightly, but statistically significantly, lower absolute brain weights were observed at 1.5 mg/kg bw per day in females (3%) and at 3 mg/kg bw per day in both sexes (4–6%). Histological examination of the animals that died in the terminal stage of the gestation period or that had total litter loss showed atrophy of the thymus and spleen, erosion of the forestomach or glandular stomach, erosion/ulcer of the small intestine and hypertrophy of the zona fasciculata of the adrenal cortex. These changes are considered to be secondary to stress. Furthermore, in these females, changes in the reproductive organs related to parturition, such as haemorrhage, inflammatory cell infiltration, necrosis and thrombus in the uterus and inflammatory cell infiltration in the vagina, were observed.

The NOAEL for parental toxicity was 1.5 mg/kg bw per day, based on moribundity, decreased body weight gain and decreased feed consumption at 3 mg/kg bw per day.

The NOAEL for offspring toxicity was 0.75 mg/kg bw per day, based on a reduction in absolute and relative thymus weights in males and females of the F_2 generation at 1.5 mg/kg bw per day.

The NOAEL for reproductive toxicity was 1.5 mg/kg bw per day, based on dystocia, prolonged parturition, prolonged gestation, increased incidence of total litter loss, low gestation index and low birth index at 3 mg/kg bw per day (Matsuura, 1999).

In a two-generation dietary reproduction study performed according to a modified OECD Test Guideline 416 protocol, SD (Crj:CD(SD)IGS, SPF) rats (15 females per dose for the F_0 generation, 36 males and 12 females per dose for the F_1 generation, 12 males per dose for the F_2 generation) were fed tolfenpyrad (purity 99.33%; lot no. 6D-01-2) in order to assess effects on immune function in the F_1 and F_2 rats. Dietary concentrations were adjusted to maintain the desired dose levels of 0, 0.75 and 3 mg/kg bw per day. The mean test substance intake for each treatment group during the premating, gestation and nursing periods was within ±20% of the target values. F_0 rats (females only) were treated with tolfenpyrad during gestation and lactation, F_1 rats were treated from weaning through mating (both sexes), gestation and lactation (females only) and F_2 rats (males only) were treated to maturation. Dietary concentrations were based on the data from the two-generation reproduction study of Matsuura (1999). F_1 clinical examination was performed daily, and body weight and feed consumption were recorded weekly and on GDs 0, 7, 14 and 20 and PNDs 0, 4,

7, 14, 17 and 21 (females). Rats were mated after 10 weeks of treatment. From GD 21 until completion of delivery, each female was observed for parturition on two occasions per day. All litters were examined (number of pups, sex of pups, number of stillbirths, number of live births, presence of gross anomalies). Pups were weighed on PNDs 0, 4, 7, 14 and 21. On PND 4, litters were culled to four pups of each sex per litter. On PND 4, one culled pup of each sex per litter was selected for measurements of organ weight and thymic and splenic lymphocyte subsets by fluorescence-activated cell sorting.

At weaning at PND 21, from each F_1 litter, one rat of each sex was selected to produce the F_2 generation, one male pup was selected for sheep red blood cell (sRBC) antibody production at 10 weeks of age, one male pup was selected for delayed-type hypersensitivity to keyhole limpet haemocyanin responsiveness at 10 weeks of age and one pup of each sex was selected for haematology and measurements of thymic and splenic lymphocyte subsets (male pup only) and organ weight measurements and necropsy (both sexes) at 3 weeks of age. At weaning at PND 21, from each F_2 litter, one male pup was selected for measurements of organ weight, fluorescence-activated cell sorting, haematology and necropsy at 10 weeks of age, one male pup was selected for sRBC antibody production at 10 weeks of age, one male pup was selected for delayed-type hypersensitivity to keyhole limpet hemocyanin responsiveness at 10 weeks of age and one pup of each sex was selected for haematology and measurements of thymic and splenic lymphocyte subsets (male pup only) and organ weight measurements and necropsy (both sexes) at 3 weeks of age. After weaning, the maternal rats were killed and necropsied. No GLP statement was provided, but the study was conducted in accordance with test laboratory standard operating procedures and quality assurance procedures.

At 3 mg/kg bw per day, there were decreases in body weight gain (up to −10% during gestation) and feed consumption (up to −22% during gestation; up to 14% during lactation) in F_0 maternal rats. In F_1 pups, no significant effects on birth weight were observed. However, body weight gain at 3 mg/kg bw per day was reduced (up to 19% in male pups, up to 20% in female pups) during lactation. After weaning, the reduced body weights quickly recovered. No effects on F_1 maternal body weight were observed during gestation or lactation. Body weights of F_2 offspring were not affected by treatment. Feed consumption was not affected in F_1 or F_2 animals. No effects on reproductive outcome were observed. The number of live F_1 pups on PND 4 was significantly lower at 3 mg/kg bw per day. No effect on postnatal survival was observed in F_2 offspring. In F_1 and F_2 pups at 3 mg/kg bw per day, black change in the peritoneal cavity due to an accumulation of dark green contents in the small intestine was observed early after birth. Haematological examination of F_1 and F_2 pups on PND 21 revealed no effects of treatment. A high count of segmented neutrophils in F_2 rats of the high-dose group at postnatal week 10 was considered incidental, as no such effects were observed at PND 21 in F_1 and F_2 pups or in other repeated-dose studies. In F_1 pups necropsied on PND 21, no effects on haematology were observed. Low thymus weights were observed on PND 4 at 0.75 mg/kg bw per day in male F_1 pups (absolute −22%; relative −20%) and at 3 mg/kg bw per day in male F_1 pups (absolute −48%; relative −38%), female F_1 pups (absolute −44%; relative −35%), male F_2 pups (absolute −41%; relative −34%) and female F_2 pups (absolute −30%; relative −22%). Low spleen weights were also observed in male (up to 44%) and female pups (up to 34%) of the F_1 and F_2 generations at 3 mg/kg bw per day. On PND 21, the reduction in thymus weight was smaller, although still statistically significant, at 3 mg/kg bw per day. No statistically significant changes were noted in the spleen weights on PND 21 or in adult thymus or spleen weights. Significantly lower values were noted in thymus and spleen cellularity in F_2 rats in the 3 mg/kg bw per day group on PNDs 4 and 21. F_2 rats in the 3 mg/kg bw per day group showed slightly, but statistically significantly, lower ratios of CD3+/CD45RA− cells and CD4+/CD8− cells in the spleen and consequently relative changes in other cell ratios on PND 21. At 3 mg/kg bw per day, slight, but statistically significant, changes were also noted in CD3−/CD45RA+ cell ratios in the spleen in F_2 rats on PND 4 and CD3+/CD45RA− cell ratios in the spleen in F_2 rats in postnatal week 10. In immune function tests, F_1 and F_2 rats in postweaning week 10 showed normal humoral immunity and cellular immune function, indicating that the observed effects on the immune system on PND 4 have no consequences for the functionality of the immune system in adult rats.

The NOAEL for maternal toxicity was 0.75 mg/kg bw per day, based on decreased body weight gain and decreased feed consumption at 3 mg/kg bw per day.

The NOAEL for offspring toxicity was 0.75 mg/kg bw per day, based on reduced body weight gain during lactation and a reduced number of live F_1 pups at PND 4, black change in the peritoneal cavity after birth in F_1 and F_2 pups, lower thymus and spleen weights early after birth in F_1 pups and in F_2 male pups, reduced thymus and spleen cellularity, and changes in immune cell ratios in the spleen in F_2 male pups at 3 mg/kg bw per day.

The NOAEL for reproductive toxicity was 3 mg/kg bw per day, the highest dose tested (Atai, 1999).

(b) Developmental toxicity

Rats

In a developmental toxicity study, groups of 21–24 pregnant female Crj:CD, SPF rats were treated orally, by gavage, with tolfenpyrad (purity 99.33%; lot no. 6D-01-2) in distilled water at a dose level of 0, 1, 3 or 4.5 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 doses were based on a range-finding study. Clinical signs and feed consumption were recorded daily. Body weight and feed consumption were measured on GDs 0, 6, 9, 12, 15 and 20. Feed consumption was measured over 2-day periods. All females were killed on day 20 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.

No clinical signs were noted. At 4.5 mg/kg bw per day, body weight was reduced at GD 9 (−2 g compared with GD 6). During this period, dams at 3 mg/kg bw per day gained 7 g, whereas control dams gained 16 g. Feed consumption was reduced at GD 9 and GD 12 at 3 mg/kg bw per day (up to 12%) and at 4.5 mg/kg bw per day (up to 28%). Necropsy of the dams revealed no treatment-related effects. Fetal weights were reduced at 4.5 mg/kg bw per day in males (−7%) and females (−9%). An increased incidence of fetuses with 14th ribs and reduced ossification of the metacarpal bones were observed at 4.5 mg/kg bw per day.

The NOAEL for maternal toxicity was 1 mg/kg bw per day, based on reduced body weight gain and feed consumption during the first days of treatment at 3 mg/kg bw per day.

The NOAEL for embryo and fetal toxicity was 3 mg/kg bw per day, based on decreased fetal weight, increased incidence of fetuses with 14th ribs and reduced ossification of the metacarpal bones observed at 4.5 mg/kg bw per day. No evidence of a teratogenic effect was observed (Hoshino, 1995).

Rabbits

In a developmental toxicity study, groups of 12–15 pregnant Kbl:JW, SPF Japanese White rabbits were treated orally, by gavage, with tolfenpyrad (purity 99.33%; lot no. 6D-01-2) in aqueous sodium carboxymethyl cellulose at a dose level of 0, 1, 3 or 6 mg/kg bw per day from days 6 through 18 of gestation (the day after artificial insemination was designated as GD 0). The doses were based on a range-finding study. Clinical signs were recorded daily. Body weight and feed consumption were measured on GDs 0, 6, 9, 12, 15, 18, 21, 25 and 28. All females were killed on day 28 of gestation. All does were examined macroscopically for abnormalities. The uterus was examined, and the numbers of live and dead fetuses, corpora lutea and implantations were counted. Body weight and sex of the fetuses were recorded. All fetuses were subjected to skeletal and visceral examinations.

One doe in the 3 mg/kg bw per day group was found dead at GD 28, and one doe at 6 mg/kg bw per day delivered prematurely on GD 27. These findings were considered to be treatment related, as both does had shown body weight loss and reduced feed consumption from GD 9 onward. At the high dose, a mild body weight loss (61 g) was observed in the does at GD 9. Body weight gain was

slightly reduced (not statistically significant) at 3 mg/kg bw per day from GD 15 to termination and at 6 mg/kg bw per day from GD 9 to termination. Feed consumption was statistically significantly reduced at 6 mg/kg bw per day at GD 9. No treatment-related clinical signs or effects on numbers of corpora lutea, implantations, or live and dead fetuses, implantation loss, live fetal weight or sex ratio were observed. However, total litter loss (nine implanted embryos) in one doe in the 6 mg/kg bw per day group was considered treatment related, as the rabbit showed body weight loss and reduced feed consumption during the treatment period. The study author reported that similar effects were observed at 6 and 9 mg/kg bw per day in a preliminary study in rabbits (data not shown). Necropsy of the other surviving does showed no treatment-related changes. No treatment-related changes were observed in individual findings after external, visceral or skeletal examinations of fetuses showed that the incidences of 13 ribs at 1 and 6 mg/kg bw per day (27.8% and 38.1%, respectively) were higher than in controls (15.9%) and the 3 mg/kg bw per day group (18.5%). The increases were not statistically significant, not dependent on dose and within the historical control range of this laboratory (33.6% [29.7–40.5] in data from the past 5 years) and were therefore not considered toxicologically significant. An increased incidence of accessory sternebrae at 1, 3 and 6 mg/kg bw per day (17.0%, 11.5% and 9.1%, respectively) compared with controls (3.3%) was also not considered toxicologically significant, as the increases were not statistically significant and lacked dose dependency. The total number of skeletal variations was significantly increased at 1 and 6 mg/kg bw per day, but not at 3 mg/kg bw per day (17.6%, 43.7%, 31.4% and 48.2% in the 0, 1, 3 and 6 mg/kg bw per day groups, respectively). However, as the values of the treatment groups were within the range of contemporary historical control values (range 34.8–51.2% in 10 studies conducted during 1991–1996, data provided by the sponsor), there was a lack of dose dependency and the control for this experiment was clearly lower than could have been expected, the finding is considered incidental and not toxicologically relevant.

The NOAEL for maternal toxicity was 1 mg/kg bw per day, based on one mortality observed at 3 mg/kg bw per day.

The NOAEL for embryo and fetal toxicity was 6 mg/kg bw per day, the highest dose tested (Hoshino, 1997).

2.6 Special studies

(a) Neurotoxicity

Rats

In an acute oral neurotoxicity study, Crl:CD(SD) rats (10 of each sex per dose) were treated by gavage with tolfenpyrad (purity 99.5%; lot no. 365-65A) in corn oil at a dose of 0, 20, 40 or 60 mg/kg bw (males) or 0, 10, 20 or 40 mg/kg bw (females). The doses were based on a range-finding study in which groups of five male and five female rats were treated by gavage with tolfenpyrad at 0, 20, 40, 60, 80 or 100 mg/kg bw and subsequently observed in an open-field arena for 1 minute at 1, 2, 3, 4, 5, 6, 7 and 8 hours post-dosing and again at 7 and 14 days after dosing. All rats were necropsied. The range-finding study indicated that the time of peak effect was 6 hours after dosing. The rats were observed daily for clinical signs. The rats were tested in a functional observational battery, including detailed clinical observations, pretest and at 6 hours and 7 and 14 days after application. Motor activity was measured pretest, on the day of dosing and at 7 and 14 days after dosing. Body weights and feed consumption were measured daily. All animals were killed on test day 15 and examined macroscopically. Five rats of each sex per dose were selected for neurohistological examination.

In the range-finding test, one male at 80 mg/kg bw and all males at 100 mg/kg bw died. All females dosed at 60 mg/kg bw or higher were found dead or were killed in a moribund condition. Clinical signs were observed at all doses, with the peak effect at 6 hours after dosing.

In the main test, one female rat at 40 mg/kg bw died on day 4. Males and females of all treatment groups lost weight after dosing (5%, 7% and 11% in males at 20, 40 and 60 mg/kg bw, respectively, and 4%, 10% and 15% in females at 10, 20 and 40 mg/kg bw, respectively). Compared with controls, reductions in body weight gain were observed in the male rats at 40 (up to 9%) and 60

mg/kg bw (up to 16%) and in female rats at 20 (up to 12%) and 40 mg/kg bw (up to 21%). A slight (6%), but statistically significant, reduction in body weight was observed on the day of dosing in males at 20 mg/kg bw. At the highest doses in males and females, body weights remained significantly reduced up to 11–13 days after dosing. At the middle doses, body weights remained decreased for 3–6 days. On the day of dosing, feed consumption was reduced by 64%, 76% and 85% in males at 20, 40 and 60 mg/kg bw, respectively, and by 30%, 44% and 63% in females at 10, 20 and 40 mg/kg bw, respectively. Feed consumption quickly recovered in males, but remained decreased for 5 and 7 days after dosing in mid- and high-dose females, respectively. Increased incidences of adverse clinical signs (including dehydration, scant faeces, soft or liquid faeces, chromorhinorrhoea and urine-stained abdominal fur) were observed in females at 40 mg/kg bw and in males at 60 mg/kg bw. Functional observational battery testing revealed no treatment-related effects, except for urine staining in high-dose females on the day of dosing. Motor activity was not affected by treatment. No test substance–related microscopic lesions were revealed by the neurohistological examination.

The LOAEL was 10 mg/kg bw, based on reductions in body weight and feed consumption observed in females on the day of dosing. No evidence of neurotoxicity was observed in rats (Barnett, 2008b).

In a 90-day neurotoxicity study, groups of 10 male and 10 female Crl:CD (SD)IGS BR rats were given tolfenpyrad (purity 99.3%; lot no. 6D-01-2) at a dietary level of 0, 15, 40 or 80 ppm (equal to 0, 1.0, 2.7 and 5.4 mg/kg bw per day for males and 0, 1.2, 3.2 and 6.0 mg/kg bw per day for females, respectively). The animals were observed daily for clinical signs. A detailed physical examination and body weight and feed consumption measurements were performed weekly. The rats were subjected to a functional observational battery and a locomotor activity test before the start of treatment and during weeks 2, 4, 8 and 12. Ophthalmological examination was perfomed pretest and in week 13. At termination, rats were killed and examined macroscopically. The brain was measured and weighed. Five rats of each sex in the control and the high-dose groups were selected for neurohistological examination of the brain, spinal cord, dorsal root ganglia, dorsal root fibres, ventral root fibres, eyes, optic nerves, skeletal muscle and sciatic and tibial nerves.

No treatment-related mortality or clinical signs were observed. Females at 80 ppm showed a reduction in body weight gain (14%) and feed consumption (17%) throughout the treatment period. There were no treatment-related effects observed in functional observational battery parameters, locomotor activity testing, ophthalmology or macroscopic and histological examination.

The NOAEL was 40 ppm (equal to 3.2 mg/kg bw per day), based on reductions in body weight gain and feed consumption in females at 80 ppm (equal to 6.0 mg/kg bw per day). No evidence of neurotoxicity was observed in rats (Kilpatrick, 2003).

(b) Studies with metabolites

Acute toxicity and genotoxicity studies with metabolites of tolfenpyrad were available.

Acute toxicity

The results of studies of acute toxicity with metabolites of tolfenpyrad are summarized in Table 10. In all of these studies, the test substance was dissolved in aqueous 0.5% carboxymethyl cellulose.

Table 10. Results of studies of acute toxicity with metabolites of tolfenpyrad[a]

Species	Strain	Sex	Route	Metabolite	Purity (%)	LD_{50} (mg/kg bw)	Reference
Rat	Sprague-Dawley (Crj:CD(SD))	M/F	Oral	OH-PT[b]	99.9	70.8 (M) 35.5 (F)	Ikeya (1999a)
Rat	Sprague-Dawley (Crj:CD(SD))	M/F	Oral	PT-CA[c]	99.5	27.4 (M) 15.4 (F)	Ikeya (1999b)
Rat	Sprague-Dawley (Crj:CD(SD))	F	Oral	PT(A)-4OH[d]	99.9	> 2 000 (F)	Oda (2012a)
Rat	Sprague-Dawley (Crj:CD(SD))	M/F	Oral	T-CA[e]	98.5	600–2 000 (M) > 2 000 (F)	Ikeya (1999c)
Rat	Sprague-Dawley (Crj:CD(SD))	M/F	Oral	T-AM[f]	99.9	> 2 000 (M/F)	Ikeya (1999d)
Rat	Sprague-Dawley (Crj:CD(SD))	M/F	Oral	CA-T-CA[g]	99.7	> 2 000 (M/F)	Ikeya (1999e)
Rat	Sprague-Dawley (Crj:CD(SD))	M/F	Oral	OH-T-CA[h]	94.6	2 024 (M) > 2 000 (F)	Ikeya (1999f)
Rat	Sprague-Dawley (Crj:CD(SD))	F	Oral	PAM[i]	100.0	300–2 000 (F)	Oda (2012b)
Rat	Sprague-Dawley (Crj:CD(SD))	M/F	Oral	OH-PAM[j]	99.1	1 095 (M/F)	Ikeya (1999g)
Rat	Sprague-Dawley (Crj:CD(SD))	M/F	Oral	PCA[k]	99.9	> 2 000 (M/F)	Ikeya (1999h)

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

[a] Statements of adherence to quality assurance and GLP were included in all studies. See Table 12 (below) for names of all metabolites included here.

[b] Lot no. Y980423. Performed according to OECD Test Guideline 401. Doses of 0, 10, 30, 100, 300 and 1000 mg/kg bw were used. Mortality and clinical signs were observed at doses of 30 mg/kg bw and higher.

[c] Lot no. Y980626. Performed according to OECD Test Guideline 401. Doses of 0, 6.25, 12.5, 25, 50 and 100 mg/kg bw were used. Mortality and clinical signs were observed at doses of 12.5 mg/kg bw and higher.

[d] Lot no. 2HO0301S. Performed according to OECD Test Guideline 423. A dose of 2000 mg/kg bw was administered to female rats. No mortality or clinical signs were observed.

[e] Lot no. Y980415. Performed according to OECD Test Guideline 401. Doses of 0, 60, 200, 600 and 2000 mg/kg bw were used. At 2000 mg/kg bw, 4/5 males died. No mortality was observed in the other groups. Clinical signs were observed at doses of 600 mg/kg bw and higher.

[f] Lot no. Y980420. Performed according to OECD Test Guideline 401. Doses of 0 and 2000 mg/kg bw were used. No mortality or clinical signs were observed.

[g] Lot no. FGC01. Performed according to OECD Test Guideline 401. Doses of 0 and 2000 mg/kg bw were used. No mortality was observed. At 2000 mg/kg bw, some rats showed diarrhoea.

[h] Lot no.˙ Y980629. Performed according to OECD Test Guideline 401. Doses of 0, 1000, 2000, 3000 and 4000 mg/kg bw were used for males, and doses of 0, 1000 and 2000 mg/kg bw were used for females. In males, mortality was observed at 2000 mg/kg bw and higher. No mortality was observed in females. Clinical signs were noted at doses of 2000 mg/kg bw and higher. Body weight loss or reduced body weight gain was observed in males at all doses and in females at 2000 mg/kg bw.

[i] Lot no. 2HO4602S. Performed according to OECD Test Guideline 423. Doses of 300 and 2000 mg/kg bw were applied to female rats (three per dose step). At 2000 mg/kg bw, all rats died. No mortality was observed at 300 mg/kg bw. Decreased body weight of the surviving rats and clinical signs were observed at both doses.

[j] Lot no. Y981207. Performed according to OECD Test Guideline 401. Doses of 0, 60, 200, 600 and 2000 mg/kg bw were used. No mortality occurred at doses up to 600 mg/kg bw. At 2000 mg/kg bw, all rats died. At doses of 600 mg/kg bw and higher, clinical signs were observed.

[k] Lot no. OL22MC01. Performed according to OECD Test Guideline 401. Doses of 0, 1000 and 2000 mg/kg bw were used. No mortality or clinical signs were observed at 1000 mg/kg bw. At 2000 mg/kg bw, 2/5 males and 1/5 females died. Surviving rats at 2000 mg/kg bw showed diarrhoea and decreased spontaneous movements.

Short-term studies of toxicity

In acute toxicity studies, the metabolites OH-PT and PT-CA were more potent than tolfenpyrad in causing mortality. Therefore, in a comparative 4-week toxicity study, the effect of treatment with tolfenpyrad (purity 99.33%; lot no. 6D-01-2), OH-PT (purity 99.9%; lot no. Y980423) and PT-CA (purity 99.5%; lot no. Y980626) was investigated. Groups of male and female F344/DuCrj (Fischer) strain SPF rats, consisting of five rats of each sex per dose, received diets containing tolfenpyrad at 0, 10, 30 or 100 ppm (equal to 0, 0.9, 2.5 and 8.0 mg/kg bw per day for males and 0, 0.9, 2.6 and 8.2 mg/kg bw per day for females, respectively), OH-PT at 0, 3, 10, 30 or 100 ppm (equal to 0, 0.2, 0.9, 2.5 and 8.4 mg/kg bw per day for males and 0, 0.3, 0.9, 2.7 and 8.8 mg/kg bw per day for females, respectively) or PT-CA at 0, 3, 10, 30 or 100 ppm (equal to 0, 0.3, 0.8, 2.5 and 8.1 mg/kg bw per day for males and 0, 0.3, 0.9, 2.7 and 8.5 mg/kg bw per day for females, respectively). Animals were checked daily for clinical signs of toxicity. Body weights and feed consumption were measured weekly. Ophthalmological examinations were carried out before dosing and at termination. Blood and urine samples were taken at termination for haematology, clinical biochemistry and urine analysis. At termination, all rats were necropsied, and brain, adrenals, thymus, spleen, lung, liver, kidney and testis/ovary were weighed. An extensive range of organs and tissues from control and high-dose rats was examined histologically. In addition, in the other dose groups, pancreas (females only), heart, lung, liver, kidney, spleen, prostate and any macroscopic lesions were examined histologically.

No treatment-related mortality or clinical signs were seen in any treatment group. For tolfenpyrad, a mild reduction in body weight gain in males at 100 ppm (up to 9%) and feed consumption in males and females at 100 ppm (about 10%) were observed. Slight reductions in body weight gain (6%) and feed consumption (7%) of high-dose females treated with PT-CA were considered not toxicologically relevant. No toxicologically relevant changes in ophthalmoscopy, urine analysis, haematology or clinical chemistry were observed. In rats treated with tolfenpyrad, there was an increase in relative weight of liver in males at 30 ppm (7%) and 100 ppm (11%) and in females at 30 ppm (5%) and 100 ppm (16%), and also an increase in relative weight of kidney in males at 30 ppm (5%) and 100 ppm (11%). In rats treated with PT-CA, an increase in absolute and relative liver weights in females at 30 ppm (absolute 3%; relative 6%) and 100 ppm (absolute 6%; relative 12%) and an increase in relative kidney weight in males at 30 ppm (7%) and 100 ppm (9%) and in females at 100 ppm (10%) were observed. In rats treated with OH-PT, there was a slight increase of relative kidney weight in males at 100 ppm (5%). Histopathological examination revealed that the increased liver weights observed in the groups treated with tolfenpyrad and PT-CA were associated with mild hepatocyte hypertrophy. The mild increases in liver weights and hepatocellular hypertrophy were considered not to be adverse. In the absence of histological changes, the mild changes in kidney weight observed in PT-CA- and OH-PT-treated rats were not considered adverse. In rats treated with tolfenpyrad, increased incidences and severity of hypertrophy of acinar cells in pancreas were found in 100 ppm females. The observation of hyaline droplets (graded as slight) in tubular epithelium in kidney observed in 3/5 males at 100 ppm was not considered toxicologically relevant.

For tolfenpyrad, the NOAEL was 30 ppm (equal to 2.5 mg/kg bw per day), based on a mild reduction in feed consumption in males and females, a mild reduction in body weight gain observed in males and increased incidences and severity of hypertrophy of acinar cells in pancreas in females at 100 ppm (equal to 8 mg/kg bw per day).

For PT-CA, the NOAEL was 100 ppm (equal to 8.1 mg/kg bw per day), the highest dose tested.

For OH-PT, the NOAEL was 100 ppm (equal to 8.4 mg/kg bw per day), the highest dose tested (Nishimura, 2001).

Genotoxicity

The results of genotoxicity studies with metabolites of tolfenpyrad are summarized in Table 11.

Table 11. Results of studies on the genotoxicity of metabolites of tolfenpyrad[a]

Metabolite	End-point	Test object	Concentration	Purity (%)	Results	Reference
In vitro						
OH-PT[b]	Gene mutation	*Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537; *Escherichia coli* WP2 *uvrA*	2.44–1 250 µg/plate (−S9) 2.44–5 000 µg/plate (+S9)	99.9	Negative	Ozaki (1999a)
PT-CA[c]	Gene mutation	*S. typhimurium* strains TA98, TA100, TA1535, TA1537; *E. coli* WP2 *uvrA*	39.1–5 000 µg/plate (±S9)	99.5	Negative	Ozaki (1999b)
T-CA[d]	Gene mutation	*S. typhimurium* strains TA98, TA100, TA1535, TA1537; *E. coli* WP2 *uvrA*	39.1–1 250 µg/plate (±S9), except for *E. coli*: 156–5 000 µg/plate (±S9)	98.5	Negative	Ozaki (1999c)
T-AM[e]	Gene mutation	*S. typhimurium* strains TA98, TA100, TA1535, TA1537; *E. coli* WP2 *uvrA*	39.1–5 000 µg/plate (±S9)	99.9	Negative	Ozaki (1999d)
CA-T-CA[f]	Gene mutation	*S. typhimurium* strains TA98, TA100, TA1535, TA1537; *E. coli* WP2 *uvrA*	156–5 000 µg/plate (±S9)	99.7	Negative	Ozaki (1999e)
OH-T-CA[g]	Gene mutation	*S. typhimurium* strains TA98, TA100, TA1535, TA1537; *E. coli* WP2 *uvrA*	39.1–5 000 µg/plate (±S9)	94.6	Negative	Ozaki (1999f)
OH-PAM[h]	Gene mutation	*S. typhimurium* strains TA98, TA100, TA1535, TA1537; *E. coli* WP2 *uvrA*	156–5 000 µg/plate (±S9)	99.1	Negative	Ozaki (1999g)
PCA[i]	Gene mutation	*S. typhimurium* strains TA98, TA100, TA1535, TA1537; *E. coli* WP2 *uvrA*	313–5 000 µg/plate (±S9)	99	Negative	Machigaki (1988)
OH-PT[j]	Chromosomal aberration	CHL cells	40–200 µg/mL (±S9), experiment 1 10–130 µg/mL (−S9), experiment 2	99.9	Negative	Ozaki (2001a)
PT-CA[k]	Chromosomal aberration	CHL cells	78.1–1 250 µg/mL (±S9), experiment 1 4.89–313 µg/mL (−S9), experiment 2	99.5	Negative	Ozaki (2001b)

Metabolite	End-point	Test object	Concentration	Purity (%)	Results	Reference
In vivo						
OH-PT[l]	Micronucleus formation	Crj:CD(SD)IGS rat bone marrow	Two gavage doses of 5, 10 or 20 mg/kg bw, separated by 24 h	99.9	Negative	Saigo (2000a)
PT-CA[m]	Micronucleus formation	Crj:CD(SD)IGS rat bone marrow	Two gavage doses of 5, 10 or 20 mg/kg bw, separated by 24 h	99.7	Negative	Saigo (2000b)

CHL: Chinese hamster lung; S9: 9000 × *g* supernatant fraction of rat liver homogenate

[a] Positive and negative (solvent) controls were included in all studies. In all studies, statements of adherence to GLP and quality assurance were included. See Table 12 (below) for names of all metabolites included here.

[b] Lot no. Y980423. Performed in accordance with OECD Test Guideline 471. Cytotoxicity in strains TA98, TA100, TA1535 and WP2 *uvrA* was observed at 1250 µg/plate and in strain TA1537 at 313 µg/plate with S9 mix. Without S9 mix, cytotoxicity was observed in strains TA98 and WP2 *uvrA* at 1250 µg/plate and in strains TA100, TA1535 and TA1537 at 313 µg/plate. Precipitation of the test compound was observed at and above 313 µg/plate with and without S9 mix.

[c] Lot no. Y980626. Performed in accordance with OECD Test Guideline 471. Bacterial growth inhibition was observed in the test group at 5000 µg/plate for *S. typhimurium* TA98 and TA1537 and *E. coli* WP2 *uvrA* and in the test group at 5000 µg/plate for TA1535 in the treatment without metabolic activation.

[d] Lot no. Y980415. Performed in accordance with OECD Test Guideline 471. Bacterial growth inhibition was observed at 625 and 1250 µg/plate for *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 and at 2500 and 5000 µg/plate for *E. coli* WP2 *uvrA*.

[e] Lot no. Y980420. Performed in accordance with OECD Test Guideline 471. Bacterial growth inhibition was observed in the test group at 5000 µg/plate for *S. typhimurium* TA98 and TA1537 and *E. coli* WP2 *uvrA* in the treatment with and without metabolic activation and in the test group at 5000 µg/plate for TA100 in the treatment with metabolic activation.

[f] Lot no. FGC01. Performed in accordance with OECD Test Guideline 471.

[g] Lot no. Y980629. Performed in accordance with OECD Test Guideline 471. Bacterial growth inhibition was observed in the test group only at 5000 µg/plate for *S. typhimurium* TA98, TA100, TA1535 and TA1537, but not for *E. coli* WP2 *uvrA*.

[h] Lot no. Y981207. Study design resembles OECD Test Guideline 471.

[i] Lot no. 88002. Performed in accordance with OECD Test Guideline 471.

[j] Lot no. Y980423. Performed in accordance with OECD Test Guideline 473. In preliminary assays, a concentration-related increase in cytotoxicity was observed in the dose range between 39.1 and 313 µg/mL following 6 hours of culture, and a concentration-related increase in cytotoxicity was observed in the dose range between 39.1 and 156 µg/mL following both 24 and 48 hours of culture. Experiment 1: Incubation with OH-PT ±S9 mix for 6 hours, followed by an 18-hour recovery period. Experiment 2: Continuous incubation with OH-PT −S9 for 24 or 48 hours.

[k] Lot no. Y980626. Performed in accordance with OECD Test Guideline 473. In preliminary assays, a concentration-related increase in cytotoxicity was observed in the dose range between 156 and 1250 µg/mL following 6 hours of culture, and a concentration-related increase in cytotoxicity was observed in the dose range between 39.1 and 313 µg/mL following 24 hours of culture and between 39.1 and 78.1 µg/mL following 48 hours of culture. Experiment 1: Incubation with PT-CA ±S9 mix for 6 hours, followed by an 18-hour recovery period. Experiment 2: Continuous incubation with PT-CA −S9 for 24 or 48 hours.

[l] Lot no. Y000328. Performed in accordance with OECD Test Guideline 474. Mortality was observed in 1/10 animals at 20 mg/kg bw per day. It was judged that the exposure of the rat bone marrow to the test article was sufficient, as the percentage of immature erythrocytes in all erythrocytes in all the test article groups was significantly lower than in the negative control group.

[m] Lot no. 0000228-1. Performed in accordance with OECD Test Guideline 474. Mortality was observed in 4/10 animals at 20 mg/kg bw per day. It was judged that the exposure of the rat bone marrow to the test article was sufficient, as the percentage of immature erythrocytes in all erythrocytes in all the test article groups was significantly lower than in the negative control group.

3. Observations in humans

No information on adverse health effects or poisoning in manufacturing plant personnel or in operators and workers exposed to tolfenpyrad was available.

Comments

Biochemical aspects

After administration of a single oral dose of radiolabelled tolfenpyrad to rats, maximum concentrations in blood and plasma were reached 2–8 hours after a low dose (1 mg/kg bw) and 4–12 hours after a high dose (20 mg/kg bw). Excretion of radioactivity in urine (2–3%) and bile (51–70%) and residual radioactivity in the carcass (5–11%) 48 hours after dosing indicate that absorption was at least 58% of the dose. Plasma half-lives were 11–28 hours. Radioactivity was widely distributed to the tissues, higher concentrations being found in liver, kidney, bone marrow and brown fat. Seven days after dosing, 88–93% and 2–3% of the radioactivity were excreted in faeces and urine, respectively. In faeces, 4–15% of the radioactivity represented tolfenpyrad, and 24–49% of the radioactivity represented the metabolite PT-CA (see Table 12 for names of metabolites). In plasma, liver and kidney, 91–100% of the radioactivity represented PT-CA, indicating extensive metabolism of tolfenpyrad. In bile, 50–67% of the administered dose was excreted within 48 hours, the major part as PT-CA-TA, PT-CA-Gluc and PT-CA, whereas low levels of Sul-OH-PT-CA and CO-PT and other, unidentified metabolites were also detected. These data indicate extensive conjugation of PT-CA in the liver and subsequent excretion into the bile. Less than 0.7% of the administered dose was present in bile as unchanged tolfenpyrad. In bile duct–cannulated rats, only 3–8% of the administered dose was excreted into faeces, predominantly as tolfenpyrad (up to 6%) and PT-CA (up to 1%). PT-CA is deconjugated following its biliary excretion and excreted in faeces. In urine, no intact tolfenpyrad was detected. Various individual metabolites (including OH-PAM and CA-T-CA) were present in urine at less than 0.5% of the administered dose, with the exception of PT-CA and PT-CA-TA (these metabolites could not be further separated), which were present at up to 1.9% of the administered dose. Observed differences in metabolite levels between sexes, doses and positions of radiolabel were minor. Following repeated dosing of [^{14}C]tolfenpyrad, plasma concentrations of radioactivity stabilized after two or three administrations at 1.5–3 times the plasma concentration found after the first dose. Tissue distribution, excretion and metabolism were similar following the single low and high doses and following single and repeated dosing.

Toxicological data

The oral LD_{50} values for tolfenpyrad dissolved in aqueous carboxymethyl cellulose were greater than or equal to 113 mg/kg bw in two rat studies, and the oral LD_{50} for tolfenpyrad dissolved in olive oil was greater than or equal to 75 mg/kg bw in one rat study. The LD_{50} for dermal toxicity was greater than 2000 mg/kg bw in rats. The acute inhalation LC_{50} was 1.50–2.21 mg/L. Tolfenpyrad was not irritating to the skin and slightly irritating to the eye of rabbits. Tolfenpyrad was not a skin sensitizer in a Magnusson and Kligman test in guinea-pigs.

In repeated-dose toxicity studies with tolfenpyrad, multiple adverse effects were observed. In a 90-day toxicity study in mice using dietary concentrations of 0, 15, 100 and 300 ppm (equal to 0, 2.4, 15.9 and 46.2 mg/kg bw per day for males and 0, 3.0, 20.2 and 57.9 mg/kg bw per day for females, respectively), the NOAEL was 100 ppm (equal to 15.9 mg/kg bw per day), based on increased ASAT activity and increased relative heart weight in males and increased relative liver weight in both sexes at 300 ppm (equal to 46.2 mg/kg bw per day). In a 90-day toxicity study in rats using dietary concentrations of 0, 15, 80 and 160 ppm (equal to 0, 0.906, 4.78 and 9.33 mg/kg bw per day for males and 0, 1.01, 5.17 and 9.32 mg/kg bw per day for females, respectively), the NOAEL was 15 ppm (equal to 0.906 mg/kg bw per day), based on changes in clinical chemistry, a reduced white blood cell count, dark brown change of the liver, hypertrophy of the proximal renal tubular epithelium and the acinar cells in the mandibular glands in females and an increase in mast cells in the

Table 12. List of abbreviations and chemical names of metabolites used in the report

Abbreviation	Chemical name
OH-PT	4-Chloro-3-(1-hydroxyethyl)-1-methyl-*N*-[4-(*p*-tolyloxy)benzyl]pyrazole-5-carboxamide
CO-PT	3-Acetyl-4-chloro-1-methyl-*N*-[4-(*p*-tolyloxy)benzyl]pyrazole-5-carboxamide
PT-CA	4-[4-[(4-Chloro-3-ethyl-1-methylpyrazol-5-yl)carbonylaminomethyl]phenoxy]benzoic acid
OH-PT-CA	4-[4-[(4-Chloro-3-(1-hydroxyethyl)-1-methylpyrazol-5-yl)carbonylaminomethyl]-phenoxy]benzoic acid
T-CA	4-(*p*-Tolyloxy)benzoic acid
CA-T-CA	4,4′-Oxydibenzoic acid
PAM	4-Chloro-3-ethyl-1-methylpyrazole-5-carboxamide
OH-PAM	4-Chloro-3-(1-hydroxyethyl)-1-methylpyrazole-5-carboxamide
Sul-OH-PT-CA	4-[4-[(4-Chloro-1-methyl-3-(1-sulfoxyethyl)pyrazol-5-yl)carbonylaminomethyl]-phenoxy]benzoic acid
PT-CA-TA	4-[4-[(4-Chloro-3-ethyl-1-methylpyrazol-5-yl)carbonylaminomethyl]phenoxy]phenyl-carbonylamino]ethane sulfonic acid
PT(A)-4OH	4-Chloro-3-ethyl-*N*-(4-hydroxybenzyl)-1-methylpyrazole-5-carboxamide
T-AM	4-(4-Tolyloxy)benzamide
PCA	4-Chloro-3-ethyl-1-methylpyrazole-5-carboxylic acid
OH-T-CA	4-[4-(Hydroxymethyl) phenoxy] benzoic acid
PT-CA-Gluc	Glucuronide conjugate of 4-[4-[(4-chloro-3-ethyl-1-methylpyrazol-5-yl)carbonylamino-methyl]phenoxy]benzoic acid

mesenteric lymph nodes, diffuse hypertrophy of hepatocytes and hypertrophy of the pancreatic acinar cells in both sexes at 80 ppm (equal to 4.78 mg/kg bw per day).

In a 28-day as well as a 90-day capsule study in dogs using doses of 0, 1, 5 and 10 mg/kg bw per day, the NOAEL was 1 mg/kg bw per day, based on the increased incidence of vomiting at 5 mg/kg bw per day. In a second 90-day capsule study in dogs with administration of tolfenpyrad at doses of 0, 10, 30 and 100 mg/kg bw per day, mild toxicity (i.e. vomiting, soft and mucoid faeces) was observed at 10 mg/kg bw per day, the lowest dose tested. Severe toxicity including mortality was observed at doses of 30 and 100 mg/kg bw per day. In a 1-year capsule study in dogs using doses of 0, 1, 5 and 20/10 mg/kg bw per day, the NOAEL was 1 mg/kg bw per day, based on increased incidences of vomiting, soft or mucoid stool and salivation and increased ALAT levels at 5 mg/kg bw per day. In all the studies in dogs, vomiting and soft stool were observed as early as the 1st day of dosing. Therefore, the overall NOAEL for these effects was 1 mg/kg bw per day, with an overall LOAEL of 5 mg/kg bw per day.

In a 78-week toxicity study in mice using dietary concentrations of 0, 15, 150 and 500/400/300 ppm (equal to 0, 2.2, 20.8 and 60.9 mg/kg bw per day for males and 0, 2.8, 27.1 and 75.9 mg/kg bw per day for females, respectively), the NOAEL was 15 ppm (equal to 2.2 mg/kg bw per day), based on decreased body weight gain and feed consumption and changes in organ weights observed in males and females at 150 ppm (equal to 20.8 mg/kg bw per day). No increased incidences of tumours were observed at doses up to 500/400/300 ppm (equal to 60.9 mg/kg bw per day), the highest concentration tested.

In a 2-year toxicity and carcinogenicity study in rats using dietary concentrations of 0, 15, 40 and 80 ppm (equal to 0, 0.56, 1.5 and 3.1 mg/kg bw per day for males and 0, 0.69, 1.9 and 3.8 mg/kg bw per day for females, respectively), the NOAEL was 15 ppm (equal to 0.56 mg/kg bw per day), based on reduced feed intake in males and females and increased severity of basophilic foci of altered hepatocytes, sinus histiocytosis of the mesenteric lymph nodes and hypertrophy of the proximal renal

tubule epithelia in females at 40 ppm (equal to 1.5 mg/kg bw per day). There was no compound-related increase in the incidence of tumours.

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

Tolfenpyrad was tested for genotoxicity in an adequate range of in vitro and in vivo assays. These assays provided no evidence of genotoxic potential.

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

A standard two-generation reproductive toxicity study in rats was performed in which dietary concentrations were adjusted to maintain the desired dose levels of 0, 0.75, 1.5 and 3 mg/kg bw per day. This was followed by a modified non-GLP two-generation reproductive toxicity study focusing on the effects of tolfenpyrad on immune function, in which the dietary concentrations were adjusted to maintain target dose levels of 0, 0.75 and 3 mg/kg bw per day. The overall NOAEL for parental toxicity from these two studies was 1.5 mg/kg bw per day, based on moribundity, decreased body weight gain and decreased feed consumption at 3 mg/kg bw per day observed in the first study. The overall NOAEL for offspring toxicity was 0.75 mg/kg bw per day, based on a reduction in absolute and relative thymus weights in males and females of the F_2 generation at 1.5 mg/kg bw per day, observed in the first study, and on reduced body weight gain during lactation and a reduced number of live F_1 pups at PND 4, black change in the peritoneal cavity after birth in F_1 and F_2 pups, lower thymus and spleen weights soon after birth in F_1 pups and in F_2 male pups, reduced thymus and spleen cellularity, and changes in immune cell ratios in the spleen in F_2 male pups at 3 mg/kg bw per day, observed in the second study. The Meeting concluded that the small reductions in absolute and relative thymus weights observed at 0.75 mg/kg bw per day, in the absence of other relevant effects, were not toxicologically significant. In the second study, humoral immunity and cellular immune function were normal in adult F_1 and F_2 rats. The overall NOAEL for reproductive toxicity was 1.5 mg/kg bw per day, based on a range of effects occurring late in gestation resulting in a reduced number of live offspring, observed in the first but not in the second study, at 3 mg/kg bw per day.

In a developmental toxicity study in rats using doses of 0, 1, 3 and 4.5 mg/kg bw per day, the NOAEL for maternal toxicity was 1 mg/kg bw per day, based on reduced body weight gain and feed consumption at 3 mg/kg bw per day, observed during the first days of treatment. The NOAEL for embryo and fetal toxicity was 3 mg/kg bw per day, based on decreased fetal weight, increased incidence of skeletal variations and delayed ossification observed at 4.5 mg/kg bw per day. No evidence of a teratogenic effect was observed.

In a developmental toxicity study in rabbits using doses of 0, 1, 3 and 6 mg/kg bw per day, the NOAEL for maternal toxicity was 1 mg/kg bw per day, based on one mortality observed at 3 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 6 mg/kg bw per day, the highest dose tested. No evidence of a teratogenic effect was observed.

The Meeting concluded that tolfenpyrad is not teratogenic in rats or rabbits.

In an acute oral (gavage) neurotoxicity study in rats using doses of 0, 20, 40 and 60 mg/kg bw in males and 0, 10, 20 and 40 mg/kg bw in females, the LOAEL was 10 mg/kg bw, the lowest dose tested, based on reductions in body weight and feed consumption observed in females on the day of dosing. Clinical signs were observed at 40 mg/kg bw in females and at 60 mg/kg bw in males. No neurotoxicity was observed.

In a 90-day neurotoxicity study in rats using dietary concentrations of 0, 15, 40 and 80 ppm (equal to 0, 1.0, 2.7 and 5.4 mg/kg bw per day for males and 0, 1.2, 3.2 and 6.0 mg/kg bw per day for females, respectively), the NOAEL was 40 ppm (equal to 3.2 mg/kg bw per day), based on reductions in body weight gain and feed consumption in females at 80 ppm (equal to 6.0 mg/kg bw per day). No clinical, functional or histological signs of neurotoxicity were observed at doses up to 80 ppm (equal to 5.4 mg/kg bw per day), the highest dose tested.

The Meeting concluded that tolfenpyrad is not neurotoxic.

In the modified two-generation reproductive toxicity study focusing on effects of tolfenpyrad on immune function (see above), tolfenpyrad caused changes in the immune system in rat pups but did not affect normal humoral immunity or cellular immune function in adult rats.

Toxicological data on metabolites and/or degradates

Studies of acute oral toxicity were performed with the tolfenpyrad metabolites OH-PT, PT-CA, PT(A)-4OH, T-CA, T-AM, CA-T-CA, OH-T-CA, PAM, OH-PAM and PCA dissolved in aqueous carboxymethyl cellulose. In general, the metabolites had low acute toxicity, except for OH-PT ($LD_{50} \geq 35.5$ mg/kg bw) and PT-CA ($LD_{50} \geq 15.4$ mg/kg bw), which were slightly more toxic than tolfenpyrad. All these metabolites showed negative results in tests for reverse mutation induction in bacteria (Note: PT(A)-4OH and PAM were not tested). OH-PT and PT-CA were also tested in a chromosomal aberration test in vitro and a micronucleus test in vivo. No genotoxicity was observed. In a 4-week dietary study in rats, the toxicity of tolfenpyrad at dietary concentrations of 0, 10, 30 and 100 ppm was compared with the toxicities of PT-CA and OH-PT at dietary concentrations of 0, 3, 10, 30 and 100 ppm. The NOAEL for tolfenpyrad was 30 ppm (equal to 2.5 mg/kg bw per day), based on a mild reduction in feed consumption in males and females, a mild reduction in body weight gain observed in males and increased incidences and severity of hypertrophy of acinar cells in pancreas in females at 100 ppm (equal to 8 mg/kg bw per day). The NOAEL for PT-CA was 100 ppm (equal to 8.1 mg/kg bw per day), the highest dose tested, and the NOAEL for OH-PT was 100 ppm (equal to 8.4 mg/kg bw per day), the highest dose tested. In view of the lower LD_{50} values for the metabolites PT-CA and OH-PT compared with tolfenpyrad, the Meeting considered these two compounds to be toxicologically relevant.

In the absence of any toxicological information on the livestock metabolite OH-PT-CA, but on consideration of the similarity of its structure to that of tolfenpyrad, it was assumed that OH-PT-CA was of similar toxic potency to tolfenpyrad. No data were available on the toxicity of the livestock metabolites, PT-CA conjugates and OH-PT-CA conjugates. However, as these are likely to be hydrolysed to PT-CA and OH-PT-CA, respectively, the Meeting concluded that their toxicities should be considered equivalent to those of PT-CA and OH-PT-CA.

Human data

No information on adverse health effects or poisoning in manufacturing plant personnel or in operators and workers exposed to tolfenpyrad was available.

The Meeting concluded that the existing database on tolfenpyrad is sufficient to characterize the potential hazards to fetuses, infants and children.

Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) for tolfenpyrad of 0–0.006 mg/kg bw on the basis of a NOAEL of 0.56 mg/kg bw per day in a 2-year rat study with tolfenpyrad, for reduced feed intake, increased severity of basophilic foci of altered hepatocytes, sinus histiocytosis of the mesenteric lymph nodes and hypertrophy of the proximal tubule epithelia in females at 1.5 mg/kg bw per day, using a safety factor of 100.

The Meeting established an acute reference dose (ARfD) of 0.01 mg/kg bw for tolfenpyrad based on a NOAEL of 1 mg/kg bw per day for reduced body weight and feed consumption observed during the first days of treatment in a developmental toxicity study with tolfenpyrad in rats at 3 mg/kg bw and an overall NOAEL of 1 mg/kg bw per day for vomiting and soft stool observed on the 1st day of treatment in 28-day, 90-day and 1-year studies with tolfenpyrad in dogs at 5 mg/kg bw per day. A

safety factor of 100 was applied. The ARfD provides a margin of exposure of 1000 over the LOAEL in the acute neurotoxicity study in rats. The Meeting considered it unlikely that the acute effects observed in rats and dogs are the result of the unpalatability of tolfenpyrad, as the effects were observed after gavage or capsule administration. The Meeting also considered it unlikely that the acute effects were secondary to local gastrointestinal irritation, as no such effects were reported in any of the studies.

The Meeting considered that the ADI and ARfD are also applicable to the metabolites PT-CA and OH-PT, which showed similar toxicity to tolfenpyrad in LD_{50} studies but lower toxicity in a 4-week dietary study. In addition, the Meeting considered the ADI and ARfD applicable to all the livestock metabolites: OH-PT-CA, PT-CA conjugates and OH-PT-CA conjugates. The Meeting noted that in the absence of data on the effects of these metabolites in long-term and developmental toxicity studies in rats and capsule studies in dogs (i.e. studies that formed the basis of the ADI and ARfD), it would not be possible to establish the relative potency of the metabolites to tolfenpyrad in order to refine the dietary exposure assessment.

Levels relevant for risk assessment of tolfenpyrad

Species	Study	Effect	NOAEL	LOAEL
Mouse	Eighteen-month study of toxicity and carcinogenicity[a]	Toxicity	15 ppm, equal to 2.2 mg/kg bw per day	150 ppm, equal to 20.8 mg/kg bw per day
		Carcinogenicity	500/400/300 ppm, equal to 60.9 mg/kg bw per day[b]	–
Rat	Thirteen-week study of toxicity[a]	Toxicity	15 ppm, equal to 0.906 mg/kg bw per day	80 ppm, equal to 4.78 mg/kg bw per day
	Two-year study of toxicity and carcinogenicity[a]	Toxicity	15 ppm, equal to 0.56 mg/kg bw per day	40 ppm, equal to 1.5 mg/kg bw per day
		Carcinogenicity	80 ppm, equal to 3.1 mg/kg bw per day[b]	–
	Two-generation studies of reproductive toxicity[a,c,d]	Parental toxicity	1.5 mg/kg bw per day	3 mg/kg bw per day
		Offspring toxicity	0.75 mg/kg bw per day	1.5 mg/kg bw per day
		Reproductive toxicity	1.5 mg/kg bw per day	3 mg/kg bw per day
	Developmental toxicity study[d]	Maternal toxicity	1 mg/kg bw per day	3 mg/kg bw per day
		Embryo and fetal toxicity	3 mg/kg bw per day	4.5 mg/kg bw per day
	Acute neurotoxicity study[e]	Neurotoxicity	40 mg/kg bw[b]	–
		Toxicity	–	10 mg/kg bw[f]
	Ninety-day neurotoxicity study[a]	Neurotoxicity	80 ppm, equal to 5.4 mg/kg bw per day[b]	–
Rabbit	Developmental toxicity study[e]	Maternal toxicity	1 mg/kg bw per day	3 mg/kg bw per day
		Embryo and fetal toxicity	6 mg/kg bw per day[b]	–
Dog	Four-week, 13-week and 1-year studies of toxicity[c,g,h]	Toxicity	1 mg/kg bw per day	5 mg/kg bw per day

LOAEL: lowest-observed-adverse-effect level; NOAEL:no-observed-adverse-effect level

[a] Dietary administration.

[b] Highest dose tested.

[c] Two or more studies combined.

[d] Dietary concentrations of tolfenpyrad were adjusted over the course of the study in order to obtain the required daily doses (mg/kg bw per day) for the different dose groups and sexes. Therefore, ppm values are not presented.
[e] Gavage administration.
[f] Lowest dose tested.
[g] Identical NOAELs and LOAELs were observed in all three dog studies. In all studies, vomiting and soft stool were observed on the 1st day of testing.
[h] Capsule administration.

Estimate of acceptable daily intake

0–0.006 mg/kg bw

Estimate of acute reference dose

0.01 mg/kg bw

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

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

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

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid (T_{max} 2–8 h); ≥ 58% (rats)
Dermal absorption	No data available (probably relatively low in view of data from LD_{50} and short-term studies with oral and dermal dosing)
Distribution	Widely distributed (rats)
Potential for accumulation	Low
Rate and extent of excretion	88–93% in faeces and 2–3% in urine; plasma half-lives at 1 and 20 mg/kg bw: 11–28 h
Metabolism in animals	Extensive, rapidly metabolized in the liver and subsequently conjugated and then excreted into bile
Toxicologically significant compounds in animals, plants and the environment	Tolfenpyrad, PT-CA, PT-CA conjugates, OH-PT, OH-PT-CA, OH-PT-CA conjugates
Acute toxicity	
Rat, LD_{50}, oral	Dissolved in aqueous carboxymethyl cellulose: ≥ 113 mg/kg bw Dissolved in olive oil: ≥ 75 mg/kg bw
Rat, LD_{50}, dermal	> 2 000 mg/kg bw
Rat, LC_{50}, inhalation	≥ 1.50 mg/L
Rabbit, dermal irritation	Not irritating
Rabbit, ocular irritation	Slightly irritating
Dermal sensitization	Not sensitizing (Magnusson and Kligman guinea-pig test)
Short-term studies of toxicity	
Target/critical effect	Many end-points affected
Lowest relevant oral NOAEL	0.906 mg/kg bw per day (rat)
Lowest relevant dermal NOAEL	200 mg/kg bw per day, the highest dose tested (rat)

Lowest relevant inhalatory NOAEC	2 mg/m^3 (rat)
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Feed intake, liver, kidneys, mesenteric lymph nodes (rats)
Lowest relevant NOAEL	0.56 mg/kg bw per day (rat)
Carcinogenicity	Not carcinogenic
Genotoxicity	
	Not genotoxic
Reproductive toxicity	
Target/critical effect	Reduced number of live offspring at parentally toxic doses
Lowest relevant parental NOAEL	1.5 mg/kg bw per day
Lowest relevant offspring NOAEL	0.75 mg/kg bw per day
Lowest relevant reproductive NOAEL	1.5 mg/kg bw per day
Developmental toxicity	
Target/critical effect	Decreased fetal weight, increased skeletal variations at maternally toxic doses
Lowest relevant maternal NOAEL	1 mg/kg bw per day (rat, rabbit)
Lowest relevant embryo/fetal NOAEL	3 mg/kg bw per day (rat)
Neurotoxicity	
Acute and subchronic neurotoxicity	Not neurotoxic
Other toxicological studies	
Immunotoxicity	Increased thymus and spleen weights and changed immune cell ratios in pups, but not in adults
Studies with PT-CA	
- Acute toxicity	$LD_{50} \geq 15.4$ mg/kg bw
- Four-week dietary toxicity	8.1 mg/kg bw per day, the highest dose tested
- Genotoxicity	Not genotoxic
Studies with OH-PT	
- Acute toxicity	$LD_{50} \geq 35.5$ mg/kg bw
- Four-week dietary toxicity	8.4 mg/kg bw per day, the highest dose tested
- Genotoxicity	Not genotoxic
Medical data	
	No data

LC_{50}: median lethal concentration; LD_{50}: median lethal dose; NOAEC: no-observed-adverse-effect concentration; NOAEL: no-observed-adverse-effect level; T_{max}: time to reach peak concentration

Summary

	Value	Study	Safety factor
ADI	0–0.006 mg/kg bw	Two-year study of toxicity in rats	100
ARfD	0.01 mg/kg bw	Developmental toxicity study in rats; 28-day, 90-day and 1-year toxicity studies in dogs	100

ADI: acceptable daily intake; ARfD: acute reference dose

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TRIFLUMIZOLE

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Explanation

Triflumizole (Fig. 1) is the common name provisionally approved by the International Organization for Standardization for (*E*)-4-chloro-α,α,α-trifluoro-*N*-(1-imidazol-1-yl-2-propoxyethylidene)-*o*-toluidine (International Union of Pure and Applied Chemistry), for which the Chemical Abstracts Service number is 68694-11-1. Triflumizole is a fungicide used for the control of powdery mildew, such as *Sphaerotheca fuliginea*, *Sphaerotheca pannosa*, *Erysiphe cichoracearum* and others. As a consequence of ergosterol biosynthesis inhibition, spore germination, mycelial growth and the spread of the fungi within the plants are inhibited.

Fig. 1. Structure of triflumizole (NF-114)

Triflumizole 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 studies evaluated in this monograph were performed by good laboratory practice (GLP)–certified laboratories and complied with the relevant Organisation for Economic Co-operation and Development (OECD) and/or United States Environmental Protection Agency test guidelines, unless indicated otherwise.

Evaluation for acceptable daily intake

1. Biochemical aspects

1.1 Absorption, distribution and excretion

Rats

The absorption, distribution and excretion of triflumizole in Sprague-Dawley rats following oral administration have been assessed in a quantitative single low-dose study, a single high-dose study and a 14-day repeated-dose study (Table 1). The experiments were performed by dosing rats with a mixture of (phenyl-U-^{14}C)-labelled triflumizole and triflumizole technical, which was subsequently diluted with dimethyl sulfoxide (DMSO). No specific information is available on biliary excretion, as bile duct–cannulated rats were not used.

Table 1. Overview of absorption, distribution and excretion studies

	Single low dose	Repeated low dose	Single high dose
Dose (mg/kg bw)	10	10 (days 1–13 unlabelled test substance, labelled test substance on day 14)	300
No. of animals	5 M, 5 F	5 M, 5 F	5 M, 5 F
Route	Oral, gavage	Oral, gavage	Oral, gavage
Purity			
- Radiochemical	98%	98%	98%
- Unlabelled	99.8%	98.3%	98.7%
Study duration	2 days	16 days	4 days
GLP	No	Yes	No
Reference	Soeda (1983)	Soeda & Mizuno (1988)	Soeda (1984)

bw: body weight; F: female; GLP: good laboratory practice; M: male

In a preliminary experiment with two male rats in which $^{14}CO_2$ was trapped, over a period of 4 days, approximately 0.07% of the administered dose was recovered from volatiles, approximately 72% from urine, approximately 27% from faeces and approximately 1% from the carcass (recovery approximately 97%). Based on these results, volatiles were not trapped in the main experiment, because the amount of radioactivity recovered from volatiles was considered to be negligible.

During the initial 48 hours (or 96 hours, single high-dose study) after dosing of labelled test substance, blood, urine and faeces were collected for analysis of the radioactivity and identification of metabolites. Blood samples were collected at 0.25, 0.5, 1, 2, 3, 4, 6, 12 and 24 hours after dose administration and daily thereafter, and urine and faeces were collected at 24-hour intervals. Two days after dose administration, the animals were killed, and various tissues were excised for analysis: fat (adipose tissue), gonads (testes and epididymides from males, ovaries and uterus from females), spleen, kidney, liver, heart, lung, thymus, brain, pancreas, adrenal, thyroid gland, urinary bladder, femoral muscle and femoral bone, a part of dorsal skin, stomach, small intestine, large intestine, blind intestine and the digestive organ contents from each of these, and the remaining body structures ("carcass"). All samples were analysed for radioactivity by (combustion) liquid scintillation counting. Pharmacokinetic analysis was based on a one-compartment model with a first-order rate. Individual 0- to 1-day urine and faecal samples from each sex were pooled and analysed for metabolites. Metabolites present in urine were concentrated, separated in fractions by column chromatography and analysed by thin-layer chromatography (TLC) after being mixed with authentic standards. The standards were detected under ultraviolet light, and the corresponding test spots were analysed for radioactivity by liquid scintillation counting. Metabolites present in faeces were extracted with methanol, concentrated under vacuum (rotary evaporator) and dissolved in water, then analysed as described for urine samples.

There were no clear sex differences in oral absorption. Based on the radiolabel recovered from urine, tissues and carcass 48 hours after administration, the oral absorption following single or repeated dosing with 10 mg/kg body weight (bw) was at least 72% (see Table 2). The oral absorption was at least 72% at 48 hours after administration of a single high dose (300 mg/kg bw), based on radiolabel recovered from urine, and at least 79% 96 hours after administration, based on radiolabel recovered from urine, tissues and carcass. The oral absorption was considerably slower after the high dose than after the low dose, as evidenced by the much longer time to reach the maximum concentration in plasma (T_{max}) (see Table 3). Excretion after 10 mg/kg bw was relatively fast following both single- and repeated-dose treatments, with approximately 90% of the administered radiolabel being excreted in the first 24 hours and about 95% of the radiolabel being excreted by 48 hours. At 300 mg/kg bw, the proportion of the administered dose that was excreted by 24 hours was 45% (males) and 35% (females) of the administered dose, increasing to 99% (males) and 92% (females) at 96 hours. The majority of the radiolabel was excreted via the urinary route: approximately 75% of the administered dose at sacrifice compared with about 20% excreted via the faeces.

The radioactivity detected in tissues and the carcass 48 hours (low dose) or 96 hours (high dose) after administration was 2% in both male and female rats (Tables 4–6). In all instances, the highest concentration of radiolabel was in the liver (approximately 1 mg equivalent [eq]/kg after the low dose in both sexes; 14.5 and 8.5 mg eq/kg after the high dose in males and females, respectively). Retention in all other tissues and organs was approximately 50% or less of the concentration detected in the liver, with well perfused tissues tending to have higher concentrations. Fat was among the tissues retaining the lowest concentration of radiolabel: approximately one tenth of the liver concentration. It should be noted that the brain retained relatively high concentrations (one third to one half of the liver concentration). This was also the case with the thyroid; however, this organ was measured only in the single low-dose group.

1.2 Biotransformation

Rats

The metabolism of triflumizole was investigated by analysing faecal and urine samples retained from the previous studies: single oral administration of 10 or 300 mg/kg bw (Soeda, 1983, 1984) and 14 consecutive oral doses of 10 mg/kg bw per day (Soeda & Mizuno, 1988).

Table 2. Cumulative excretion and retention of total radioactivity following oral dosing with triflumizole in rats

Fraction	Cumulative excretion/retention (% of administered radiolabel) in males/females						
	0–24 h			0–48 h			0–96 h[a]
	Single oral 10 mg/kg bw	Repeated oral 10 mg/kg bw per day	Single oral 300 mg/kg bw	Single oral 10 mg/kg bw	Repeated oral 10 mg/kg bw per day	Single oral 300 mg/kg bw	Single oral 300 mg/kg bw
Urine	71/70	72/66	36/29	74/75	74/70	77/72	79/77
Faeces	18/18	20/21	9/6	19/19	22/22	19/13	21/15
Total excreted[b]	*89/89*	*92/87*	*45/35*	*93/94*	*96/91*	*99/85*	*99/92*
Tissues & carcass	NC	NC	NC	2/2	2/2	NC	1/2
Total recovered[b]	*89/89*	*92/87*	*45/35*	*95/96*	*98/94*	*99/85*	*101/94*

NC: not calculated

[a] Animals exposed to low dose were sacrificed after 48 hours; those exposed to high dose were sacrificed after 96 hours.

[b] Due to rounding, totals may differ slightly from additions in the table.

Source: Soeda (1983, 1984); Soeda & Mizuno (1988)

Table 3. Toxicokinetic parameters: plasma

Parameter	Single oral low (males/females)	Repeated oral low (males/females)	Single oral high (males/females)
T_{max} (h)	1.8/1.7	~1	~24
C_{max} (μg eq/mL)	3.3/2.4	2.3/2.2	19/21
Approximate terminal half-life (h)	13/15	NC	NC
Volume of distribution (mL)	657/715	NC	NC

C_{max}: maximum concentration in plasma; eq: equivalent; NC: not calculated; T_{max}: time to reach C_{max}

Source: Soeda (1983, 1984); Soeda & Mizuno (1988)

Table 4 . Levels of triflumizole equivalent in rat tissue at 2 days after administration of 10 mg/kg bw (single low dose)

Organ/tissue	Levels (ppm) of triflumizole equivalent (mean ± SE)	
	Males	Females
Plasma	0.26 ± 0.03	0.20 -
Brain	0.42 ± 0.02	0.30 -
Liver	1.22 ± 0.04	0.92 ± 0.06
Kidney	0.50 -	0.26 ± 0.03
Femoral muscle	0.10 -	0.10 -
Femoral bone	0.10 -	0.10 -
Fat	0.10 -	0.02 ± 0.02
Dorsal skin	0.18 ± 0.07	0.18 ± 0.11
Spleen	0.30 -	0.22 ± 0.02
Heart	0.30 -	0.20 -
Lung	0.40 -	0.30 -
Pancreas	0.22 ± 0.02	0.18 ± 0.02

Organ/tissue	Levels (ppm) of triflumizole equivalent (mean ± SE)	
	Males	Females
Thyroid	0.62 ± 0.28	0.64 ± 0.17
Bladder	0.44 ± 0.11	0.29 ± 0.08
Thymus	0.10 -	0.10 -
Adrenal	0.36 ± 0.05	0.30 -
Carcass	0.12 ± 0.02	0.14 ± 0.03
Testis	0.16 ± 0.03	–
Epididymis	0.32 ± 0.04	–
Ovary	–	0.16 ± 0.03
Uterus	–	0.10 -
Stomach	0.34 ± 0.06	0.22 ± 0.04
Small intestine	0.10 -	0.08 ± 0.02
Large intestine	0.50 ± 0.19	0.10 -
Blind intestine	0.70 ± 0.06	0.06 ± 0.03
Stomach contents	0.40 ± 0.26	0.01 -
Small intestinal contents	0.36 ± 0.05	0.16 ± 0.05
Large intestinal contents	0.74 ± 0.09	0.58 ± 0.08
Blind intestinal contents	0.44 ± 0.05	0.46 ± 0.08

- indicates a standard error of < 0.01; ppm: parts per million; SE: standard error
Source: Soeda (1983)

Table 5. Levels of triflumizole equivalent in rat tissue at 2 days after administration of 300 mg/kg bw (single high dose)

Organ/tissue	Levels (ppm) of triflumizole equivalent (mean ± SE)	
	Males	Females
Plasma	2.04 ± 0.16	0.90 ± 0.18
Brain	5.18 ± 0.41	4.40 ± 1.30
Liver	14.52 ± 0.95	8.48 ± 1.50
Kidney	5.58 ± 0.25	4.18 ± 0.42
Femoral muscle	1.20 ± 0.15	1.04 ± 0.29
Femoral bone	0.78 ± 0.08	0.56 ± 0.07
Fat	1.36 ± 0.12	1.08 ± 0.28
Spleen	2.74 ± 0.26	2.36 ± 0.56
Heart	3.82 ± 0.50	2.76 ± 0.69
Lung	3.52 ± 0.22	2.44 ± 0.34
Pancreas	2.46 ± 0.25	1.92 ± 0.42
Adrenal	2.90 ± 0.29	2.76 ± 0.44
Carcass	2.28 ± 0.14	4.14 ± 0.61
Testis	1.46 ± 0.18	–
Ovary	–	1.24 ± 0.27
Uterus	–	1.36 ± 0.51
Digestive organs & their contents	1.82 ± 0.27	3.02 ± 1.05

ppm: parts per million; SE: standard error
Source: Soeda (1984)

Table 6. Levels of triflumizole equivalent in rat tissue at 2 days after administration of 10 mg/kg bw (repeated low dose)

Organ/tissue	Levels (ppm) of triflumizole equivalent (mean ± SE)	
	Males	Females
Plasma	0.18 -	0.13 -
Brain	0.42 ± 0.02	0.43 ± 0.04
Liver	1.01 ± 0.03	1.14 ± 0.05
Kidney	0.39 -	0.45 ± 0.02
Femoral muscle	0.08 -	0.10 -
Femoral bone	0.06 -	0.07 -
Fat	0.07 -	0.11 -
Spleen	0.20 -	0.24 ± 0.02
Heart	0.24 -	0.25 ± 0.02
Lung	0.31 -	0.32 ± 0.02
Pancreas	0.18 -	0.19 ± 0.02
Adrenal	0.27 -	0.28 ± 0.02
Carcass	0.11 -	0.16 ± 0.04
Testis	0.12 -	–
Ovary	–	0.14 -
Uterus	–	0.11 -
Digestive organs and their contents	0.28 -	0.33 ± 0.03

- indicates a standard error of < 0.01; ppm: parts per million; SE: standard error
Source: Soeda & Mizuno (1988)

Triflumizole is extensively metabolized: less than 2% of the radiolabel recovered from urine or faeces was identified as parent compound (Table 7). A few differences in metabolite pattern were observed between males and females after repeated low and single high doses, but not after a single low dose. The major urinary metabolites are the sulfate conjugates of FM-8-1 and FA-1-5, each representing approximately 20% of the radiolabel recovered after the single low dose from that matrix and, respectively, approximately 11% and 20% after the high dose. In faeces, FD-2-1 is a major metabolite in all dose regimens (~6–10% of the recovered radiolabel). Considerable differences between dose regimens exist with respect to other major metabolites. FM-2-1 is the major metabolite after a single oral low dose (~9% of radiolabel recovered in faeces), but represents less than 2% for the other dose regimens. FA-1-1 is a major metabolite after single and repeated low dosing (~5–10%), but not after single oral high dosing (< 3%), whereas FD-1-1 is the major metabolite after a single oral high dose (~16%) and a minor metabolite after single and repeated low doses (< 2%). The metabolite FM-6-1 was tentatively identified by TLC co-chromatography, and the radioactive band corresponding to the authentic FM-6-1 was obscure, because of its low radioactivity.

There was also a supplemental, non-GLP study to the main single-dose metabolism studies (Soeda, 1983, 1984). The purpose of this study was to confirm the presence of the metabolite FM-6-1 in rat faeces and urine recovered during the main metabolism studies. Portions of the methanol fraction of 0- to 2-day urine and methanol extract of 0- to 2-day faeces (~74 kBq) in each experimental group or sex of the high and low single-dose studies were combined and purified using silica gel column chromatography, fractioned according to the radioactive chromatograms and analysed by TLC. The fraction corresponding to FM-6-1 was subjected to preparative TLC, and the radioactive band was extracted with acetone and then analysed using gas chromatography–mass spectrometry (GC-MS) and compared with the FM-6-1 standard. The GC-MS results confirmed the presence of FM-6-1 in the urine and faecal samples (Soeda, 1985).

Table 7. Distribution of radiolabel from the parent compound and major metabolites

Compound (code)	% of radiolabel recovered from excreta in males/females								
	Single oral low			Repeated oral low			Single oral high		
	Urine[a]	Faeces[a]	Total[b]	Urine[a]	Faeces[a]	Total[b]	Urine[a]	Faeces[a]	Total[b]
NF-114 (parent compound)	0.7/0.6	1.5/1.5	0.9/0.7	0.1/0.1	0.3/0.4	0.1/0.2	0.8/1.4	0.9/0.8	0.8/1.3
FM-2-1	0.6/0.5	8.3/9.3	2.1/2.3	0.1/0.1	0.5/0.3	0.2/0.1	0.4/0.6	1.8/1.2	0.7/0.6
FM-6-1	0.4/0.3	1.3/1.5	0.6/0.5	0.1/0.2	1.4/1.0	0.4/0.4	0.5/2.1	0.9/1.2	0.5/1.9
FM-7-1	0.3/0.5	1.1/1.0	0.5/0.6	0.1/0.1	0.1/0.2	0.1/0.1	0.1/0.2	0.6/0.4	0.2/0.2
FM-8-1	2.6/2.4	4.2/4.5	2.9/2.8	3.0/2.9	5.6/4.8	3.5/3.2	0.4/0.5	2.2/1.8	0.8/0.7
FM-8-1-S	19/20	4.2/3.6	15/16	19/21	4.4/11	15/18	13/9	1.6/2.3	11/7.5
FD-1-1	0.1/0.2	1.8/1.5	0.5/0.5	0.1/0.1	0.7/0.8	0.2/0.3	3.0/3.2	14/18	5.2/5.4
FD-2-1	1.8/1.4	6.1/5.5	2.6/2.2	1.4/1.7	10/10	3.3/3.6	0.7/1.0	9.8/5.2	2.5/1.7
FD-2-1-S	2.1/2.3	3.1/3.1	2.3/2.5	5.4/5.1	3.0/4.2	4.8/4.8	5.2/4.8	0.7/0.6	4.2/4.0
FD-2-1-G	5.6/6.4	1.3/1.1	4.6/5.2	3.0/4.3	2.2/1.6	2.7/3.5	4.9/1.6	1.5/1.6	4.2/3.2
FD-4-1	0.8/0.8	1.0/1.1	0.8/0.8	0.6/0.6	1.4/1.2	0.7/0.8	0.4/1.7	0.6/1.7	0.5/1.0
FD-6-1	1.0/1.0	3.4/2.2	1.4/1.3	0.6/1.0	5.5/3.7	1.7/1.6	0.8/0.8	4.7/1.9	1.6/0.9
FD-7-1	7.5/6.4	1.8/1.7	6.2/5.3	5.7/5.1	1.7/2.8	4.7/4.4	3.5/0.9	1.4/1.7	3.0/1.0
FA-1-1	1.0/0.7	7.8/7.6	2.3/2.1	4.0/0.4	9.6/4.6	5.2/1.4	0.6/1.2	2.7/1.0	1.0/1.2
FA-1-5	0.5/1.7	2.4/3.8	0.9/1.8	2.8/4.5	1.5/4.4	2.5/4.4	0.6/1.2	2.7/1.0	1.0/1.2
FA-1-5-S	20/19	–	16/15	24/23	2.7/2.8	5.9/5.9	17/24	2.8/4.3	14/21
FA-1-5-G	3.6/2.7	1.1/1.3	3.0/2.4	7.0/6.7	2.7/4.1	5.9/5.9	2.5/1.6	1.7/1.7	2.3/1.6
Unidentified metabolites	33/34	50/50	36/37	23/23	47/41	27/27	45/42	51/52	46/43
Total	*100/ 100*	*100/ 100*	*98.0/ 98.2*	*100/ 100*	*100/ 100*	*98.0/ 97.5*	*100/ 100*	*100/ 100*	*98.8/ 98.3*

G: glucuronide conjugate; S: sulfate conjugate
[a] Percentage of radioactivity found in urine or faeces.
[b] Percentage of radioactivity administered.
Source: Soeda (1983, 1984); Soeda & Mizuno (1988)

In summary, all metabolites listed in Table 8 were identified in urine as well as in faeces of the rat. In total, 16 metabolites were identified in urine and faeces and represented 60–75% of the administered radiolabel dose.

The proposed metabolic pathway for triflumizole is illustrated in Fig. 2.

2. Toxicological studies

2.1 Acute toxicity

The acute toxicity of triflumizole is summarized in Table 9.

Table 8. Metabolites identified in urine and faeces of rat

Code	Metabolite
FM-2-1	2-(4-Chloro-2-trifluoromethylphenylimino)-2-imidazol-1-yl-ethanol
FM-6-1	*N*-(4-Chloro-2-trifluoromethylphenyl)-2-propoxy-acetamidine
FM-7-1	*N*-(4-Chloro-2-trifluoromethylphenyl)-2-propoxy-acetimidic acid methyl ester
FM-8-1	*N*-(4-Chloro-2-trifluoromethylphenyl)-2-hydroxy-acetamidine
FM-8-1-S	*N*-(4-Chloro-2-trifluoromethylphenyl)-2-hydroxy-acetamidine sulfate conjugate
FD-1-1	*N*-(4-Chloro-2-trifluoromethylphenyl)-2-propoxy-acetamide
FD-2-1	*N*-(4-Chloro-2-trifluoromethylphenyl)-2-hydroxy-acetamide
FD-2-1-S	*N*-(4-Chloro-2-trifluoromethylphenyl)-2-hydroxy-acetamide sulfate conjugate
FD-2-1-G	*N*-(4-Chloro-2-trifluoromethylphenyl)-2-hydroxy-acetamide glucuronide conjugate
FD-4-1	*N*-(4-Chloro-2-trifluoromethylphenyl)-formamide
FD-6-1	*N*-(4-Chloro-2-trifluoromethylphenyl)-2-(2-hydroxypropoxy)-acetamidine
FD-7-1	*N*-(4-Chloro-2-trifluoromethylphenyl)-oxalamic acid
FA-1-1	4-Chloro-2-trifluoromethylphenylamine
FA-1-5	2-Amino-5-chloro-3-trifluoromethylphenol
FA-1-5-S	2-Amino-5-chloro-3-trifluoromethylphenol sulfate conjugate
FA-1-5-G	2-Amino-5-chloro-3-trifluoromethylphenol glucuronide conjugate

Table 9. Acute toxicity of triflumizole

Species	Strain	Sex	Route	LD_{50} (mg/kg bw)	LC_{50} (mg/L)	Reference	GLP
Rat	Wistar-SLC	M	Oral	1 057	–	Nishibe et al. (1983a)	No
		F		1 780	–		
Rat	SLC:SD	M	Dermal	> 5 000	–	Nishibe et al. (1983b)	No
		F		> 5 000	–		
Rat	Wistar Crl: WI	M & F	Inhalation	–	> 3.6	Janssen (2005)	Yes

F: female; GLP: good laboratory practice; LC_{50}: median lethal concentration; LD_{50}: median lethal dose; M: male

(a) Oral administration

An acute oral toxicity test for triflumizole (purity 98.7%; lot no. YS-200) was conducted in rats. Mortality of both male and female rats was observed in the highest dose groups. Signs of toxicity from triflumizole observed in rats included ataxia, hypotonia, ventral position, lacrimation, urinary incontinence, decreased body temperature, decreased heart rate and respiration rate and ptosis. Body weights decreased slightly in the high-dose groups (2000 mg/kg bw and up) on the 1st day, although they had recovered by the 2nd day after dosing. Haemorrhages of intestinal mucosa, thymus and stomach mucosa and dark reddish lung were observed in dead rats. However, no gross pathological change was observed in the rats surviving for 14 days. The acute oral median lethal dose (LD_{50}) of the test substance was calculated to be 1057 (863–1297) mg/kg bw for male rats and 1780 (1369–2314) mg/kg bw for female rats (Nishibe et al., 1983a).

(b) Dermal application

An acute dermal toxicity test for triflumizole (purity 98.7%; lot no. YS-200) was conducted in rats. No deaths were observed during the study, nor were any gross pathological changes observed at necropsy. No signs of toxicity were observed in male rats. Urinary incontinence was observed only in female rats on the 2nd and 3rd days. In all groups, body weight decreased on the 1st day after

application, but recovered thereafter. No dose–response relationship was seen in these two observed signs. Therefore, it was concluded that these were not caused by triflumizole. No effects on feed consumption were observed. The acute dermal LD_{50} of the test substance was found to be greater than 5000 mg/kg bw for male and female rats (Nishibe et al., 1983b).

Fig. 2. Proposed metabolic pathway

[ ] = tentatively identified

(c) Exposure by inhalation

An acute inhalation toxicity test for triflumizole (purity 99.7%; batch no. TDL-577) was conducted in rats. No mortalities occurred. Symptoms of toxicity included hunched posture, lethargy and chromodacryorrhoea (head and/or snout) among the majority of the animals between day 1 and day 4. Rales occurred in one animal on day 1, and periorbital alopecia occurred in another animal between days 4 and 13. No effects were seen on body weight or body weight gain; the body weight gain shown by the animals over the study period was considered to be normal for rats of this age and strain in this type of study. Macroscopic postmortem examination of the animals did not reveal abnormalities.

The study is considered acceptable. The acute 4-hour median lethal concentration (LC_{50}) in rats is greater than 3.6 mg/L, the maximum attainable exposure concentration (Janssen, 2005).

(d) Dermal and ocular irritation

A non-GLP dermal irritation study with triflumizole (purity 98.7%; lot no. YS-200) was performed using male Angola rabbits. No signs of erythema or oedema were observed after application of triflumizole on either intact or abraded skin (combined score = 0). Taking into account the absence of any skin reactions when applied to 9 cm^2, it is not expected that applying the same dose to 6 cm^2 will change the classification of the test substance (Nishibe et al., 1983c).

A non-GLP eye irritation study with triflumizole (purity 98.7%; lot no. YS-200) was performed using male Japanese White rabbits. At 24 hours after administration, conjunctival redness and discharge (grades 1–2) were observed in 5/6 animals. At 48 hours, no effects were observed except for conjunctival redness (grade 1) in 2/6 animals. Based upon the findings in this study, the test substance is mildly irritating to the eye of rabbits (Nishibe et al., 1983d).

(e) Dermal sensitization

In a non-GLP Magnusson and Kligman maximization test, triflumizole (purity 98.2%; lot no. YS-0155) was tested using 12 Hartley guinea-pigs per group. The study was performed partly in accordance with OECD Test Guideline 406; a negative control group should have been included. No results of the intradermal injection/topical induction are presented. Following challenge with triflumizole at 25% weight per weight (w/w), dermal responses were observed in 8/12 test animals. Sensitization of this strain of animals was positively tested with *N*-phenyl-*p*-phenylenediamine (positive control), which gave very severe allergic reactions in all areas treated.

Compared with the results found in the positive control group, the reactions elicited by triflumizole were very slight, and some of these disappeared after 48 hours. However, as no negative control group was included and no information on the dose selection was presented, it cannot be excluded that the reactions were due to sensitization instead of skin irritation. It is therefore concluded that triflumizole is sensitizing to the skin of guinea-pigs in this maximization study (Nishibe et al., 1983e).

2.2 Short-term studies of toxicity

(a) Oral administration

Mice

A 28-day feeding study in mice was not submitted. In the European Union monograph (The Netherlands, 2009), such a study is evaluated (Nishibe et al., 1980c). The no-observed-adverse-effect level (NOAEL) of that study was 200 ppm (equal to 40 mg/kg bw per day), which is comparable to the NOAEL in the 90-day feeding study described below.

In a non-GLP 90-day feeding study, groups of 20 male and 20 female Charles River ICR mice were treated with doses of triflumizole (purity 98.7%; lot no. YS-200) in the diet. The concentrations

in the feed were 0, 20, 200 and 2000 parts per million (ppm) (equal to 0, 3.2, 33 and 381 mg/kg bw per day for males and 0, 4.2, 43 and 466 mg/kg bw per day for females, respectively). An additional group of 10 animals of each sex was used for haematology and blood chemistry tests at the start of the dosing period. The procedure for obtaining blood for the blood chemistry tests was terminal. It should be noted that no cholinesterase activity was measured. The study was performed partly in accordance with OECD Test Guideline 408. Deviations from the guideline were that the animals were checked for morbidity and mortality once instead of twice a day and that no sensory stimuli tests or ophthalmological and functional observations were included. Further, blood clotting potential, urea and creatinine were not determined, and histopathological examinations of the spinal cord, aorta, female mammary gland and peripheral nerve were not performed. However, these deviations are not considered to have influenced the conclusions of the study.

In this study, a reduction in body weight gain (27% in males, 15% in females compared with controls; Table 10) and a slight increase in feed consumption (< 10% in males and females) was found in the highest dose group. Haemoglobin (males) and mean corpuscular haemoglobin concentrations (MCHC) (both sexes) were also significantly decreased (4–6%). Statistically increased levels of potassium were measured in both sexes (12–17%). In females, an increase in relative kidney weight (11% at 200 ppm and 8% at 2000 ppm) and ketone bodies in urine in 4/10 females at 2000 ppm were observed. Relative adrenal weights were also increased in females of all treated groups, but not in a dose-related manner (19–21% increase). Absolute and relative liver weights were increased in both sexes (Table 11), which corresponded with the microscopic finding of swelling of cytoplasm in the central zone of all male livers, at 2000 ppm. At 200 ppm, liver weights of males (absolute and relative weights) and relative liver, adrenal and kidney weights of females were increased, and haemoglobin levels of males were significantly decreased. Relative liver and adrenal weights were also increased in females at 20 ppm. The changes in liver weight at the middle and low doses are not considered toxicologically relevant, as the increases in relation to controls were less than 10%. The changes in blood parameters at the middle and high doses are also considered to be toxicologically irrelevant, as the relative changes are small, and the decrease in haemoglobin level is not more severe at the high dose. The significance of effects on adrenal weight observed in all short-term toxicity studies with rodents was evaluated, taking into account all submitted data, and it was concluded that the effects are not considered to be toxicologically relevant.

Table 10. Mean body weight gain in mice following dietary exposure for 13 weeks

	Mean body weight gain (g)							
	0 ppm		20 ppm		200 ppm		2 000 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
Mean	18.0	14.2	14.9*	13.3	16.5	12.9	13.1*** (−27%)	12.1 (−15%)
Standard deviation	4.4	4.2	4.3	4.4	4.2	3.8	2.4	3.8

*: $P < 0.05$; ***: $P < 0.001$

Source: Nishibe et al. (1980a)

In conclusion, based on decreased body weight gain, increased feed consumption and effects on the liver (increased liver weight, swelling of cytoplasm) at 2000 ppm (equal to 381 mg/kg bw per day), the NOAEL for triflumizole in mice was 200 ppm (equal to 33 mg/kg bw per day) (Nishibe et al., 1980a).

Rats

A 28-day feeding study in rats was not submitted for evaluation. However, in the European Union monograph (The Netherlands, 2009), such a study was evaluated (Nishibe et al., 1980d); the NOAEL was 20 ppm (equal to 2.3 mg/kg bw per day), based on increased relative ovary weight at 200 ppm (equal to 22 mg/kg bw per day), which is lower than the NOAEL achieved in the 90-day feeding study described below.

Table 11. Liver weight in mice following dietary exposure for 13 weeks

	0 ppm		20 ppm		200 ppm		2 000 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
Absolute liver weight (g)								
Mean	1.658	1.399	1.630	1.407	1.788* (+8%)	1.423	1.969*** (+19%)	1.677*** (+20%)
Standard deviation	0.193	0.204	0.171	0.158	0.212	0.230	0.200	0.226
Relative liver weight (%)								
Mean	3.632	3.604	3.770	3.852* (+7%)	3.972*** (+9%)	3.937** (+9%)	4.703*** (+29%)	4.750*** (+32%)
Standard deviation	0.253	0.329	0.181	0.333	0.250	0.368	0.310	0.413

*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$
Source: Nishibe et al. (1980a)

In a non-GLP 90-day feeding study, groups of 20 male and 20 female Charles River Sprague-Dawley rats were treated with doses of triflumizole (purity 98.7%; lot no. YS-200) in the diet. The concentrations in the feed were 0, 20, 200 and 2000 ppm (equal to 0, 1.4, 15 and 177 mg/kg bw per day for males and 0, 1.8, 17 and 218 mg/kg bw per day for females, respectively). An additional group of 10 animals of each sex was used for haematology and blood chemistry tests at the start of the dosing period. At the end of the study, all animals in the test were subjected to haematology and blood chemistry tests. Blood for the blood chemistry tests was sampled after overnight fasting. The procedure for obtaining blood for the blood chemistry tests was terminal. Storage of the samples until testing and the time elapsed between sampling and testing were not specified. Plasma cholinesterase activity was measured using the 5,5′-dithiobis-2-nitrobenzoic acid method with *S*-butyrylthiocholine iodide as substrate. The study was performed partly in accordance with OECD Test Guideline 408. Deviations from the guideline were that the animals were checked for morbidity and mortality once instead of twice a day and that no sensory stimuli tests or ophthalmological and functional observations were included. Further, blood clotting potential, urea and creatinine were not determined, and histopathological examinations of the spinal cord, aorta, female mammary gland and peripheral nerve were not performed. However, these deviations are not considered to have influenced the conclusions of the study.

Oral exposure of rats to triflumizole at a concentration of 2000 ppm for 13 weeks resulted in a significantly lower body weight gain of females over the entire study period (16% at week 13; Table 12) and of males during weeks 1, 2 and 3 of dosing (10% at week 3) and an increased feed consumption in both sexes, mainly in the first weeks of the study (up to 14% in males and 45% in females in weeks 1–4). This may indicate a rise in catabolism, which may explain the increased concentrations of blood urea nitrogen, cholesterol, total protein and albumin observed in females at the highest dose levels. Further, female red blood cell parameters (decrease in red blood cells, haemoglobin and MCHC and increase in mean cell volume [MCV]) and plasma cholinesterase activity were affected at the high dose (Table 13). Kidney weights were increased in both sexes. Decreased adrenal weights were found in males, and decreased thymus and increased spleen weights were seen in females. Absolute and relative weights of the liver were increased in both sexes, which correlated with the microscopic finding of fatty metamorphosis in the livers of all animals in this dose group (Table 14).

In males, increased liver weights were also seen in the 20 and 200 ppm groups, as well as increased kidney weights at 200 ppm. The increases in absolute weights may be attributable to the higher body weights (~5%) in these dose groups compared with controls. The increases in relative weights were low (< 10%), and no changes in related parameters were present. Therefore, these deviations are not considered to be toxicologically relevant in the low- and mid-dose groups.

Table 12. Mean body weight gain in rats following dietary exposure for 13 weeks

	Mean body weight gain (g)							
	0 ppm		20 ppm		200 ppm		2 000 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
Mean	335.1	171.7	361.5	164.0	360.5	158.3 (−8%)	321.0	144.9*** (−16%)
Standard deviation	35.0	25.0	54.0	21.0	49.3	16.4	46.6	22.0

***: $P < 0.001$
Source: Nishibe et al. (1980b)

Table 13. Plasma cholinesterase activity in rats following dietary exposure for 13 weeks

Dose (ppm)	Activity (U/mL)			
	0 months		3 months	
	Males	Females	Males	Females
0	0.18 ± 0.03	0.77 ± 0.31	0.10 ± 0.02	1.02 ± 0.24
20	–	–	0.12 ± 0.03	1.03 ± 0.33
200	–	–	0.10 ± 0.03	0.99 ± 0.27
2 000	–	–	0.10 ± 0.02	0.76 ± 0.27**

**: $P < 0.01$
Source: Nishibe et al. (1980b)

Table 14. Liver weight in rats following dietary exposure for 13 weeks

	0 ppm		20 ppm		200 ppm		2 000 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
Absolute liver weight (g)								
Mean	11.69	7.17	13.10* (+12%)	7.31	13.03* (+11%)	7.08	14.89*** (+27%)	8.49*** (+18%)
Standard deviation	1.45	0.56	1.99	0.92	1.93	0.84	2.73	1.00
Relative liver weight (%)								
Mean	2.29	2.34	2.47	2.42	2.44* (+7%)	2.38	3.00*** (+31%)	3.02*** (+29%)
Standard deviation	0.18	0.17	0.35	0.23	0.22	0.24	0.31	0.20

*: $P < 0.05$; *** P: < 0.001
Source: Nishibe et al. (1980b)

Based on the decreased body weight gain, increased feed consumption, liver effects (increased liver weight, fatty changes) and increased kidney weights at 2000 ppm (equal to 177 mg/kg bw per day), the NOAEL for triflumizole in rats was 200 ppm (equal to 15 mg/kg bw per day) (Nishibe et al., 1980b).

Dogs

In a 1-year toxicity study, groups of six male and six female Beagle dogs were treated with doses of triflumizole (purity 98.7%; lot no. TK-1114) in the diet. The study was performed in accordance with OECD Test Guideline 409. In this study, groups of purebred Beagle dogs (six of each sex per dose) were dosed with triflumizole at a dietary concentration of 0, 100, 300 or 1000 ppm

(equal to 0, 3, 10 and 34 mg/kg bw per day for males and 0, 3, 11 and 35 mg/kg bw per day for females, respectively). Feed consumption was determined each working day, and body weight once a week. At least once a day, animals were observed for signs of toxicity. Ophthalmoscopic examinations were carried out prior to treatment and during weeks 12 and 51. Clinical chemistry, haematological examinations and urine analyses were carried out once before treatment and then during dose weeks 6, 12, 26 and 51. At interim sacrifice (at 13 weeks), two animals of each sex per dose were killed. The remaining animals were killed after 52 weeks. After necropsy, all animals were subjected to gross pathological assessment, followed by histopathological examination.

Oral exposure of dogs to triflumizole at a concentration of 1000 ppm for 1 year resulted in slightly but statistically significantly decreased packed cell volume, haemoglobin and red blood cells and increased MCV levels in males only. Alkaline phosphatase levels were statistically significantly increased in males (79%) and females (63%), and liver weights were increased in males (18%) and females (25%) (Table 15). Moreover, macroscopic changes in the liver, defined as lobular pattern and granular texture, were observed in one animal in each of the male and female high-dose main and interim groups. At a dose level of 300 ppm, no adverse effects were observed. Therefore, the NOAEL was 300 ppm (equal to 10 mg/kg bw per day) (Chesterman et al., 1984).

Table 15. Liver weight in dogs[a] following 1-year dietary exposure

	0 ppm		100 ppm		300 ppm		1 000 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
Absolute liver weight (g)								
Interim (13 weeks)[a,b]	343.3		333.2		375.3		399.8*	
Interim (13 weeks)[b,c]	362.2	304.9	358.7	302.1	398.9	363.5	430.7	382.4
Terminal (52 weeks)	388.7	321.9	421.1	375.8	419.3	380.2	460.0**	402.5*
Relative liver weight (%)[a]								
Interim (13 weeks)[a,c]	3.13		3.02		3.42		3.66	
Interim (13 weeks)[c]	3.10	3.16	3.12	2.94	3.52	3.32	3.72	3.75
Terminal (52 weeks)[c]	3.15	2.97	3.43	2.96	3.42	3.30	3.77	3.50

*: $P < 0.05$; **: $P < 0.01$

[a] $n = 2$ of each sex at 13 weeks and 4 of each sex at 52 weeks. For meaningful statistics, the males and females were taken together at 13 weeks.

[b] Adjusted for body weight.

[c] Statistical analysis was not performed.

Source: Chesterman et al. (1984)

(b) Dermal application

Rats

In a 21-day dermal toxicity study, groups of six male and six female Charles River CD rats were treated with doses of triflumizole (purity 97%; lot no. 2112) on the skin. The test substance was moistened with distilled water into a paste. This was applied to the clipped dorsal skin (10% of the body surface) of the rats, at dose levels of 10, 100 and 1000 mg/kg bw per day. The application site was then wrapped with porous gauze bandages fastened with non-irritating tape. Following the 6-hour period of daily exposure, the bandages were removed, and the dosed skin areas were washed with tap water. A control group was handled in a similar way, except that no test substance was administered. The total dose administered to each rat was adjusted weekly based on the most recently recorded body weights. Daily observations on mortality and clinical signs and weekly recordings of body weights and feed consumption were performed. At the end of the study (21 days), blood and urine samples were collected, and macropathological and micropathological examinations took place. The study was

performed in accordance with OECD Test Guideline 410, except that only the treated skin, liver and kidney were examined histopathologically. As the liver is the target organ in the short-term oral toxicity studies, this deviation probably did not affect the derivation of a NOAEL.

Dermal exposure of rats to triflumizole at a concentration of 1000 mg/kg bw per day for 21 days resulted in a significant increase (16%) in relative liver weight of males. A slight increase in the incidence of vacuolar fatty change in the livers of females of the high-dose group was seen, as well as an increase in the severity of the effect. However, as the effects were not accompanied by any other effects in the liver in the same sex and as the effects in females were restricted to only mild fatty change, these liver effects in males and females are considered not to be adverse. The number of animals with skin inflammation (score: trace) was slightly higher in the high-dose groups than in the control groups. One low-dose female showed macroscopic skin effects; the skin was therefore examined microscopically and scored as mild inflammation. There is no clear dose–response relationship in severity, and the effects observed in the high-dose group were marginal (trace). The effects could be due to the application procedure. They are not considered to be related to the test substance (Table 16).

Table 16. Summary of effects in rats following dermal exposure for 6 hours/day for 21 days

	0 mg/kg bw per day		10 mg/kg bw per day		100 mg/kg bw per day		1 000 mg/kg bw per day	
	Males	Females	Males	Females	Males	Females	Males	Females
Organ weights								
Liver								
- Absolute (g)	12.78	7.92	14.42	7.64	14.67	7.60	14.60	8.97
- Relative (%)	4.18	4.49	4.54	4.23	4.67	4.45	4.83* (+16%)	4.94
Microscopic pathology								
Liver								
- Vacuolar fatty change (trace–mild)	0/6	1/6	–	–	–	–	0/6	3/6
Skin								
- Inflammation (trace)	2/6	1/6	–	1/1	–	–	3/6	3/6

*: $P < 0.05$
Source: Goldenthal (1990)

Based on the absence of adverse effects in the highest dose group, the NOAEL for triflumizole in rats was 1000 mg/kg bw per day, the highest dose tested (Goldenthal, 1990).

2.3 Long-term studies of toxicity and carcinogenicity

Mice

In a combined chronic toxicity and carcinogenicity study, groups of 50 male and 50 female B6C3F1 mice were treated with doses of triflumizole (purity 98.6%; lot no. TK-1116) in the diet for 104 weeks. The study was performed mainly in accordance with OECD Test Guideline 453. Triflumizole was administered in feed for 104 consecutive weeks at four concentrations: 0, 100, 400 and 1600 ppm (equal to 0, 16, 67 and 296 mg/kg bw per day for males and 0, 22, 88 and 362 mg/kg bw per day for females, respectively). Additional groups of 10 male and 10 female mice per dose were used for each of three interim kills, performed in weeks 26, 52 and 78 after initiation of

exposure. Baseline blood parameters were established at the start of the study, using an additional 20 males and 20 females. Clinical observations and measurements of body weight, feed consumption and water consumption were performed during the test period. Clinical chemistry parameters were measured in 6–10 of the sacrificed animals in weeks 0, 26, 52 and 78 after the study started. No blood samples or urine samples were taken 3 months after dosing started. No ophthalmoscopy was performed. Gross pathology and histopathology were performed on all animals dying spontaneously or killed in a moribund condition, as well as those killed at the scheduled necropsies. Organs of all sacrificed animals at the scheduled necropsies were weighed.

Chronic oral administration of triflumizole to mice at doses of 400 ppm and above caused primarily liver effects. A decrease in body weight gain was noted at the highest dose in males (significant, −44%) and females (not significant, −10%). Effects on liver enzymes were observed as increased levels of alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) in males administered 1600 ppm. The absolute and/or relative liver weights were increased in animals in the mid-dose and high-dose groups (Table 17). An increased number of animals in the high-dose group, compared with the control group, had macroscopic liver effects, such as nodules, white zone and enlargement. Some of these effects were also seen to a lesser degree in males and/or females in the 400 ppm group. The histopathological non-neoplastic lesions were found primarily in the liver, where several effects increased in a dose-related manner in the mid- and high-dose groups (Table 18). These histological findings included hepatic nodules and fatty metamorphosis (change) at all dose levels. Additionally, cytological alterations, pigmentation and necrosis of the liver were observed in males at 1600 ppm and, to a lesser degree, at 400 ppm.

The increase in liver fatty metamorphosis observed in males administered 100 ppm is not considered to be a toxicologically relevant effect, in the absence of additional liver effects. The decreases in the number of white blood cells in males of all dose groups do not show a consistent pattern across time or dose groups. In the absence of effects on lymphoid organs, its toxicological significance is not clear. As neither its relation to treatment nor its toxicological significance is clear, the reduction in the number of white blood cells observed in this study is not considered relevant.

An increase in the number of hepatic nodules was observed in males in all dose groups and in females in the mid- and high-dose groups, in comparison with the control group. The author of the study report considered it a non-neoplastic lesion. In view of the absence of a clear dose–effect relationship in males at the two lower doses and the non-significant increase in incidence observed in females of these dose groups, only the highest dose is considered to have resulted in a treatment-related increase in hepatic nodules. No increase in tumour incidence was observed (Tables 19 and 20).

In conclusion, the NOAEL was 100 ppm (equal to 16 mg/kg bw per day), based on non-neoplastic liver effects, such as increased organ weight and histopathological findings (nodules and fatty changes in both sexes as well as granulomatous inflammation, cytological alterations, pigmentation and necrosis in some males), in mice at 400 ppm (equal to 67 mg/kg bw per day) (Inoue, 1984).

Rats

In a combined chronic toxicity and carcinogenicity study, groups of 80 male and 80 female CD rats were treated with doses of triflumizole (purity 98.6%; lot no. TK-1116) in the diet for 104 weeks. The groups of 80 animals were split into an oncogenicity group (50 rats of each sex) and a toxicity group (30 rats of each sex, of which 10 were killed at 52 weeks). Triflumizole was administered at four concentrations: 0, 100, 400 and 1600 ppm (equal to 0, 3.5, 14 and 59 mg/kg bw per day for males and 0, 4.5, 18 and 77 mg/kg bw per day for females, respectively). Clinical observations and measurements of body weight, feed consumption and water consumption were performed during the test period. Clinical pathology parameters and urine samples were measured in 10 of the sacrificed animals of each sex in weeks 0, 26, 50 and 77/78 after the study started. Ophthalmoscopy was performed in controls and high-dose animals before treatment and in weeks 6, 13, 27, 51, 78 and 102. Gross pathology and histopathology were performed on all animals dying spontaneously or killed in a moribund condition, as well as those killed at the scheduled necropsies.

Table 17. Liver weight findings in mice following chronic dietary exposure

	0 ppm		100 ppm		400 ppm		1 600 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
Absolute liver weight (g)								
Week 26								
- Mean	1.33	1.12	1.37	1.22	1.36	1.27*	1.83***	1.57***
- Standard deviation	0.08	0.12	0.12	0.11	0.10	0.13	0.21	0.16
Week 52								
- Mean	1.57	1.38	1.59	1.40	1.79	1.50*	2.22***	1.83***
- Standard deviation	0.20	0.11	0.28	0.16	0.37	0.10	0.21	0.09
Week 78								
- Mean	1.74	1.46	1.90	1.48	1.57	1.51	2.32**	1.93***
- Standard deviation	0.51	0.30	1.16	0.13	0.12	0.17	0.31	0.21
Week 104								
- Mean	1.80	1.70	1.83	1.48	1.91	1.51	2.51***	1.86
- Standard deviation	0.82	0.81	1.01	0.40	0.69	0.37	0.80	0.53
Relative liver weight (%)								
Week 26								
- Mean	3.545	3.947	3.587	3.880	4.030***	4.139	5.651***	5.515***
- Standard deviation	0.160	0.335	0.157	0.204	0.213	0.337	0.513	0.303
Week 52								
- Mean	3.678	3.959	3.654	3.801	4.093*	4.137	5.905***	5.512***
- Standard deviation	0.323	0.492	0.293	0.451	0.376	0.628	0.252	0.140
Week 78								
- Mean	4.547	4.178	4.721	4.040	4.018	4.309	6.629**	6.086***
- Standard deviation	1.541	1.018	3.612	0.470	0.352	0.651	0.579	0.648
Week 104								
- Mean	4.455	5.104	4.551	4.266*	4.738	4.549	7.496***	5.882
- Standard deviation	2.564	2.503	3.101	0.906	1.853	1.121	2.410	1.892

*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$
Source: Inoue (1984)

Table 18. Histopathology: non-neoplastic lesions in mice following chronic dietary exposure

	0 ppm		100 ppm		400 ppm		1 600 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
Liver								
Hepatic nodule	7/60	5/60	16/60	7/60	13/60	9/60	22/60	17/60
Fatty metamorphosis	17/60	9/60	23/60	10/60	27/60	17/60	40/60	25/60

Table 19. Time-related occurrence of tumours in male mice following chronic dietary exposure

Summary of histopathological findings	Weeks 0–26				Weeks 27–52				Weeks 53–78				Weeks 79–104			
	A[a]	B[a]	C[a]	D[a]	A	B	C	D	A	B	C	D	A	B	C	D
No. of benign tumours	0	0	0	0	0	0	0	1	0	0	1	0	21	33	27	30
No. of malignant tumours	0	0	0	0	0	0	0	0	1	1	1	0	9	7	13	10
No. of total tumours	0	0	0	0	0	0	0	1	1	1	2	0	30	40	40	40
No. of animals with a single tumour	0	0	0	0	0	0	0	1	1	1	2	0	22	20	16	27
No. of animals with multiple tumours	0	0	0	0	0	0	0	0	0	0	0	0	4	8	11	6
No. of animals examined	11	10	10	10	10	10	10	10	11	12	12	10	48	48	48	50

[a] A: 0 ppm; B: 100 ppm; C: 400 ppm; D: 1600 ppm.
Source: Inoue (1984)

Table 20. Time-related occurrence of tumours in female mice following chronic dietary exposure

Summary of histopathological findings	Weeks 0–26				Weeks 27–52				Weeks 53–78				Weeks 79–104			
	A[a]	B[a]	C[a]	D[a]	A	B	C	D	A	B	C	D	A	B	C	D
No. of benign tumours	0	0	0	0	1	0	0	0	0	1	0	0	27	16	19	6
No. of malignant tumours	0	0	0	0	0	0	0	0	0	0	0	1	9	8	8	14
No. of total tumours	0	0	0	0	1	0	0	0	0	1	0	1	36	24	27	20
No. of animals with a single tumour	0	0	0	0	1	0	0	0	0	1	0	1	15	15	16	14
No. of animals with multiple tumours	0	0	0	0	0	0	0	0	0	0	0	0	10	4	5	2
No. of animals examined	10	10	10	10	10	10	10	10	11	11	10	11	49	49	50	49

[a] A: 0 ppm; B: 100 ppm; C: 400 ppm; D: 1600 ppm.
Source: Inoue (1984)

Organs of all sacrificed animals at the scheduled necropsies were weighed. The study was mainly conducted in accordance with OECD Test Guideline 453, with the following exceptions. The mortality exceeded 50% in the male control, low-dose and medium-dose groups and the female low-dose group. No blood samples or urine samples were taken 3 months after dosing started. Cholinesterase activity was measured immediately after blood sampling and centrifugation, using the Technicon method no. 354-75 P/A. Plasma cholinesterase activity was measured using acetyl and butyryl substrate, erythrocyte cholinesterase activity using acetyl substrate only. Brain cholinesterase activities were measured in the fresh homogenates of the mid-line sagittal section of the brain, using the same assays as for plasma cholinesterases. Rats were apparently not fasted prior to blood sampling or sacrifice.

The study is considered acceptable, even though the mortality was higher than 50% (OECD Test Guideline 453) in male rats in the control, low-dose and mid-dose groups and in female rats in the low-dose group (Table 21). First, comparisons with historical control data indicate that the mortality was within the background range for these rats in the laboratory where the study was performed, except for male rats of the high-dose group, where the mortality was low, and female rats of the low-dose group, where the mortality was rather high. Additionally, there was no indication of an increase in tumour incidence even when including the results of the animals that died between 18 and 24 months. Moreover, the survival rate increased with increasing dose and was not too high at the highest dose level.

Table 21. Mortality rates in rats following chronic dietary exposure

	0 ppm		100 ppm		400 ppm		1 600 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
Mortality	52/70	31/70	50/70	47/70	46/70	25/70	32/70	22/70

Source: Virgo (1984)

Chronic oral administration of triflumizole to rats also provoked neurotoxic effects at the high dose. Convulsions (violent jerking movement, ataxia, tremors) were noted in all dose groups except the female controls (Table 22). When looking at the historical control incidences of convulsions in CD rats for this laboratory, the incidences in males ranged from 0% to 6.7% and in females ranged from 0% to 2.0%. At doses of 0, 100, 400 and 1600 ppm, the percentages affected in males were 3.8%, 5.0%, 2.5% and 7.5%, respectively, and in females were 0%, 2.5%, 2.5% and 18.8%, respectively. Therefore, the incidence of convulsive episodes among controls and animals receiving 100 or 400 ppm was consistent with that reported in a range of similar studies. However, the incidence of convulsions at the highest concentration of 1600 ppm was above the background range, particularly in females, and is considered an adverse effect of treatment.

Table 22. Convulsive episodes in rats following chronic dietary exposure

Sex	Dose (ppm)	Incidence (number of convulsing animals/total number) (% affected)	Week of onset of convulsions (mean ± SD)
M	0	3/80 (3.8%)	81 ± 31
	100	4/80 (5.0%)	52 ± 20
	400	2/80 (2.5%)	58 ± 30
	1 600	6/80 (7.5%)	42 ± 24
F	0	0/80	–
	100	2/80 (2.5%)	62 ± 31
	400	2/80 (2.5%)	65 ± 51
	1 600	15/80* (18.8%)	30 ± 17

F: females; M: males; SD: standard deviation; *: $P < 0.05$
Source: Virgo (1984)

Body weight gain and feed consumption were decreased in high-dose animals. Erythrocyte acetylcholinesterase activity was decreased in males (400 and 1600 ppm) and in females (100 and 400 ppm) transiently (one or two occasions) without a dose–response relationship (Table 23). Brain acetylcholinesterase activity (Table 24) was not changed on any occasions in any dose groups, except for a transient decrease in males from the 400 ppm group. It is considered that triflumizole does not inhibit acetylcholinesterase activities because of no dose-related response or no more severe change with time. Brain butyrylcholinesterase (butyrylcholine as a substrate) was decreased compared with controls in males (400 and 1600 ppm) and in females (all dose groups) at week 54 (Table 24). After administration for 104 weeks, its activity was increased in both sexes (significant in female) of the 1600 ppm group. Due to the lack of consistency and lack of a dose–response relationship, these changes are assessed as being incidental.

Plasma acetylcholinesterase (acetylcholine as a substrate) was increased in males (1600 ppm) and decreased in females (1600 ppm) at week 26. This was not observed at week 77 or 102 (Table 25). Plasma butyrylcholinesterase (butyrylcholine as a substrate) revealed a similar trend. As no consistent decrease was observed, it is concluded that triflumizole does not inhibit brain, erythrocyte or plasma cholinesterases.

Table 23. Erythrocyte acetylcholinesterase activity in rats following chronic dietary exposure

Dose (ppm)	Activity (IU/L)							
	26 weeks		50 weeks		77 weeks		102 weeks	
	Males	Females	Males	Females	Males	Females	Males	Females
0	1 265 ± 139	1 013 ± 143	658 ± 61	587 ± 48	946 ± 124	984 ± 86	1 175 ± 146	987 ± 156
100	1 212 ± 139	1 031 ± 158	691 ± 89	573 ± 38	838 ± 205	799 ± 106*	1 130 ± 186	1 140 ± 164
400	1 153 ± 134	1 001 ± 142	584 ± 80*	539 ± 56*	924 ± 112	802 ± 93*	1 048 ± 238	1 084 ± 226
1 600	1 090 ± 174*	1 054 ± 170	565 ± 68*	614 ± 63	995 ± 137	951 ± 92	1 234 ± 294	1 148 ± 188

IU: International Units; *: $P < 0.05$
Source: Virgo (1984)

Table 24. Brain acetylcholinesterase and butyrylcholinesterase activity in rats following chronic dietary exposure

Dose (ppm)	Activity (10^3 IU/L)							
	54 weeks		104 weeks		54 weeks		104 weeks	
	Acetylcholinesterase				Butyrylcholinesterase			
	Males	Females	Males	Females	Males	Females	Males	Females
0	10.9 ± 0.7	10.0 ± 0.7	8.1 ± 1.1	8.5 ± 0.5	2.0 ± 0.2	3.2 ± 0.3	1.1 ± 0.2	1.1 ± 0.2
100	10.6 ± 1.0	10.0 ± 0.5	8.2 ± 0.4	8.2 ± 0.8	2.1 ± 0.4	2.4 ± 0.3*	1.2 ± 0.1	1.0 ± 0.2
400	10.0 ± 0.6*	10.1 ± 0.6	8.5 ± 0.6	8.2 ± 0.7	1.4 ± 0.2*	2.5 ± 0.3*	1.2 ± 0.2	1.2 ± 0.2
1 600	10.7 ± 1.3	9.7 ± 0.7	8.5 ± 0.6	8.6 ± 0.4	1.5 ± 0.4*	2.7 ± 0.2*	1.3 ± 0.2	1.3 ± 0.3*

IU: International Units; *: $P < 0.05$
Source: Virgo (1984)

Table 25. Plasma acetylcholinesterase activity in rats following chronic dietary exposure

Dose (ppm)	Activity (IU/L)							
	26 weeks		50 weeks		77 weeks		102 weeks	
	Males	Females	Males	Females	Males	Females	Males	Females
0	639 ± 78	3 638 ± 656	429 ± 69	1 506 ± 259	804 ± 285	2 575 ± 543	1 264 ± 741	1 790 ± 624
100	639 ± 80	3 108 ± 283*	370 ± 68	1 545 ± 221	698 ± 124	2 324 ± 524	780 ± 184	1 972 ± 676
400	717 ± 112	3 152 ± 583	494 ± 87	1 435 ± 213	660 ± 114	2 281 ± 428	1 262 ± 441	2 266 ± 573
1 600	765 ± 201*	2 599 ± 590*	457 ± 126	1 445 ± 243	774 ± 303	2 470 ± 462	1 121 ± 801	2 076 ± 420

IU: International Units; *: $P < 0.05$
Source: Virgo (1984)

The main target organ was the liver. Relative liver weight was increased in both males and females in the high-dose groups and in males administered 400 ppm (Table 26). Females administered 400 or 1600 ppm had more macroscopic liver lesions than females in the control group, including swollen or pale livers and dark, depressed areas in the liver. Microscopically, the number of observed effects increased with increasing dose, and the effects were more prominent in females than in males. The incidence of diffuse hepatocytic fatty vacuolation, as shown in Table 27, indicated that the liver damage increased with time. At 104 weeks, the severity also increased with dose. Centriacinar, periacinar and midzonal vacuolation was also observed, but without a clear dose-related pattern. The effects were reflected in the liver enzyme levels, notably an increase in ALAT in males in the high-dose group. Other findings in the livers of females at the high dose and, to a lesser extent, at the middle dose were periacinar hepatocytic hypertrophy, basophilic and eosinophilic foci, focal inflammation/necrosis and hyaline degradation/fibrosis of the bile ducts. Additionally, females at 1600 ppm also showed an increased incidence of cystic ovaries macroscopically, which was confirmed histopathologically by follicular cysts. No increase in tumour incidence was observed (Fig. 3). In the absence of a clear dose–response relationship and additional adverse effects, the periacinar hepatocytic hypertrophy observed in females given 100 ppm (Table 27) is not considered to be toxicologically relevant. The other non-neoplastic effects observed at 100 ppm in males and females were considered comparable with those seen in controls.

Table 26. Liver weight in rats following chronic dietary exposure

	0 ppm		100 ppm		400 ppm		1 600 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
Absolute liver weight (g)								
Week 54								
- Mean	27.9	15.1	30.0	16.2	33.6**	16.5	35.3***	18.3
- Standard deviation	4.0	2.5	4.4	4.1	4.3	4.1	3.7	5.1
Week 104 onco[a]								
- Mean	27.8	23.2	29.5	22.3	29.5	24.9	30.3	24.7
- Standard deviation	3.6	5.6	4.5	5.6	6.6	6.6	4.6	4.6
Week 104 tox[b]								
- Mean	31.0	21.1	28.7	22.3	30.3	23.8	31.0	22.0
- Standard deviation	6.9	3.3	0.6	5.7	3.4	4.4	6.5	4.8
Relative liver weight (%)								
Week 54								
- Mean	3.3	3.3	3.5	3.5	4.2***	4.2**	4.8***	4.8***
- Standard deviation	0.4	0.2	0.4	0.5	0.7	0.9	0.5	0.9
Week 104 onco[a]								
- Mean	3.1	3.8	3.4	3.8	3.7*	4.1	4.5***	5.2***
- Standard deviation	0.6	0.6	0.4	0.5	0.8	0.7	1.0	0.7
Week 104 tox[b]								
- Mean	3.8	3.6	3.7	3.2	3.8	4.1	4.7*	5.2***
- Standard deviation	1.0	0.7	0.6	0.3	0.9	0.9	0.5	0.6

*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$

[a] Oncogenicity study.

[b] Toxicity study.

Source: Virgo (1984)

Table 27. Liver histopathology findings in rats following chronic dietary exposure

	Males				Females			
	Control	100 ppm	400 ppm	1 600 ppm	Control	100 ppm	400 ppm	1 600 ppm
Diffuse hepatocytic fatty vacuolation								
- Week 54	0/10	1/10	3/10	4/10	0/10	0/10	2/10	3/10
- Week 104	10/69	15/70	26/70	35/70	13/70	15/70	43/70	55/70
Periacinar hepatocytic hypertrophy	0/69	5/70	10/70	17/70	0/70	18/70	13/70	28/70

Source: Virgo (1984)

After 54 weeks, 10 animals of each sex per group were killed (satellite group). In these small groups, no treatment-related effect on neoplasm incidence was found. In addition, in animals that were killed or that died during the treatment period, no treatment-related effects on tumour incidence occurred. There was a lower incidence of fibromas in the subcutis of males that had received the highest dose of triflumizole ($P < 0.01$) compared with controls. In animals killed after 104 weeks of treatment, the incidence of neoplasms did not suggest an effect of the administration of triflumizole. There were, however, lower than control incidences of both benign and malignant mammary gland fibroepithelial tumours and pituitary adenomas in females that had received the highest dose of triflumizole (statistically significant in all cases).

It is concluded that triflumizole, when fed to CD rats for 104 weeks, did not increase the incidence of tumours, shorten the induction period of tumours or alter the type of tumours found; indeed, there were generally fewer tumours in rats treated at 1600 ppm than in controls. This is probably a result of the lower body weight gain and feed consumption observed in rats that had received the highest dose of triflumizole.

In conclusion, the NOAEL was 100 ppm (equal to 3.5 mg/kg bw per day), based on hepatotoxicity (increased liver weight and macroscopic and microscopic hepatic changes) observed at 400 ppm (equal to 14 mg/kg bw per day). No carcinogenic potential of triflumizole was observed in this study (Virgo, 1984).

2.4 Genotoxicity

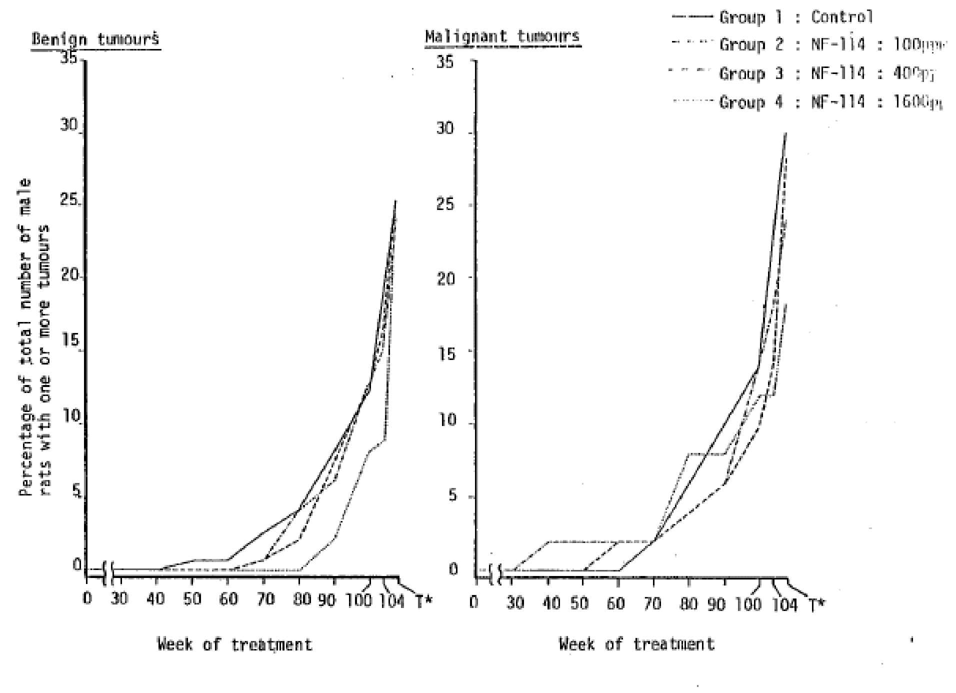
Triflumizole has been tested in a battery of genotoxicity studies comprising several in vitro assays and two in vivo assays, the results of which are summarized in Table 28. Triflumizole was not genotoxic or mutagenic in any of the assays tested.

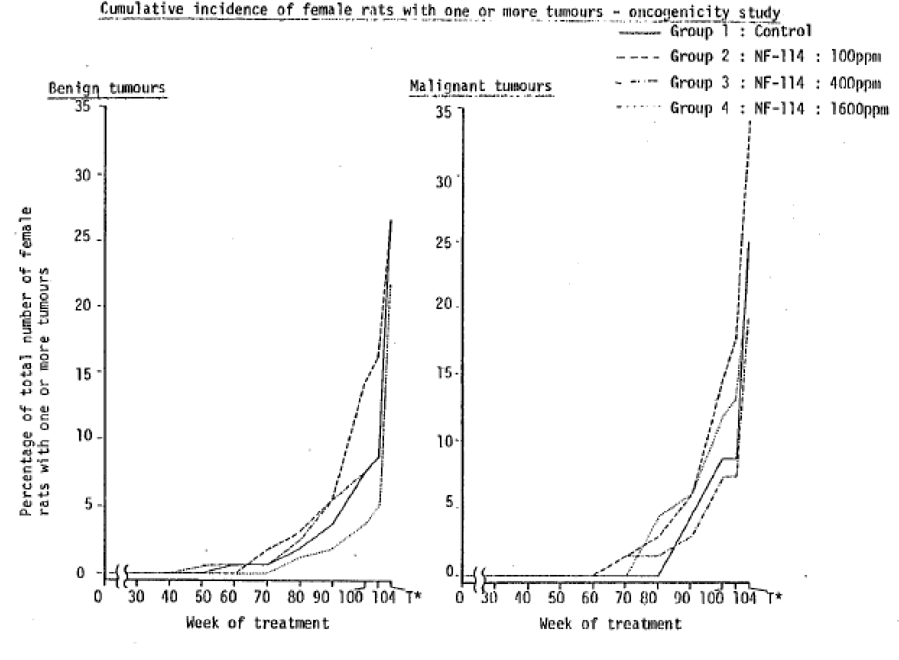
2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

In a non-GLP range-finding reproductive toxicity study, groups of six male and six female Sprague-Dawley rats were treated with doses of triflumizole (purity not stated; batch no. TK-1116) in the diet at a dose level of 0, 400 or 1200 ppm (equal to approximately 0, 20 and 60 mg/kg bw per day, respectively) for 2 weeks prior to mating, throughout the mating period, gestation and lactation, and up to termination after day 21 postpartum. Rats were paired for mating on day 15 of treatment, and females were checked for evidence of mating each morning after pairing. The day on which evidence of mating was found was designated as day 1 of gestation. Animals were examined daily for clinical signs. Males and females were weighed at twice weekly intervals until mating occurred; females were additionally weighed every 2–3 days postcoitum and every 3–4 days postpartum. All does were allowed to deliver their litters and rear their offspring until postpartum day 21. Litter observations were recorded at birth and/or throughout the postpartum interval. On postpartum day 21, female rats

Fig. 3. Cumulative incidence of rats with one or more tumours in the carcinogenicity study





Source: Virgo (1984)

Table 28. Results of genotoxicity studies on triflumizole

End-point	Test system	Concentration range	Purity (%)	Results	Reference
In vitro					
Reverse mutation[a]	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537	5–5 000 µg/plate in DMSO	98.2	Negative (±S9 mix)	Nishibe (1987)
Reverse mutation[b]	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538; and *Escherichia coli* WP2*uvrA*	8–8 000 µg/plate in DMSO	98.6	Negative (±S9 mix)	Inoue (1983)
Gene mutation (HPRT)[c]	CHL cells (V79)	1.22–19.5 µg/mL (−S9 mix) in DMSO 9.75–156 µg/mL (+S9 mix) in DMSO	98.3	Negative (±S9 mix)	Seeberg & Forster (1989)
Chromosomal aberration[d]	CHL cells	5–40 µg/mL in DMSO	98.2	Negative (±S9 mix)	Nishibe (1988)
DNA repair (unscheduled DNA synthesis)[e]	Primary rat hepatocytes (male Fischer 344)	12.5–40.0 µg/mL in DMSO	98.7	Negative	Cifone (1984)
In vivo					
Micronuclei (bone marrow)[f]	Mouse (CD-1)	160–1 600 mg/kg bw in DMSO	98.7	Negative	Ivett (1984)
Chromosomal aberrations (bone marrow)[g]	Chinese hamster	1 000–4 000 mg/kg bw in 0.5% carboxymethyl cellulose sodium salt	98.3	Negative	Mosesso (1989)

CHL: Chinese hamster lung; DMSO: dimethyl sulfoxide; DNA: deoxyribonucleic acid; HPRT: hypoxanthine–guanine phosphoribosyltransferase; S9: 9000 × *g* supernatant fraction from rat liver homogenate

[a] Test in duplicate; positive controls included; S9 fraction of phenobarbital and 5,6-benzoflavone-induced rat liver; GLP and quality assurance (QA) statements included. Cytotoxicity observed at ≥ 50 µg/plate; precipitation observed at ≥ 1575 µg/plate.

[b] Test in duplicate; positive controls included; S9 fraction of phenobarbital and 5,6-benzoflavone-induced rat (Sprague-Dawley) liver. Cytotoxicity observed at ≥ 80 µg/plate.

[c] Test in duplicate; positive controls included; S9 fraction of phenobarbital and 5,6-benzoflavone-induced rat (Sprague-Dawley) liver; GLP and QA statements included. Cytotoxicity observed at ≥ 39.1 µg/mL (−S9 mix) and ≥ 313 µg/mL (+S9 mix), observed in a separate toxicity test using dose levels of 1–2500 µg/mL; precipitation observed at ≥ 2500 µg/mL.

[d] Test in duplicate; positive controls included; S9 fraction of phenobarbital and 5,6-benzoflavone-induced rat liver; GLP and QA statements included. Only 100 cells/dose scored instead of 200 cells/dose as required by OECD. Cytotoxicity observed at ≥ 40 µg/mL.

[e] Single test; positive control included; GLP and QA statements included. Cytotoxicity observed at ≥ 40 µg/mL.

[f] Administered by single oral gavage in three dose groups with five male and five female animals per dose; positive control included; sacrifice 24 or 48 hours after dosing; GLP and QA statements included. Clinical signs: 1st hour after dosing, the mice in the medium- and high-dose groups had difficulties breathing, one female animal in the high-dose group was observed to convulse after 24 hours, two male mice in the high-dose group died before the second sacrifice, and all other animals in the high-dose group seemed barely alive.

[g] Administered by single oral application in three dose groups with five male and five female animals per dose; positive control included; sacrifice at 12, 24 or 48 hours after dosing; GLP and QA statements included. Clinical signs: decreased activity in all treatment groups. Only 50 cells scored instead of the 100 cells/animal required by OECD.

and their litters were killed and subjected to gross necropsy. Males were killed and subjected to gross necropsy after the females had successfully littered.

Some evidence of toxicity was observed in rats fed diet containing 400 ppm triflumizole. Body weight gain in females was reduced compared with controls over gestation days (GDs) 0–13 (approximately 25% less than control body weight gain; $P < 0.01$). Gestation length was increased by 1 day compared with controls, but gestation index (number of live litters born/number pregnant × 100) was not affected. The mean litter size was slightly reduced (10.5 versus 13.3 for controls; statistical significance not known), but the number of live births and viability were the same as for controls. No other differences were noted in the 400 ppm group compared with controls.

Treatment with 1200 ppm triflumizole in the diet produced significant toxicity. Males showed a significant reduction in body weight gain compared with controls in the interval before pairing (−39%; $P < 0.05$); body weight gain was normal in males for the remainder of the study. Females had a decrease in body weight gain compared with controls before pairing (−29%; not statistically significant) and over GDs 0–13 (−47%; $P < 0.001$). Body weight gain increased for the remainder of gestation and was comparable to or greater than that of controls during the lactation period. The precoital interval (time between initial pairing and evidence of mating) was increased in 3/6 females. Controls mated within 4 days. In the high-dose group, three females mated within 4 days, whereas two mated within 5–8 days and one within 9–12 days. The estrous cycle appeared to have shifted to a 5-day cycle instead of the normal 4-day cycle in the high-dose group. Gestation length was extended by approximately 1 day, and the gestation index (the number of live litters born/number pregnant × 100) was reduced (33% versus 100% for controls). Of the six females in the high-dose group, only one gave birth to live fetuses and survived. Of the three female rats that gave birth, one gave birth to live fetuses but was killed in extremis on postpartum day 1; necropsy revealed retained dead fetuses in utero. The other female gave birth to dead fetuses. Of the three females that did not give birth, one female was found dead on day 23 postcoitum, and one female was killed in extremis on day 24 postcoitum. Both of these females had dead fetuses in utero. The remaining female did not deliver a litter, and necropsy revealed evidence of one early resorption. Necropsy of animals that died or were killed in extremis did not reveal any abnormalities other than the dead fetuses. In surviving fetuses in the high-dose group, fetal birth weight was reduced compared with controls, but increased during lactation.

In conclusion, the parental systemic lowest-observed-adverse-effect level (LOAEL) was 400 ppm (equal to approximately 20 mg/kg bw per day in males and females), the lowest dose tested, based on reduced maternal weight gain over GDs 0–13. A parental systemic NOAEL could not be identified.

The offspring LOAEL was 1200 ppm (equal to approximately 60 mg/kg bw per day), based on dead fetuses and reduced body weight at birth. The offspring NOAEL was 400 ppm (equal to approximately 20 mg/kg bw per day).

The reproductive LOAEL was 1200 ppm (equal to approximately 60 mg/kg bw per day), based on increased gestation length, reduced gestation index and retention of dead fetuses. The reproductive NOAEL was 400 ppm (equal to approximately 20 mg/kg bw per day) (Tesh & Willoughby, 1982).

In a three-generation reproductive toxicity study, groups of 30 male and 30 female CD rats were treated with doses of triflumizole (purity not stated; lot no. TK 1116) in the diet at a dose level of 0, 70, 170 or 420 ppm (equal to 0, 4.8, 11.7 and 29.0 mg/kg bw per day for F_0 males and 0, 5.5, 13.5 and 33.3 mg/kg bw per day for F_0 females, respectively). Thirty F_0 animals were treated for 13 weeks before pairing and continuously until premature termination of the study after weaning of the F_{1A} litters, following adverse reaction at the highest dose level. Feed consumption and water consumption were recorded weekly for both sexes until animals were paired for mating. Males were weighed weekly throughout the study. Females were weighed weekly until mating occurred, on days 1, 3, 7, 14 and 21 postcoitum and on days 1, 4, 10, 14, 21 and 25 postpartum. Observations were made for estrous cycle length, mating performance, fertility, gestation length and parturition. Litter size and

offspring viability were recorded. Offspring were weighed as litters on days 1, 4, 10, 14, 21 and 25 postpartum. All animals were examined externally and internally at necropsy for macroscopic abnormalities. Limited histopathology was conducted on selected F_0 adults.

Continuous dietary administration of triflumizole at 420 ppm resulted in slight decreases in body weight gain, increased length of estrous cycles, reduced vaginal cornification and extended precoital interval. Eventual mating performance and conception rate were unaffected, but gestation length was extended, and severe parturition difficulties resulted in maternal death and high perinatal mortality of offspring. In surviving offspring, body weight gain to weaning was reduced. At a dose of 170 ppm, similar but less severe effects on gestation length, parturition and perinatal mortality were observed. At a dose of 70 ppm, only slight increases in gestation length were observed. There were no organ weight changes or pathological findings in males at any dose that were considered related to treatment. There was a marginal increase in relative liver weights in females in the 420 ppm dose group, but no microscopic findings. The only treatment-related macroscopic finding in offspring was a statistically significant increase in the incidence of hydronephrosis at 420 ppm.

In conclusion, the NOAEL for parental and reproductive effects was 70 ppm (equal to 4.8 mg/kg bw per day). Based on the results observed at 170 and 420 ppm, the decision was made to terminate this study after weaning of the F_{1A} litters and to conduct a second study at levels of 0, 30, 70 and 170 ppm in the diet (see below) (Tesh, Willoughby & Whitney, 1984).

In a three-generation reproductive toxicity study, groups of 30 male and 30 female CD rats were treated with doses of triflumizole (purity 98.6%; lot no. TK 1116) in the diet at a dose level of 0, 30, 70 or 170 ppm (equal to 0, 2.1, 4.8 and 12 mg/kg bw per day for F_0 males and 0, 2.5, 5.8 and 14 mg/kg bw per day for F_0 females; 0, 2.6, 5.8 and 13 mg/kg bw per day for F_1 males and 0, 2.8, 6.6, and 16 mg/kg bw per day for F_1 females; and 0, 2.6, 6.0 and 15 mg/kg bw per day for F_2 males and 0, 3.0, 6.9 and 16 mg/kg bw per day for F_2 females, respectively).

Thirty F_0 animals were treated for 13 weeks before pairing twice in succession. The first pairing produced the F_{1A} litters, which were discarded at weaning. After the second pairing, half of the females (*n* = 15) were killed on day 21 postcoitum to permit teratological examination, and the remainder were allowed to litter (F_{1B} litters), from which the F_1 generation was selected. This procedure was repeated for the F_1 generation: 30 F_1 animals were treated for 13 weeks before pairing twice in succession. The first pairing produced the F_{2A} litters, which were discarded at weaning. After the second pairing, half of the females were killed on day 21 postcoitum to permit teratological examination, and the remainder of the females were allowed to litter (F_{2B} litters). From the F_{2B} litter, 10 animals of each sex per dose were selected and treated for 13 weeks after weaning. Another group of 30 animals of each sex per dose from the F_{2B} litter underwent the above-mentioned procedure. Physical development and auditory and visual function were examined in the F_{1B}, F_{2B} and F_{3B} litters. The data on the F_0, F_1 and F_2 females (*n* = 15) selected for the teratogenicity part of the study are included in the summary/conclusions below; however, litter data on these females were omitted. Selected animals (10 of each sex per dose) from the F_0 and F_1 parents (after 29 weeks of treatment) and all F_2 parents, as well as some of the F_2 adults (13 weeks after weaning), were subjected to a detailed necropsy procedure, and several organs were weighed. Microscopic examination was performed as shown in Table 29.

All other animals were examined externally and internally for macroscopic abnormalities. The study was performed partly in accordance with OECD Test Guideline 416 (1983); the main deviations were that 1) the reproductive organs of not all parental animals were subjected to a full histopathological examination and 2) feed consumption was measured only until the animals were mated for the first pairing. In addition to OECD Test Guideline 416 from 1983, organ weights of several organs were determined, and more tissues were examined than indicated in the guideline.

Table 29. Summary of schedule for microscopic examination of tissues of rats in a three-generation study

	Number/sex/dose	Dose groups	Tissues examined
F_0 (parent)	5	Control, high-dose group	Full tissue list
	5	Control, high-dose group	Reproductive organs
F_1 (parent)	5	Control, high-dose group	Full tissue list
	5	Control, high-dose group	Reproductive organs
F_2 (13 weeks)	10	All dose groups	Full tissue list
F_2 (parent)	5	Control, high-dose group	Full tissue list
	25 males/dose 12 females/dose	Control, high-dose group	Reproductive organs
F_{3B} (weanlings)	3	Control, high-dose group	Full tissue list

Source: Tesh, Willoughby & Secker (1986)

Parental toxicity consisted of increased kidney weights at 70 and 170 ppm and increased liver weights at 170 ppm (Table 30). Moreover, placental weights were increased at 170 ppm in all generations (Table 30). One pregnant female in the high-dose group died of dystocia, and a relationship to treatment cannot be excluded. The parental effects at 70 ppm were rather slight, significantly affecting only absolute kidney weights of females of the F_1 generation. Therefore, effects at this dose level are not considered adverse.

At 170 ppm, an increased gestation length was observed in the first two generations. In each generation, there tended to be decreased conception rate, fertility and/or percentage mating at the highest dose, although sometimes without clear dose–response relationships (Table 31). At 170 ppm, the litter size of the F_{1A} generation was decreased; however, this was not seen in any generation thereafter (Table 32).

At 170 ppm in the F_3 generation, several statistically significant effects were seen on birth weight, live birth index and viability index (Table 32). However, these effects were seen only in this third generation and were not consistent or dose related in the other generations, or the incidences were within the range observed in other generations.

The NOAEL for parental toxicity was 70 ppm (equal to 4.8 mg/kg bw per day), based on increased placental weights and increased liver and kidney weights at the high dose (170 ppm, equal to 12 mg/kg bw per day). The NOAEL for reproductive toxicity was 70 ppm (equal to 4.8 mg/kg bw per day), based on reduced mating, fertility and litter size. The NOAEL for offspring toxicity was 170 ppm (equal to 12 mg/kg bw per day), the highest dose tested (Tesh, Willoughby & Secker, 1986).

(b) Developmental toxicity

Rats

In a non-GLP developmental toxicity study in rats, groups of 24 pregnant female Sprague-Dawley (Crj:CD) rats were treated with doses of triflumizole (purity 98.7%; lot no. YS200) by gavage at a dose level of 0, 10, 35 or 120 mg/kg bw per day. The study was performed partly in accordance with OECD Test Guideline 414. Rats were dosed up to GD 16, but caesarean sections were not performed until GD 20. OECD guidelines state that doses should be administered daily from implantation to the day before caesarean section. Females were examined daily from GD 6 to GD 20 for mortality and clinical signs. Body weights and feed consumption were recorded daily on GDs 1–20. Water consumption was recorded on GDs 1, 6, 11, 16 and 20. On GD 20, the rats were killed and subjected to a caesarean section and gross necropsy. The uterus and ovaries were exposed, and the number and location of viable and non-viable fetuses, number of resorptions and total numbers of implantations and corpora lutea were recorded. The uterus was removed and weighed, and fetuses were removed. The uterus of each apparently non-pregnant female was opened and stained with

Table 30. Organ weights in adult rats in a three-generation reproductive toxicity study (n = 10)

	0 ppm		30 ppm		70 ppm		170 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
F_0 animals								
Placental weight (g)								
- F_{1A}	–		–		–		–	
- F_{1B}	0.49		0.52		0.51		0.56**	
F_1 animals								
Liver weight								
- Absolute (g)	21.7	13.8	22.7	14.5	23.2	14.6	24.0 (+11%)	15.8* (+14%)
- Relative (%)	3.3	4.0	3.5	4.1	3.5	3.9	3.8 (+15%)	4.4* (+10%)
Kidney weight								
- Absolute (g)	4.11	2.53	4.29	2.62	4.17	2.83* (+12%)	4.16 (+9%)	2.86* (+13%)
- Relative (%)	0.62	0.72	0.66	0.73	0.63	0.77	0.66 (+10%)	0.80* (+11%)
Placental weight (g)								
- F_{2A}	–		–		–		–	
- F_{2B}	0.53		0.53		0.54		0.59*	
F_2 animals (13 weeks)								
Kidney weight								
- Absolute (g)	3.55	2.18	3.54	2.08	3.34	2.03	3.77 (+6%)	2.45 (+12%)
- Relative (%)	0.67	0.74	0.69	0.73	0.70	0.73	0.73* (+9%)	0.80 (+8%)
F_2 animals (parent)								
Liver weight								
- Absolute (g)	23.9	13.0	22.9	13.4	24.3	13.1	26.3* (+10%)	13.7
- Relative (%)	3.4	3.7	3.5	3.8	3.5	3.7	3.8** (+11%)	3.8
Kidney weight								
- Absolute (g)	4.17	2.44	3.94	2.51	4.20	2.57	4.56* (+9%)	2.67* (+9%)
- Relative (%)	0.60	0.70	0.60	0.71	0.61	0.71	0.66** (+10%)	0.75 (+7%)
Placental weight (g)								
- F_{3A}	–		–		–		–	
- F_{3B}	0.54		0.53		0.56		0.66**	

–: not assessed; *: $P < 0.05$; **: $P < 0.01$
Source: Tesh, Willoughby & Secker (1986)

Table 31. Effects on mating/fertility/gestation in rats in a three-generation reproductive toxicity study

	0 ppm		30 ppm		70 ppm		170 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
F_0 animals								
% mating								
- First pairing (F_{1A})	100	100	97	100	97	97	93	97
- Second pairing (F_{1B})	100	100	100	100	97	100	97	100
Conception rate (%)								
- First pairing (F_{1A})	97	97	97	97	90	90	93	93
- Second pairing (F_{1B})	100	100	100	100	97	97	93	93
Fertility index (%)								
- First pairing (F_{1A})	97	97	93	97	87	87	87	90
- Second pairing (F_{1B})	100	100	100	100	93	97	90	93
Mean gestation length (days)								
- First pairing (F_{1A})	–	22.6	–	22.7	–	22.6	–	23.0***
- Second pairing (F_{1B})	–	22.6	–	22.7	–	22.7	–	22.8
F_1 animals								
% mating								
- First pairing (F_{2A})	97	100	97	97	100	100	93	97
- Second pairing (F_{2B})	97	100	87	90	97	97	86	97
Conception rate (%)								
- First pairing (F_{2A})	83	83	79	79	87	87	71	72
- Second pairing (F_{2B})	89	90	96	96	90	90	92	89
Fertility index (%)								
- First pairing (F_{2A})	80	83	77	77	87	87	67	70
- Second pairing (F_{2B})	86	90	83	87	87	87	79	86
Mean gestation length (days)								
- First pairing (F_{2A})	–	22.7	–	22.6	–	22.8	–	23.0
- Second pairing (F_{2B})	–	22.6	–	22.6	–	22.7	–	22.7
F_2 animals								
Bleeding from vagina before parturition (first pairing F_{3A})	–	1	–	2	–	2	–	6
% mating								
- First pairing (F_{3A})	93	97	90	100	90	100	87	97
- Second pairing (F_{3B})	83	93	83	90	97	100	70	90
Conception rate (%)								
- First pairing (F_{3A})	89	90	100	100	93	93	81	83
- Second pairing (F_{3B})	92	86	83	81	86	83	100	100
Fertility index (%)								
- First pairing (F_{3A})	83	87	90	100	83	93	70	80
- Second pairing (F_{3B})	77	80	69	73	83	83	70	90

Table 31 (continued)

	0 ppm		30 ppm		70 ppm		170 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
Mean gestation length (days)								
- First pairing (F_{3A})	–	22.6	–	22.9	–	22.9	–	23.0
- Second pairing (F_{3B})	–	22.6	–	22.7	–	22.8	–	22.8

–: not applicable; *** P: < 0.001
Source: Tesh, Willoughby & Secker (1986)

Table 32. Effects in rat pups in a three-generation reproductive toxicity study

	0 ppm		30 ppm		70 ppm		170 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
F_1 pups								
Litter size (day 1)								
- F_{1A}	13.6		13.1		13.1		12.0*	
- F_{1B}	13.6		12.9		12.8		14.2	
Live birth index (%)								
- F_{1A}	98		98		97		93	
- F_{1B}	93		93		97		98	
Viability index day 25 (%)								
- F_{1A}	84		86		92		90	
- F_{1B}	77		94		81		84	
Birth weight (g)								
- F_{1A}	5.8		5.8		5.9		6.1	
- F_{1B}	5.8		6.0		6.1		6.0	
F_2 pups								
Litter size (day 1)								
- F_{2A}	12.1		12.6		12.9		12.6	
- F_{2B}	12.9		12.6		12.7		12.6	
Live birth index (%)								
- F_{2A}	97		95		92		95	
- F_{2B}	100		100		93		89	
Viability index day 25 (%)								
- F_{2A}	82		79		69		82	
- F_{2B}	87		85		80		90	
Birth weight (g)								
- F_{2A}	6.3		6.3		6.2		6.2	
- F_{2B}	6.1		5.9		5.9		6.3	
F_3 pups								
Litter size (day 1)								
- F_{3A}	11.8		11.7		12.5		11.9	
- F_{3B}	11.5		12.5		12.3		12.4	

	0 ppm		30 ppm		70 ppm		170 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
Live birth index (%)								
- F_{3A}	99		95		91 (−8%)		72*** (−27%)	
- F_{3B}	97		99		92 (−5%)		85 (−12%)	
Viability index day 25 (%)								
- F_{3A}	94		90		80 (−13%)		57*** (−37%)	
- F_{3B}	88		93		97		66* (−25%)	
Birth weight (g)								
- F_{3A}	6.2		6.4		5.9 (−5%)		5.7* (−8%)	
- F_{3B}	6.7		6.4		6.4 (−4%)		5.8* (−13%)	

*: $P < 0.05$; ***: $P < 0.001$
Source: Tesh, Willoughby & Secker (1986)

ammonium sulfide to detect implantations. All viable fetuses were weighed, sexed and examined for external malformations and variations. Approximately half of each litter was placed in Bouin's fixative and examined for visceral malformations and variations, and the remaining half of the fetuses were fixed in ethanol and stained for examination for skeletal malformations and variations. In addition, the following organs of the dams were weighed: liver, kidney, spleen, ovaries and adrenals.

Maternal effects were observed at dose levels of 35 and 120 mg/kg bw per day and consisted of significant reductions in body weight gain, feed consumption and water intake and significant increases in spleen and liver weights (Table 33). In the 120 mg/kg bw per day dose group, a statistically significant decrease in body weight gain was observed for several days immediately after the start of dosing (GD 6). Such an effect was not observed at 35 mg/kg bw per day. At these dose levels, a reduction in the number of viable fetuses, a reduction in fetal weight, an increase in the number of late resorptions and increased placental weight were also observed. At the highest dose, there was an increase in fetuses with 14th rudimentary rib; in the low- and mid-dose groups, the percentage of fetuses affected was comparable to that in controls. The incidences of renal pelvic dilatation showed no dose–response relationship in the percentage of fetuses affected. Therefore, these histopathological findings were not considered adverse.

The NOAEL for maternal toxicity was 10 mg/kg bw per day, based on reduced body weight gain and feed consumption, increased placental weight and increased liver and spleen weights at 35 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 10 mg/kg bw per day, based on a reduction in the number of viable fetuses, a reduction in fetal weight and an increase in the number of late resorptions at 35 mg/kg bw per day.

The total litter loss, mainly due to late resorptions in one high-dose female, might potentially have been caused by a teratological effect in the fetuses. However, as placental weight and the number of late resorptions increased as the dose level increased, it was considered that the late resorption had occurred as a consequence of damage to the placenta by the test substance, rather than a direct effect on fetuses. Therefore, it is considered that triflumizole did not produce a teratogenic response in this study (Nishibe et al., 1983f).

In a developmental toxicity study in rats, groups of 24 pregnant female Sprague-Dawley (Crj:CD) rats were treated with triflumizole (purity 98.3%; lot no. TK4121) by gavage at a dose level of 0, 3, 7 or 35 mg/kg bw per day. The study was performed partly in accordance with OECD Test Guideline 414. Rats were dosed from GD 6 up to GD 16, but a caesarean section was not performed until GD 20. OECD guidelines state that doses should be administered daily from implantation to the day before caesarean section. Females were examined daily throughout the study for mortality and clinical signs. Body weight and feed consumption were recorded daily on GDs 0–20. Water intake

Table 33. Maternal and fetal toxicity in a developmental toxicity study in rats

Parameter	0 mg/kg bw per day	10 mg/kg bw per day	35 mg/kg bw per day	120 mg/kg bw per day
Maternal effects				
Body weight gain days 0–20 (g)	139.4	137.1 (−2%)	117.1*** (−16%)	111.5*** (−20%)
Organ weights				
- Liver, absolute (g)	14.93	14.91	14.97	15.38
- Liver, relative (%)	4.55	4.62	4.81*	5.07***
- Spleen, absolute (g)	0.680	0.659	0.751*	0.774***
- Spleen, relative (%)	0.207	0.204	0.242***	0.256***
Litter response				
Live fetuses/ pregnant female	14.3	15.0	11.5** (−20%)	11.5** (−20%)
Fetal weight (g)				
- Males	3.8	3.7	3.5** (−6%)	3.5** (−7%)
- Females	3.5	3.5	3.3 (−5%)	3.2** (−8%)
Placental weight (g)	0.46	0.49 (+7%)	0.79*** (+73%)	0.85*** (+86%)
Dead or resorbed fetuses[a]	15 (4.2%)	16 (4.3%)	83*** (23.2%)	87*** (24.0%)
- Early deaths	14 (3.9%)	16 (4.3%)	17 (4.7%)	14 (3.9%)
- Late deaths	1 (0.3%)	0 (0%)	66*** (18.4%)	73*** (20.1%)
Examination of the fetuses[a]				
Skeletal findings				
- 14th rudimentary rib (% of fetuses)	11 (6%)	16 (9%)	16 (11%)	55* (37%)
Visceral findings				
- Renal pelvic dilatation (% of fetuses)	13 (8%)	29* (17%)	24 (19%)	25* (20%)

*: $P < 0.05$; ** P: < 0.01; *** P: < 0.001

[a] Number of fetuses, with percentage of fetuses in parentheses.

Source: Nishibe et al. (1983f)

was measured on GDs 1, 6, 11, 16 and 20. On GD 20, females were killed and subjected to a caesarean section and gross necropsy. The uterus was removed and opened, and fetuses were removed. Numbers of viable fetuses, early and late resorptions, total implantations and number of corpora lutea were recorded. Uteri from apparently non-pregnant females were stained with ammonium sulfide solution for confirmation of pregnancy status. All viable fetuses were weighed, sexed and examined for external malformations and variations. The total numbers of fetuses examined (number of litters) were 339 (24), 343 (23), 342 (24) and 317 (24) for the 0, 3, 7 and 35 mg/kg bw per day groups, respectively. Approximately half of the fetuses were placed in Bouin's fixative and examined for visceral malformations and variations, and the remaining half of the fetuses were fixed in ethanol and stained for examination for skeletal malformations and variations.

Maternal toxicity was evident at 35 mg/kg bw per day. Body weight gain was reduced compared with controls over GDs 17–18 and over the dosing interval (−18% and −15%, respectively; $P < 0.05$). The reduction in mean body weight gain was accompanied by reductions in feed consumption on GD 7 and daily over GDs 12–19, ranging from −9% to −16% of control values. No statistically significant differences were noted in absolute body weight or water intake. Changes in organ weights were not definitive of adverse effects of treatment. The mean absolute right adrenal weight was significantly decreased, but was not accompanied by changes in relative weight. The mean left ovary weight relative to body weight was significantly increased, but the right relative ovary weight was comparable to that of controls. The placental weight was significantly increased compared with controls (+45%; $P < 0.01$). Gross necropsy did not reveal any adverse effects of treatment. No adverse effects of treatment were observed in females treated with 3 or 7 mg/kg bw per day.

Evidence of developmental toxicity was seen at 35 mg/kg bw per day. The incidence of late resorptions/dead fetuses was significantly ($P < 0.05$) increased (17 versus 0 for controls; 4.8% versus 0% for controls). Although the number of viable fetuses was slightly reduced compared with controls (13.2 versus 14.1 for controls), this reduction did not attain statistical significance. Fetal weight was not affected (Table 34). No statistically significant, treatment-related external, visceral or skeletal malformations or variations were noted.

Table 34. Fetal toxicity in a developmental toxicity study in rats

	0 mg/kg bw per day	3 mg/kg bw per day	7 mg/kg bw per day	35 mg/kg bw per day
Litter response				
Live fetuses/ pregnant female	14.1	14.9	14.3	13.2 (−6%)
Dead or resorbed fetuses	23	13 (−43%)	24	35 (+52%)
- Early deaths	23	12	21	18
- Late deaths	0	1	3	17*

*: $P < 0.05$; ** P: < 0.01
Source: Gotoh (1986)

The maternal toxicity NOAEL was 7 mg/kg bw per day, based on reductions in body weight gain and feed consumption and increased placental weights at 35 mg/kg bw per day. The embryo/fetal toxicity NOAEL was 7 mg/kg bw per day, based on increased numbers of late resorptions/dead fetuses at 35 mg/kg bw per day (Gotoh, 1986).

Rabbits

In a non-GLP developmental toxicity study in rabbits, groups of 15 pregnant female New Zealand White rabbits were treated with triflumizole (purity 98.7%; lot no. TK3081) by gavage at a dose level of 0, 50, 100 or 200 mg/kg bw per day. The study was performed partly in accordance with OECD Test Guideline 414. Deviations from the guideline were the following: 1) only 15 female rabbits per group were used instead of the recommended 20; and 2) guidelines state that doses should be administered daily from implantation to the day before caesarean section; in this study, the last day of dosing was GD 18, but the caesarean section was not conducted until GD 29. The rabbits were observed for clinical signs and weighed every 2 days, except during GDs 6–18, when they were weighed daily. Feed consumption was generally measured over 3-day intervals spanning GDs 1–29, and water consumption was generally measured over 2-day intervals between GDs 2 and 29. On GD 29, the rabbits were killed and subjected to a caesarean section and gross necropsy. The uterus was removed and opened, and the types of implantations (live and dead fetuses, resorptions) were counted and their relative positions recorded. The ovaries were also removed, and corpora lutea were counted and recorded. Viability of the fetuses was monitored for 24 hours after the caesarean section. Then all

fetuses were processed and examined for visceral and skeletal malformations and variations. The total numbers of fetuses examined (number of litters) were 100 (13), 87 (14), 105 (13) and 109 (14) for the 0, 50, 100 and 200 mg/kg bw per day groups, respectively.

Treatment with triflumizole produced maternal toxicity, as shown by reduced body weight and feed consumption at 200 mg/kg bw per day. A slight, temporary depression of feed consumption was also recorded at 100 mg/kg bw per day. Absolute body weight in the high-dose group was significantly decreased ($P \leq 0.05$) compared with controls on GD 8 and again on GD 11, continuing to study termination on GD 29. The reductions in absolute body weight ranged from 4% on GD 11 to a maximum of 6% on GD 18. Body weight gain over the study period of GDs 0–29 was reduced by 25% compared with that of controls (not statistically significant). Calculation of body weight gain over the dosing interval of GDs 6–18 revealed a 57% reduction in body weight gain of high-dose females compared with the controls. The reductions in absolute body weight and body weight gain in high-dose females were accompanied by reduced feed consumption over the dosing intervals of GDs 8–10, 12–14 and 16–18 (22%, 30% and 35%, respectively). Feed consumption was also reduced in the 50 mg/kg bw per day group on GDs 8–10 (13%) and in the 100 mg/kg bw per day group over GDs 12–14 (32%), but these reductions are not considered an adverse effect of treatment because they were not correlated with reductions in body weight. Gross necropsy revealed changes in the high-dose group, including increased absolute liver weight (15%), reduced absolute ovary weights (22%) and reduced placental weights (−16%) (Table 35).

Table 35. Maternal and fetal toxicity in a developmental toxicity study in rabbits

	0 mg/kg bw per day	50 mg/kg bw per day	100 mg/kg bw per day	200 mg/kg bw per day
Maternal effects				
Weight (g)				
- Final body weight	3 853	3 725	3 753	3 645**
- Liver weight	92	94	92	106** (+15%)
- Ovary weight	0.96	0.83	0.90	0.75** (−22%)
Placental weight (g)				
- Male fetuses	5.1	4.7	4.5	4.3* (−16%)
- Female fetuses	4.9	4.7	4.4	4.1* (−16%)
Litter response				
Pup weight (g)				
- Males	37.5	35.9	36.1	32.3** (−13%)
- Females	36.2	35.5	34.3	31.6* (−14%)
24 h survival rate (%)	98.0	90.8	86.7	77.1* (−21%)

*: $P < 0.05$; **: $P < 0.01$
Source: Furuhashi (1985)

Treatment with triflumizole did not result in any external, visceral or skeletal malformations. Pups from the high-dose group had a reduced 24-hour survival rate (77% compared with 98% for controls) and statistically significantly reduced pup weight ($P < 0.01$ for males; $P < 0.05$ for females)

when compared with controls (Table 35). Values were also low when compared with the laboratory historical control data. No other effects on reproduction or fetal development were noted.

In conclusion, the maternal toxicity NOAEL was 100 mg/kg bw per day, based on reductions in body weight gain and feed consumption, increased liver weight and decreased ovary and placental weights compared with the controls at 200 mg/kg bw per day. The embryo/fetal toxicity NOAEL was 100 mg/kg bw per day, based on a reduced 24-hour survival rate and reductions in pup weight compared with controls at 200 mg/kg bw per day (Furuhashi, 1985).

In a developmental toxicity study in rabbits, groups of 16 pregnant female New Zealand White rabbits were treated with triflumizole (purity 98.3%; lot no. TK4121) by gavage at a dose level of 0, 5, 25 or 50 mg/kg bw per day at GDs 7–19. The study was performed partly in accordance with OECD Test Guideline 414. Deviations from the guideline included the following: 1) only 16 female rabbits per group were used instead of the recommended 20; 2) feed consumption was not measured; and 3) OECD guidelines state that doses should be administered daily from implantation to the day before caesarean section; in this study, the last day of dosing was GD 19, with a caesarean section conducted on GD 29. Females were examined twice daily throughout the study for mortality and clinical signs. Body weights were recorded on GDs 0, 7, 13, 20, 24 and 29; feed consumption was not recorded. On GD 29, the rabbits were killed and subjected to a caesarean section and gross necropsy. The uterus and ovaries were exposed, and the number and location of viable and non-viable fetuses, resorptions and total numbers of implantations and corpora lutea were recorded. The uterus was removed and weighed, and fetuses were removed. The uterus of each apparently non-pregnant female was opened and stained with ammonium sulfide to detect implantations. All fetuses were weighed, sexed and examined for external malformations and variations. Viable fetuses were retained for 24 hours for viability determination, after which they were sacrificed for examination. All fetuses were then processed and examined for visceral and skeletal malformations/variations. The total numbers of fetuses examined (number of litters) were 128 (15), 110 (15), 116 (15) and 118 (16) for the 0, 5, 25 and 50 mg/kg bw per day groups, respectively.

There was no clear evidence of maternal toxicity at any of the doses tested. All the female rabbits survived to study termination, and no adverse clinical signs were observed; although there was evidence of reduction in body weight gain in the high-dose group over days 7–13, this was not statistically significant. Overall, no statistically significant differences were noted in mean absolute body weight or body weight gain of treated female rabbits; feed consumption was not recorded. No changes were evident during examination at gross necropsy.

Treatment with triflumizole did not result in any adverse effects on reproduction and did not produce external, visceral or skeletal malformations. Two rabbits from the low-dose group and one rabbit from the high-dose group delivered early, but this was not considered to be related to treatment because of the lack of a dose–response relationship and low incidence. No effects on fetal development were noted.

In conclusion, based on the lack of statistically significant effects seen in this limited study, the maternal toxicity NOAEL was set at 50 mg/kg bw per day, the highest dose tested; a LOAEL could not be determined. The embryo/fetal toxicity NOAEL was 50 mg/kg bw per day, the highest dose tested; a LOAEL could not be determined (Keller, 1988).

2.6 *Special studies*

(a) Neurotoxicity

In an acute neurotoxicity study, groups of 11 male and 11 female CD (Crl: CD® (SD)IGS BR) rats were treated with a single dose of triflumizole (purity 99.2%; lot no. TBC-343) by gavage. The study was performed in accordance with OECD Test Guideline 424. The rats were segregated by sex, divided into groups of 11 and administered the following doses orally: 25, 100 and 400 mg/kg bw for males and 25, 100 and 200 mg/kg bw for females. Detailed clinical observations and functional

observational battery evaluations were performed on days −4, 1 (before and 2 hours after test substance administration), 8 and 15. Motor activity evaluations were conducted immediately following each functional observational battery interval. Individual body weights were measured on day −1 and weekly during the study. Following neurobehavioural evaluations, six animals of each sex per dose were randomly selected for neuropathology.

One male in the 400 mg/kg bw group and one female in the 200 mg/kg bw group were found dead on day 4 of the study (Table 36). Postmortem evaluations did not reveal the cause of death of the two deceased animals.

A few animals at 100 mg/kg bw had decreased activity within 3–6 hours after dosing. At 400 mg/kg bw in males and 200 mg/kg bw in females, decreased activity, shallow/slow breathing and black/yellow discoloured hair in the anogenital or scrotal region were observed in some animals within 3–6 hours after dosing, and some males were noted with skin cold to touch. The male that died had clinical signs similar to those of other males in that dose group on days 1 and 2. The female that died had similar clinical signs; in addition, impaired limb function and impaired righting reflex were found at 3 hours post-dosing on days 1 and 2. One other female at 200 mg/kg bw had impaired limb function at 3 hours after dosing. One male at 400 mg/kg bw had decreased activity on day 8 (Table 36). No other clinical findings were seen. Body weight was not affected by exposure to triflumizole.

Functional observational battery evaluations showed some statistically significant and dose-dependent changes on day 1. At 100 mg/kg bw, the following signs were observed: decreased rearing counts (females), decreases in mean forelimb and/or hindlimb grip strength (males and females) and decreased body temperature (females). Effects in the high-dose males and females (400 mg/kg bw for males and 200 mg/kg bw for females) were decreased rearing counts, changes in gait, mobility and righting reflex, decreases in mean forelimb and/or hindlimb grip strength, increase in thermal response and decreased body temperature. Motor activity evaluations revealed statistically significant and dose-dependent decreases in all motor activity parameters (horizontal activity, vertical activity, stereotypy activity, total distance) in mid- and high-dose animals on day 1. On day 8, high-dose males demonstrated increases in motor activity parameters (Table 36).

No clinical signs of acute neurotoxicity were observed in animals dosed with 25 mg/kg bw. The NOAEL was 25 mg/kg bw, based on the clinical findings and the functional and motor activity effects observed at the next higher dose (Goldenthal, 2003).

In a repeated-dose neurotoxicity study, groups of 16 male and 16 female CD (Crl: CD® (SD)IGS BR) rats were treated with doses of triflumizole (purity 99.2%; lot no. TBC-343) in the diet for 13 weeks. The study was performed in accordance with OECD Test Guideline 424. Dose levels were 0, 70, 700 and 2000 ppm (equal to 0, 4.1, 41 and 117 mg/kg bw per day for males and 0, 4.9, 48 and 133 mg/kg bw per day for females, respectively) and were based on the results of unspecified previous studies. All animals were observed twice a day for morbidity, injury and availability of food and water. A detailed clinical examination of each animal was performed once during each study week. Ophthalmoscopic examinations were performed pretest and prior to scheduled euthanasia, and body weight and feed consumption measurements were conducted weekly. Blood was collected pretest, in week 4 and at study termination from 10 animals of each sex per group. Functional observational battery examinations were conducted on 10 animals of each sex per group prior to initiation of exposure to the test article and during weeks 4, 8 and 13 of test article administration. The same animals were tested at all time points. All 10 animals of each sex per group designated for behavioural testing were also tested for motor activity prior to initiation of exposure to the test article and during weeks 4, 8 and 13. Following the functional observational battery and motor activity evaluations, six rats of each sex per group were randomly selected for neuropathological evaluation from the 10 rats of each sex per group designated as behavioural test animals. Complete necropsies were performed on these animals. The brain (including cerebrum, cerebellum/pons and medulla

Table 36. Effects in rats following single oral administration

	0 mg/kg bw per day		25 mg/kg bw per day		100 mg/kg bw per day		400 mg/kg bw per day	200 mg/kg bw per day
	Males	Females	Males	Females	Males	Females	Males	Females
Mortality	0/11	0/11	0/11	0/11	0/11	0/11	1/11	1/11
Clinical signs[a]								
Decreased activity	1/11	0/11	1/11	0/11	3/11	4/11	9/11	9/11
Shallow breathing	0/11	0/11	0/11	0/11	0/11	0/11	5/11	3/11
Slow breathing	0/11	0/11	0/11	0/11	0/11	0/11	4/11	4/11
Discoloured hair	0/11	0/11	1/11	1/11	0/11	0/11	4/11	2/11
Skin cold to touch	0/11	0/11	0/11	0/11	0/11	0/11	4/11	0/11
Righting reflex impaired	0/11	0/11	0/11	0/11	0/11	0/11	0/11	1/11
Limb function impaired	0/11	0/11	0/11	0/11	0/11	0/11	0/11	2/11
Functional observational battery[a]								
Rearing counts	–	–	No effect	No effect	No effect	dc (28%)	d (77%)	dc (63%)
Gait, mobility, righting reflex	0.0	0.0	0.0	0.0	0.0	0.0	1.0	1.0
Grip strength forelimbs	–	–	No effect	No effect	dc (27%)	dc (19%)	dc (68%)	dc (38%)
Grip strength hindlimbs	–	–	No effect	No effect	dc (35%)	d (11%)	dc (46%)	d (21%)
Thermal response time	–	–	No effect	No effect	No effect	No effect	ic (132%)	ic (158%)
Tail pinch response	1.0	1.0	1.0	1.0	1.0	1.0	0.0	0.0
Body temperature	–	–	No effect	No effect	No effect	dc (5%)	dc (7%)	dc (8%)
Motor activity measurements								
Day 1								
All parameters[b]	–	–	No effect	No effect	dc (~43–84%)	dc (~50–70%)	dc (~80–97%)	dc (~80–95%)
Day 8								
Horizontal activity (11–30 min)	–	–	No effect	No effect	No effect	No effect	ic (~79%)	No effect
Stereotypy (11–30 min)	–	–	No effect	No effect	No effect	No effect	ic (~86%)	No effect
Total distance (11–20 min)	–	–	No effect	No effect	No effect	No effect	ic (~119%)	No effect
Day 15	No treatment-related effects							

d: decreased, but not statistically significantly, compared with the controls; dc: statistically significantly decreased compared with the controls; ic: statistically significantly increased compared with the controls

[a] Incidences on day 1, hardly any effects on days 8 and 15.

[b] Horizontal and vertical activity, stereotypy and total distance.

oblongata), proximal sciatic nerve (2), sural nerve (2), tibial nerve (2), spinal cord including cervical swelling (C3–C6) and lumbar swelling (L1–L4), trigeminal ganglia (2), dorsal root ganglia (C3–C6, L1–L4) and dorsal and ventral root fibres (C3–C6, L1–L4) were collected. After fixation, both sciatic nerves with tibial, fibular and sural extensions were dissected free from the carcass from control and high-dose animals. No neuropathology animals died on study or were euthanized in extremis. For the remaining animals (10 of each sex per group), complete necropsy examinations were performed. No cholinesterase activities were measured.

All rats survived to scheduled termination. Body weights were slightly higher (10%) in males at 700 ppm and lower (8%) in females at 2000 ppm. Feed consumption was lower (9%) in females at 2000 ppm. Slight decreases in red blood cells, haemoglobin and haematocrit were observed in males and females at 2000 ppm at both intervals of analysis and in females at 700 ppm only in week 4. Increases in reticulocytes were observed in males and females at 2000 ppm at 4 and 13 weeks and at 700 ppm in week 4. Changes in locomotor activity, general arousal and rearing in males were observed without a clear dose–response relationship (Table 37). Increased liver weights were observed in males and females at 700 and 2000 ppm (Table 38). The decrease (> 10%) in uterus weight observed in the mid- and high-dose groups could not be explained by a change in body weight, as only the body weight in the high-dose group was decreased. In contrast, none of the other repeated-dose studies in rats showed an effect on the uterus. Therefore, this decrease in uterus weight, in the absence of any histopathology, is considered not treatment related. The increase in relative kidney weight was only slight and therefore not considered treatment related. Liver hypertrophy was seen in all males at 70, 700 and 2000 ppm and in females at 700 and 2000 ppm. Periportal hepatocellular vacuolation was observed at 700 and 2000 ppm (Table 39).

Table 37. Effects in rats following 13-week dietary exposure

Parameter	0 ppm		70 ppm		700 ppm		2 000 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
Functional observational battery								
Rearing								
- Week 4	4.2 ± 3.5	No effects	8.4 ± 5.4	No effects	9.3 ± 6.0	No effects	12.6 ± 6.3*	No effects
- Week 13	3.7 ± 4.8	No effects	6.4 ± 4.0	No effects	12.9 ± 2.6*	No effects	8.5 ± 5.7	No effects
Motor activity measurements[a]								
Horizontal activity	–	–	ic (87%)	No effects	ic (86%)	No effects	ic (92%)	No effects
Stereotypy activity	–	–	ic (94%)	No effects	ic (92%)	No effects	ic (88%)	No effects
Total distance	–	–	ic (136%)	No effects	ic (119%)	No effects	ic (157%)	No effects
Vertical activity	–	–	ic (58%)	ic (20%)	ic (58%)	ic (27%)	ic (46%)	ic (22%)

ic: statistically significantly increased compared with the controls; *: $P < 0.05$

[a] Data from week 13, consistent statistically significant changes observed only in week 13.

Source: Goldenthal (2004)

Table 38. Liver weights in rats following 13-week dietary exposure

	0 ppm		70 ppm		700 ppm		2 000 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
Absolute liver weight (g)								
Week 13								
- Mean	14.378	8.194	16.291	9.033	19.810** (+38%)	9.762** (+19%)	22.767** (+58%)	10.812** (+32%)
- Standard deviation	2.857	0.483	2.075	1.233	2.766	0.972	3.839	1.353
Relative liver weight (%)								
Week 13								
- Mean	2.971	3.137	3.050	3.312	3.589** (+21%)	3.623** (+15%)	4.504** (+53%)	4.439** (+41%)
- Standard deviation	0.275	0.170	0.261	0.278	0.364	0.294	0.533	0.389

**: $P < 0.01$
Source: Goldenthal (2004)

Table 39. Pathology findings in rats following 13-week dietary exposure

	0 ppm		70 ppm		700 ppm		2 000 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
Pathology								
Kidney								
Chronic progressive nephropathy								
- Minimal	1/10	0/10	3/10	2/10	5/10	0/10	5/10	0/10
Liver								
Hypertrophy centrilobular								
- Minimal	0/10	0/10	10/10	0/10	5/10	8/10	0/10	0/10
- Mild	0/10	0/10	0/10	0/10	5/10	2/10	0/10	10/10
Hypertrophy panlobular								
- Minimal	0/10	0/10	0/10	0/10	0/10	0/10	2/10	0/10
- Mild	0/10	0/10	0/10	0/10	0/10	0/10	8/10	0/10
Vacuolation periportal								
- Minimal	0/10	0/10	0/10	0/10	1/10	3/10	7/10	6/10
- Mild	0/10	0/10	0/10	0/10	0/10	1/10	3/10	2/10
Neuropathology								
Tibial nerve								
Axonal/myelin degeneration								
- Minimal	2/6	0/6	Not assessed		Not assessed		2/6	1/6

Source: Goldenthal (2004)

When the results of the motor activity measurement are viewed, it is apparent that habituation has occurred. Habituation, defined as a decrement in some natural response to a stimulus after repeated presentations, is apparent when an animal's locomotor behaviour declines with experience in the test cage within a single test session. However, habituation is also apparent with a decline in locomotion between sessions. This inter-session decline in activity counts provides evidence of previous experience in the test environment. In this study, there appears to be both within-session habituation as well as across-session habituation in the series of four sessions (especially in control rats). The male treatment groups showed a within-session decrement in activity. However, in contrast to male and female control rats or to the female test article–treated rats, male test article–treated rats failed to show a very robust across-session habituation. A clear inter-session habituation was, however, also not observed in the female control and treatment groups.

For motor activity measurements, consistent statistically significant changes were observed only in week 13. However, although the dose level increased from 70 to 2000 ppm, there was no dose–response relationship with regard to increased motor activity. Additionally, historical control data from other laboratories (no historical control data available from the performing laboratory) indicate that the motor activity of treated rats falls within the historical control range.

A statistically significant change in rearing was noted in males at 700 and 2000 ppm at weeks 13 and 4, respectively. These effects were most likely related to the pretest difference in total number of rears between control rats and do not reflect test article–related changes in the functional behaviour. There was no trend in the data, and there was no dose or time dependency. These effects, although statistically significant, are not considered to be physiologically relevant. In conclusion, the effects on motor activity are considered not adverse because there is no dose–response relationship, there is no effect in females and the locomotor changes are within the normal range of behaviour. Furthermore, it should be noted that in the 2-year rat study, female rats appeared to be more sensitive than males to the neurotoxic effects of triflumizole: a statistically significant increase of convulsive episodes was observed in females of the highest-dose group, but not in males.

The study is generally considered acceptable, as it was designed and executed in accordance with OECD Test Guideline 424, except that cholinesterase activity was not measured. Additionally, electroencephalograms have been not made, which could give an early indication of the occurrence of convulsions. It should be noted that overt convulsions were observed only in the chronic study more than 13 weeks after initiation of exposure.

Based on the liver effects at 700 ppm (equal to 41 mg/kg bw per day), the NOAEL for non-neurotoxic effects was 70 ppm (equal to 4.1 mg/kg bw per day). The NOAEL for neurotoxicity was 2000 ppm (equal to 117 mg/kg bw per day), the highest dose tested (Goldenthal, 2004).

(b) Immunotoxicity

In an immunotoxicity study, triflumizole (purity 99.8%; lot no. THJ-933) was administered for 28 days to BALB/c female mice in the diet at a dose level of 0, 20, 200 or 2000 ppm (equal to 0, 4.4, 43 and 413 mg/kg bw per day). Groups of 10 female mice per dose were used in the sheep red blood cell (sRBC) assay, whereas groups of 8 female mice per dose were used for the natural killer (NK) cell assay. The 1st day of dietary administration was designated as day 1. Mice were monitored daily for toxicity and were weighed on day 1 and weekly thereafter. Mice in the sRBC cohort were immunized on day 24 with sRBCs (0.2 mL/mouse of 1×10^8 sRBCs/mL). On day 29, all animals were euthanized, and body, spleen and thymus weights were recorded. Mice in the sRBC cohort had blood collected in tubes without anticoagulant. Blood was allowed to clot for approximately 30 minutes, followed by centrifugation at $2000 \times g$ for 10 minutes. Serum was collected and stored at −20 ºC until used for the assay. For mice in the NK cell assay cohort, the spleen was collected and prepared for evaluation of NK activity. Positive controls for the sRBC assay were treated with cyclophosphamide monohydrate at 15 mg/kg bw by intraperitoneal administration once daily for 5 days (days 24–28). Positive controls for the NK assay were treated with 10 µL reconstituted polyclonal antibody in 0.9% saline (per 2 mL dose) by intravenous administration on day 28.

All animals survived to study termination, and no clinical signs were noted. There did not appear to be any definitive treatment-related changes in water consumption; it appeared that significant amounts of spillage occurred during the movement of the cages or water bottles. Water consumption in the high-dose group was slightly reduced compared with controls, ranging from −8% to −13% over weeks 2–4, but it is not known if these values are statistically significant. Feed consumption was not affected by treatment. Mean body weight was slightly reduced in the high-dose group compared with controls over days 8–29, but the decreases were not biologically significant, ranging from −3% to −7% of controls. Although differences in mean absolute body weight did not appear to be biologically significant, body weight gain of mice in the high-dose group was biologically significant, with a reduction of −53% when compared with control weight gain. Absolute and relative spleen and thymus weights were not affected by treatment with triflumizole. Anti-sRBC immunoglobulin M (IgM) was significantly decreased in the high-dose group (−45%, $P < 0.05$). Although IgM was also decreased in the 200 ppm group, the difference did not attain statistical significance. NK cell activity was not affected by treatment. The cyclophosphamide positive control group had significantly decreased absolute and relative spleen and thymus weights and reduced IgM. The polyclonal antibody positive control group had significantly decreased absolute and relative spleen weights and reduced NK cell activity.

In conclusion, the NOAEL for systemic toxicity was 200 ppm (equal to 43 mg/kg bw per day), based on significantly reduced body weight gain at 2000 ppm (equal to 413 mg/kg bw per day). The NOAEL for immunotoxicity was 200 ppm (equal to 43 mg/kg bw per day), based on a significant reduction in anti-sRBC IgM in mice immunized with sRBCs at 2000 ppm (equal to 413 mg/kg bw per day) (Burleson, 2010).

(c) Effects on enzymes and other biochemical parameters

In a study designed to assess hepatic microsomal enzyme induction in rats, groups of five rats (rat strain not provided) of each sex per dose were administered triflumizole (purity 98.3%; lot no. TK-4121) in 0.5% carboxymethyl cellulose by oral gavage for 5 consecutive days at a dose of 0 or 200 mg/kg bw per day. Groups of five positive control rats of each sex were administered phenobarbital sodium (PB) dissolved in water at 75 mg/kg bw per day or 3-methylcholanthrene (3-MC) dissolved in sesame oil at 25 mg/kg bw per day by intraperitoneal injection on 4 consecutive days. Following the treatment period, each rat was anaesthetized and the liver perfused in situ for microsomal assays. Following perfusion, the liver was removed and weighed, and a section was removed from the left lateral lobe and weighed for use in hepatic microsomal determinations. Measurements included total amount of microsomal protein, amounts of cytochrome P448/P450 on a per milligram protein basis as well as a per gram liver basis, and activities of the following enzymes: *p*-nitroanisole *O*-demethylase (PNAS), 7-ethoxycoumarin *O*-deethylase (7-EC) and 7-ethoxyresorufin *O*-deethylase (EROD).

No mortality was observed in any of the groups. Ataxia was noted on days 1–3 of treatment in the PB group. No clinical signs were noted in any other groups. Treatment with triflumizole did not result in any definitive adverse effects on body weight.

Mean absolute and relative liver weights were increased ($P < 0.05$) in the triflumizole-treated males and females (+32% and +24%, respectively), males and females in the PB group (+17% and +24%, respectively) and males and females in the 3-MC group (+31% and +38%, respectively), compared with the control group (Table 40).

Other increases in the triflumizole, PB and 3-MC groups compared with the control group included microsomal P448/P450 concentrations per milligram protein (males: +73%, +67% and +60%, respectively; females: +15%, +39% and +58%, respectively); concentration of P450 per gram liver (males: +90%, +122% and +75%, respectively; females: +86%, +108% and +88%, respectively); and concentration of P450 on a total liver basis (males: +142%, +176% and +136%, respectively; females: +135%, +175% and +169%, respectively) (Table 41).

Table 40. Body and liver weights in rats

	Control		Triflumizole 200 mg/kg bw per day		PB 75 mg/kg bw per day		3-MC 25 mg/kg bw per day	
	Males	Females	Males	Females	Males	Females	Males	Females
Body weight (g)	299	226	289	228	319	241	309	234
Absolute liver weight (g)	12.36	9.34	15.62**	11.81*	15.36**	12.37*	16.66*	13.37*
Liver weight relative to body weight (%)	4.11	4.14	5.41*	5.15*	4.81**	5.13*	5.39*	5.72*

3-MC: 3-methylcholanthrene; PB: phenobarbital sodium; *: $P < 0.01$; **: $P < 0.05$
Source: Johnson (1988)

Table 41. Hepatic microsomal enzyme induction in rats

	Control		Triflumizole 200 mg/kg bw per day		PB 75 mg/kg bw per day		3-MC 25 mg/kg bw per day	
	Males	Females	Males	Females	Males	Females	Males	Females
Lowry protein (mg/mL)	2.24	2.15	3.34	3.32*	3.32	3.39*	3.40	2.57
Cytochrome P448/450 (µmol/mg protein)	0.63	0.65	1.09	0.85	1.05	1.03	1.01	1.17
P450 (µmol/g liver)	3.75	3.14	7.15	6.35	8.31	7.10	6.53	6.40

3-MC: 3-methylcholanthrene; PB: phenobarbital sodium; *: $P < 0.01$
Source: Johnson (1988)

When the effects of treatment on the activities of the enzymes are examined, triflumizole, PB and 3-MC increased the activity levels of PNAS (overall group activity increased by 2.1-, 2.4- and 2.8-fold, respectively) and 7-EC (increased by 1.9-, 2.2- and 8.3-fold, respectively). Triflumizole and PB did not increase the activity of EROD, whereas 3-MC treatment increased the overall activity by 48-fold (Table 42).

In summary, the results of this study demonstrated that treatment with triflumizole at 200 mg/kg bw per day for 5 days increased liver weight and microsomal P448/P450 levels, although the increases in microsomal levels on a per milligram protein, per gram of liver and per total liver basis did not often attain statistical significance. The increased liver weight and P448/P450 activity levels indicate that triflumizole treatment increased metabolic capacity. PNAS activity, indicative of P450, and 7-EC activity, indicative of P448/P450, were significantly increased, whereas triflumizole did not affect the activity levels of EROD (a P448 indicator). This suggests that triflumizole acts more like a PB-type inducer than a 3-MC-type inducer (Johnson, 1988).

(d) Studies on metabolites

The acute oral toxicity of 10 metabolites of triflumizole was assessed in Slc:SD rats in a non-GLP study. The test substances were administered in a single dose by gavage. The tested metabolites were FD-1-1, FD-2-1, FD-6-1, FD-7-1, FM-2-1, FM-5-1, FM-6-1, FM-8-1, FA-1-1 and FA-1-5. Of these metabolites, FA-1-1 and FD-1-1 were defined as metabolites relevant for the environment. Metabolites FM-5-1, FM-6-1, FD-1-1, FM-8-1, FD-6-1, FD-2-1, FD-7-1 and FA-1-1 were identified in plants (Table 43).

***Table 42. Mean enzyme activities in rats*[a]**

Group	Sex	PNAS	7-EC	EROD
Control	M	48.1 ± 13.1	231.6 ± 106.1	0.99 ± 0.62
	F	34.3 ± 5.1	106.8 ± 32.2	0.90 ± 0.48
Overall		41.2 ± 11.9	169.2 ± 98.9	0.95 ± 0.55
Triflumizole 200 mg/kg bw per day	M	113.8 ± 6.8	504.7 ± 114.3	1.21 ± 0.36
	F	59.9 ± 9.7	150.6 ± 41.2	1.25 ± 0.63
Overall		86.8* ± 29.5	327.6* ± 203.4	1.23 ± 0.49
PB 75 mg/kg bw per day	M	125.5 ± 24.9	534.8 ± 132.1	1.50 ± 0.55
	F	72.7 ± 17.0	217.8 ± 86.7	1.06 ± 6.27
Overall		99.1* ± 34.3	376.3* ± 197.5	1.28 ± 0.47
3-MC 25 mg/kg bw per day	M	130.1 ± 16.0	1 477.2 ± 375.4	52.72 ± 15.22
	F	103.1 ± 10.6	1 339.5 ± 295.2	38.06 ± 7.83
Overall		116.6* ± 19.2	1 408.3* ± 325.7	45.39* ± 13.78

7-EC: 7-ethoxycoumarin *O*-deethylase; EROD: 7-ethoxyresorufin *O*-deethylase; F: females; M: males; 3-MC: 3-methylcholanthrene; PB: phenobarbital sodium; PNAS: *p*-nitroanisole *O*-demethylase; *: $P \leq 0.05$

[a] Data presented as mean ± standard deviation of nanomoles per hour per milligram protein.

Source: Johnson (1988)

Table 43. Overview of major metabolites of triflumizole

Metabolite	Source	Chemical name
FD-1-1	Environment, plant	*N*-(4-Chloro-2-trifluoromethylphenyl)-2-propoxy-acetamide
FD-2-1	Plant	*N*-(4-Chloro-2-trifluoromethylphenyl)-2-hydroxy-acetamide
FD-6-1	Plant	*N*-(4-Chloro-2-trifluoromethylphenyl)-2-(2-hydroxypropoxy)-acetamidine
FD-7-1	Plant	*N*-(4-Chloro-2-trifluoromethylphenyl)-oxalamic acid
FM-2-1	–	2-(4-Chloro-2-trifluoromethylphenylimino)-2-imidazol-1-yl-ethanol
FM-5-1	Plant	(*E*)-*N*-(4-Chloro-2-trifluoromethylphenyl)-*N*′-formylpropoxy-acetamidine
FM-6-1	Plant	*N*-(4-Chloro-2-trifluoromethylphenyl)-2-propoxy-acetamidine
FM-8-1	Plant	*N*-(4-Chloro-2-trifluoromethylphenyl)-2-hydroxy-acetamidine
FA-1-1	Environment, plant	4-Chloro-2-trifluoromethylphenylamine
FA-1-5	–	2-Amino-5-chloro-3-trifluoromethylphenol

The studies were performed in accordance with OECD Test Guideline 401. For doses, refer to Table 44 below.

FD-1-1

Mortality: Death occurred in almost all groups of both sexes on the 1st and 3rd days.

Symptoms of toxicity: Decreased motor activity, ventral position, hypotonia, ataxia, decreased body temperature, lacrimation and haematuria were observed in both sexes. In addition to these toxic signs, decreased righting reflex, low sensitivity, urinary incontinence and bradypnoea in female rats and diarrhoea and decreased heart rate in male rats were observed.

Body weight: A temporary body weight decrease was found in both sexes.

Pathology: Haemorrhages in intestinal and bladder mucosa were observed in both sexes. In addition, dilated bladder was observed in dead female rats.

FD-2-1

Mortality: No deaths occurred in male rats. One death was observed on the 1st day in females.

Symptoms of toxicity: Decreased motor activity, low sensitivity, ventral position, hypotonia, bradypnoea, decreased righting reflex, decreased body temperature and ataxia were observed in both sexes. In addition to these toxic signs, lacrimation was observed in female rats.

Body weight: No unusual changes in body weight were found in either sex.

Pathology: No abnormality was observed in either sex.

FD-6-1

Mortality: No deaths occurred in male rats. In females, death occurred in the 3000 mg/kg bw dose group on the 1st or 2nd day.

Symptoms of toxicity: Decreased motor activity, ventral position, hypotonia, low sensitivity, decreased righting reflex, ataxia, bradypnoea and decreased body temperature were observed in both sexes. In addition to these toxic signs, lacrimation, ptosis, urinary incontinence, salivation and decreased heart rate were observed in female rats.

Body weight: A temporary body weight decrease was found in female rats.

Pathology: Haemorrhages in intestinal mucosa were found in dead female rats.

FD-7-1

Mortality: Death occurred in the 1000 mg/kg bw and higher dose groups in males and in all groups in females on the 1st or 2nd day.

Symptoms of toxicity: Decreased motor activity, ventral position, hypotonia, ataxia, low sensitivity, decreased righting reflex, urinary incontinence, bradypnoea and decreased body temperature were observed in both sexes.

Body weight: A temporary body weight decrease was found in both sexes.

Pathology: Haemorrhages in intestinal and stomach mucosa were observed in dead rats of both sexes.

FM-2-1

Mortality: No death occurred in either sex.

Symptoms of toxicity: Decreased motor activity, hypotonia and ataxia were observed in both sexes. In addition to these toxic signs, ventral position, low sensitivity, decreased righting reflex, decreased body temperature and urinary incontinence were observed in female rats.

Body weight: A temporary body weight decrease was found in both sexes.

Pathology: No abnormality was observed in either sex.

FM-5-1

Mortality: No deaths occurred in male rats. In females, a death was observed in the 3000 mg/kg bw dose group on the 2nd day.

Symptoms of toxicity: Decreased motor activity, ventral position, hypotonia, ataxia, low sensitivity and decreased righting reflex were observed in both sexes. In addition to these toxic signs, urinary incontinence, bradypnoea and decreased body temperature were observed in female rats.

Body weight: A temporary body weight decrease was found in both sexes.

Pathology: No abnormality was observed in either sex.

FM-6-1

Mortality: Death occurred in male rats of the 4167 mg/kg bw and higher dose groups on the 1st to 5th days. In females, deaths occurred on the 1st and 3rd days in all groups.

Symptoms of toxicity: Decreased motor activity, hypotonia, ventral position, ataxia, lacrimation and decreased body temperature were observed in both sexes. In addition to these toxic signs, low sensitivity, decreased righting reflex and bradypnoea in female rats and haematuria in male rats were observed.

Body weight: A temporary body weight decrease was found in both sexes.

Pathology: Haemorrhages in intestinal and stomach mucosa were observed in both sexes. In addition, haemorrhages in bladder mucosa in males and dilated bladder in females were observed.

FM-8-1

Mortality: Death occurred in the 1400 mg/kg bw and higher dose groups in males and the 1593 mg/kg bw and higher dose groups in females on the 1st to 3rd days.

Symptoms of toxicity: Decreased motor activity, ventral position, hypotonia, ataxia, low sensitivity, decreased righting reflex, bradypnoea, lacrimation, urinary incontinence and decreased body temperature were observed in both sexes.

Body weight: A temporary body weight decrease was found in both sexes.

Pathology: Haemorrhages in intestinal mucosa were observed in dead rats of both sexes. Haemorrhages in stomach mucosa and ascites were observed in dead male rats.

FA-1-1

Mortality: Deaths occurred in the 819 mg/kg bw and higher dose groups in males on the 1st to 3rd days and the 579 mg/kg bw and higher dose groups in females on the 1st to 3rd days.

Symptoms of toxicity: Decreased motor activity, low sensitivity, decreased righting reflex, hypotonia, ventral position, ataxia, bradypnoea and lacrimation were observed in both sexes. In addition to these toxic signs, decreased body temperature and diarrhoea in male rats and salivation, urinary incontinence and vocalization in female rats were observed.

Body weight: A temporary body weight decrease was found in male rats.

Pathology: Haemorrhages in intestinal mucosa were observed in dead rats of both sexes. In addition, dilated bladder and ascites were observed in dead male rats.

FA-1-5

Mortality: No deaths were seen in males. In females, a death was observed in the 3000 mg/kg bw dose group on the 1st day.

Symptoms of toxicity: Decreased motor activity, hypotonia, ataxia, urinary incontinence and low sensitivity were observed in both sexes. In addition to these toxic signs, decreased righting reflex was observed in female rats.

Body weight: A temporary body weight decrease was found in male rats.

Pathology: No abnormality was observed in either sex.

Conclusions on acute toxicity of metabolites

The acute oral LD_{50}s of the test substances are given in **Error! Reference source not found.**44. Compared with the parent compound (triflumizole), for which the LD_{50} values were 1057 mg/kg bw in males and 1780 mg/kg bw in females, FA-1-1 and FD-7-1 are slightly more toxic, whereas the other metabolites FD-1-1, FD-2-1, FD-6-1, FM-2-1, FM-5-1, FM-6-1, FM-8-1 and FA-1-5 are less toxic (Nishibe et al., 1985).

Table 44. Summary of acute oral toxicity of triflumizole metabolites

Test substance	Purity (%)	Lot no.	Appearance	Group size	Doses (mg/kg bw)	LD_{50} (mg/kg bw)
FD-1-1	99.1	GLP 82-1	Colourless liquid	10 M/dose	3 471, 4 167, 5 000, 6 000, 7 200, 8 640	5 882 (5 729–6 132)
				5 F/dose	2 785, 3 482, 4 352, 5 440, 6 800, 8 500	3 405 (2 965–3 910)
FD-2-1	99.9	31-2294-SY	White crystal	5 M/dose	500, 1 000, 2 000	> 2 000
				5 F/dose	1 000, 2 000, 3 000	> 3 000
FD-6-1	> 98	31-8329-MH	Yellow crystal	5 M/dose	500, 1 000, 2 000	> 2 000
				5 F/dose	1 000, 2 000, 3 000	2 000–3 000
FD-7-1	> 96	31-3158	White crystal	5 M/dose	500, 1 000, 2 000	~1 000
				5 F/dose	1 000, 2 000, 3 000	~1 000
FM-2-1	99.9	31-2299-SY	Colourless crystal	5 M/dose	500, 1 000, 2 000	> 2 000
				5 F/dose	1 000, 2 000, 3 000	> 3 000
FM-5-1	98.8	31-2381-YK	Colourless liquid	5 M/dose	500, 1 000, 2 000	> 2 000
				5 F/dose	1 000, 2 000, 3 000	> 3 000
FM-6-1	99.8	GLP 82-2	White crystal	10 M/dose	3 471, 4 167, 5 000, 6 000, 7 200	4 987 (4 887–5 087)
FM-6-1	> 99	MH-8319	White crystal	10 F/dose	1 561, 2 107, 2 845, 3 841, 5 185, 7 000	2 131 (1 398–2 874)
FM-8-1	99.9	31-2349-YK	Colourless crystal	10 M/dose	1 077, 1 400, 1 820, 2 367, 3 077, 4 000	1 935 (1 651–2 261)
FM-8-1	> 99	31-4187-MH	Light yellow crystal	10 F/dose	942, 1 225, 1 593, 2 071, 2 629, 3 500	2 144 (1 894–2 429)
FA-1-1	98.5	33764	Colourless liquid	10 M/dose	655, 819, 1 024, 1 280, 1 600, 2 000	961 (872–1 060)
				10 F/dose	402, 482, 579, 694, 833, 1 000	771 (711–839)
FA-1-5	99.7	31-2384-YK	Light brown crystal	5 M/dose	500, 1 000, 2 000	> 2 000
				5 F/dose	1 000, 2 000, 3 000	> 3 000

F: females; M: males
Source: Nishibe et al. (1985)

3. Observations in humans

A report on the results of its yearly health examination of the personnel involved in the production of triflumizole at the Takaoka plant (Japan) in the period May 1996 to May 2002 was prepared as a consequence of the Japanese "Occupational Safety and Health Law" and has been submitted to the regulatory authority. Commercial production of triflumizole started in 1985 at this plant. The health examination consisted of physical examination, haematology, urine analysis and blood chemistry. No adverse health effects attributable to chemical exposure were observed. In

addition, it was reported that in the period covered, no events of acute poisoning by exposure or skin and/or eye irritation were observed (Takami, 2002).

Comments

Biochemical aspects

The absorption of (phenyl-U-^{14}C)-labelled triflumizole in rats was at least 72% of the administered single and repeated doses at 10 mg/kg bw after 48 hours and about 80% of the applied single dose at 300 mg/kg bw after 96 hours. Absorption was considerably slower after the high dose than after the low dose, as evidenced by the much longer time to reach C_{max} (T_{max}). The radioactivity detected in tissues was generally low (~2%), with highest concentrations found in the liver. Excretion of triflumizole occurred rapidly and independently of sex. Most of the administered low dose (~90%) was recovered within 24 hours. At the single high dose of 300 mg/kg bw, 35–45% of the administered dose was excreted by 24 hours, increasing to 92–99% at 96 hours. The majority of the radiolabel was excreted via the urinary route (~75% of the administered dose compared with ~20% excreted via the faeces).

Triflumizole is extensively metabolized. A few differences in metabolite pattern were observed between males and females after repeated low and single high doses, but not after a single low dose. The major urinary metabolites are the sulfate conjugates of *N*-(4-chloro-2-trifluoromethyl-phenyl)-2-hydroxy-acetamidine (FM-8-1) and 2-amino-5-chloro-3-trifluoromethylphenol (FA-1-5). In faeces, *N*-(4-chloro-2-trifluoromethylphenyl)-2-hydroxy-acetamide (FD-2-1) is a major metabolite. Differences between dose regimens exist with respect to other major metabolites. 2-(4-Chloro-2-trifluoromethylphenylimino)-2-imidazol-1-yl-ethanol (FM-2-1) is the major metabolite after a single oral low dose. 4-Chloro-2-trifluoromethylphenylamine (FA-1-1) is a major metabolite after single and repeated low dosing, whereas *N*-(4-chloro-2-trifluoromethylphenyl)-2-propoxy-acetamide (FD-1-1) is the major metabolite after a single high dose The presence of the metabolite *N*-(4-chloro-2-trifluoromethylphenyl)-2-propoxy-acetamidine (FM-6-1) was confirmed in rat faeces and urine.

Toxicological data

Triflumizole has an oral LD_{50} of 1057 mg/kg bw in rats. The dermal LD_{50} is greater than 5000 mg/kg bw, and the inhalation LC_{50} is greater than 3.6 mg/L. No skin irritation and only mild eye irritation were observed. Based on a maximization test in guinea-pigs, triflumizole is considered a skin sensitizer. These studies were conducted prior to GLP, except for the acute inhalation study, but they were conducted to an acceptable standard.

In all short-term studies, decreased body weight gain combined with increased feed consumption and increased liver weights were observed. In a non-GLP 90-day dietary toxicity study, mice were exposed to 0, 20, 200 or 2000 ppm (equal to 0, 3.2, 33 and 381 mg/kg bw per day for males and 0, 4.2, 43 and 466 mg/kg bw per day for females, respectively). The NOAEL was 200 ppm (equal to 33 mg/kg bw per day), based on decreased body weight gain, increased feed consumption and liver effects (increased liver weight, swelling of cytoplasm) observed at 2000 ppm (equal to 381 mg/kg bw per day).

In a second non-GLP 90-day dietary toxicity study, rats were exposed to 0, 20, 200 or 2000 ppm (equal to 0, 1.4, 15 and 177 mg/kg bw per day for males and 0, 1.8, 17 and 218 mg/kg bw per day for females, respectively). Decreased body weight gain, increased feed consumption, liver effects (increased liver weight, fatty changes) and increased kidney weights were also observed at 2000 ppm in both sexes. Based on these effects at 2000 ppm (equal to 177 mg/kg bw per day), the NOAEL was 200 ppm (equal to 15 mg/kg bw per day).

In a 1-year oral toxicity study with dogs exposed to 0, 100, 300 or 1000 ppm (equal to 0, 3, 10 and 34 mg/kg bw per day for males and 0, 3, 11 and 35 mg/kg bw per day for females, respectively), the NOAEL was 300 ppm (equal to 10 mg/kg bw per day), based on liver effects (increased weights,

macroscopic changes) and increased alkaline phosphatase levels at 1000 ppm (equal to 34 mg/kg bw per day).

In a combined chronic toxicity and carcinogenicity study in mice, animals were exposed through the diet to triflumizole at a concentration of 0, 100, 400 or 1600 ppm (equal to 0, 16, 67 and 296 mg/kg bw per day for males and 0, 22, 88 and 362 mg/kg bw per day for females, respectively). At 400 ppm (equal to 67 mg/kg bw per day) and above, liver effects were observed, as demonstrated by increased organ weight and histopathological findings (nodules and fatty changes in both sexes as well as granulomatous inflammation, cytological alterations, pigmentation and necrosis in some males). Based on these liver effects, the NOAEL was 100 ppm (equal to 16 mg/kg bw per day).

In a combined chronic toxicity and carcinogenicity study in which rats were exposed through the diet to triflumizole at a concentration of 0, 100, 400 or 1600 ppm (equal to 0, 3.5, 14 and 59 mg/kg bw per day for males and 0, 4.5, 18 and 77 mg/kg bw per day for females, respectively), the liver was an important target organ, as demonstrated by increased organ weight and a wide range of histopathological findings at 400 ppm (equal to 14 mg/kg bw per day) and above. The highest dose group of females also showed an increased incidence of convulsive episodes and an increased incidence of ovarian follicular cysts. Based on the liver effects observed, the NOAEL was 100 ppm (equal to 3.5 mg/kg bw per day).

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

The genotoxic potential of triflumizole was tested in an adequate range of in vitro and in vivo studies. Triflumizole showed no evidence of genotoxicity in any assays.

The Meeting concluded that triflumizole is unlikely to be genotoxic.

Based on the lack of genotoxicity and the absence of carcinogenicity in mice and rats, the Meeting concluded that triflumizole is unlikely to be carcinogenic in humans.

In a three-generation reproductive toxicity study, rats were exposed through the diet to triflumizole at a concentration of 0, 30, 70 or 170 ppm (equal to 0, 2.1, 4.8 and 12 mg/kg bw per day for males and 0, 2.5, 5.8 and 14 mg/kg bw per day for females, respectively). The NOAEL for parental toxicity was 70 ppm (equal to 4.8 mg/kg bw per day), based on increased placental weights and increased liver and kidney weights at the high dose (170 ppm, equal to 12 mg/kg bw per day). The NOAEL for reproductive toxicity was 70 ppm (equal to 4.8 mg/kg bw per day), based on reduced mating, fertility and litter size. The NOAEL for offspring toxicity was 170 ppm (equal to 12 mg/kg bw per day), the highest dose tested.

Two developmental toxicity studies were conducted in rats. In a non-GLP study, rats were exposed by gavage to 0, 10, 35 or 120 mg/kg bw per day, and in a GLP study, the dose levels were 0, 3, 7 and 35 mg/kg bw per day. Maternal toxic effects were similar in the two studies and comprised reduced body weight gain and feed consumption and increased placental weight at 35 mg/kg bw per day as well as increased liver and spleen weights at the same dose in the non-GLP study. Based on these effects, the overall NOAEL for maternal toxicity was 10 mg/kg bw per day, and the overall LOAEL was 35 mg/kg bw per day. The observed developmental effects were a reduction in the number of viable fetuses, a reduction in fetal weight and an increase in the number of late resorptions at 35 mg/kg bw per day. The overall NOAEL for embryo and fetal toxicity was 10 mg/kg bw per day, with an overall LOAEL of 35 mg/kg bw per day.

Two developmental toxicity studies were conducted in rabbits. In a non-GLP study, rabbits were exposed by gavage to 0, 50, 100 or 200 mg/kg bw per day, and in a GLP study, the dose levels were 0, 5, 25 and 50 mg/kg bw per day. Maternal toxic effects were reduced body weight gain and feed consumption, increased liver weights, decreased ovary weights and decreased placental weight at 200 mg/kg bw per day. The overall NOAEL for maternal toxicity was 100 mg/kg bw per day, with an overall LOAEL of 200 mg/kg bw per day. The observed developmental effects were a lower 24-hour pup survival rate and decreased pup weight at 200 mg/kg bw per day. Based on these effects, the overall NOAEL for embryo and fetal toxicity was 100 mg/kg bw per day, with an overall LOAEL of 200 mg/kg bw per day.

The Meeting concluded that triflumizole is not teratogenic in rats or rabbits.

The Meeting was aware of several mechanistic studies and unpublished reports that have been submitted to a regulatory authority. These studies aimed to clarify the effects seen in the reproductive and developmental toxicity studies in rats. These studies were not made available to the Meeting.

In an acute neurotoxicity study, rats were administered triflumizole by gavage at 0, 25, 100 or 400 (males)/200 (females) mg/kg bw. Based on the clinical findings and the functional and motor activity effects observed at the next higher dose (100 mg/kg bw), the NOAEL was 25 mg/kg bw.

In a 13-week neurotoxicity study, rats were exposed to triflumizole at 0, 70, 700 or 2000 ppm (equal to 0, 4.1, 41 and 117 mg/kg bw per day for males and 0, 4.9, 48 and 133 mg/kg bw per day for females, respectively). The systemic findings were similar to those obtained in the 90-day repeated-dose toxicity study, the liver clearly being the main target organ. Based on the liver effects at 700 ppm (equal to 41 mg/kg bw per day), the NOAEL for non-neurotoxic effects was 70 ppm (equal to 4.1 mg/kg bw per day). Observed effects on motor activity were considered not adverse because there was no dose–response relationship, there were no effects in females and the locomotor changes were within the normal range of behaviour. The NOAEL for neurotoxicity was 2000 ppm (equal to 117 mg/kg bw per day), the highest dose tested.

In an immunotoxicity study, triflumizole was administered for 28 days to female mice in diet containing concentrations of 0, 20, 200 and 2000 ppm (equal to 0, 4.4, 43 and 413 mg/kg bw per day, respectively). The NOAEL for systemic toxicity was 200 ppm (equal to 43 mg/kg bw per day), based on significantly reduced body weight gain at 2000 ppm (equal to 413 mg/kg bw per day). The NOAEL for immunotoxicity was 200 ppm (equal to 43 mg/kg bw per day), based on a significant reduction in anti-sRBC IgM in mice immunized with sRBCs at 2000 ppm (equal to 413 mg/kg bw per day).

Toxicological data on metabolites and/or degradates

Acute oral toxicity studies were performed with a number of triflumizole metabolites. For metabolites FD-1-1, FD-2-1, FD-6-1, FM-2-1, FM-5-1, FM-6-1, FM-8-1 and FA-1-5, the oral LD_{50}s were similar to or higher than that of the parent (1057 mg/kg bw). Only metabolites FA-1-1 and FD-7-1 were slightly more toxic than the parent (LD_{50} = 771 mg/kg bw and ~1000 mg/kg bw, respectively).

Human data

A report on the health examination of production workers in the period May 1996 – May 2002 did not reveal any adverse health effects. No cases of acute poisoning or skin/eye irritation were observed in the same period.

The Meeting concluded that the database on triflumizole was adequate to characterize the potential hazards to fetuses, infants and children.

Toxicological evaluation

An acceptable daily intake (ADI) of 0–0.04 mg/kg bw was established on the basis of the NOAEL of 3.5 mg/kg bw per day for hepatotoxicity (increased liver weight, macroscopic and microscopic hepatic changes) in the chronic toxicity study in the rat. A safety factor of 100 was applied. This is supported by the NOAEL of 4.8 mg/kg bw per day in the multigeneration study of reproductive toxicity in rats.

An acute reference dose (ARfD) of 0.3 mg/kg bw was established on the basis of the NOAEL of 25 mg/kg bw in the acute neurotoxicity study in the rat, based on clinical findings and effects on

function and motor activity at 100 mg/kg bw. A safety factor of 100 was applied. The effects observed in developmental toxicity studies in rats were not considered to be a consequence of a single dose.

The ADI and the ARfD are also applicable to the metabolites containing the 4-chloro-2-(trifluoromethyl)phenyl group.

Levels relevant to risk assessment of triflumizole

Species	Study	Effect	NOAEL	LOAEL
Mouse	Ninety-day study of toxicity[a]	Toxicity	200 ppm, equal to 33 mg/kg bw per day	2 000 ppm, equal to 381 mg/kg bw per day
	Two-year study of toxicity and carcinogenicity[a]	Toxicity	100 ppm, equal to 16 mg/kg bw per day	400 ppm, equal to 67 mg/kg bw per day
		Carcinogenicity	1 600 ppm, equal to 296 mg/kg bw per day[b]	–
Rat	Acute neurotoxicity study[c]	Neurotoxicity	25 mg/kg bw	100 mg/kg bw
	Ninety-day studies of toxicity[a,d]	Toxicity	200 ppm, equal to 15 mg/kg bw per day	700 ppm, equal to 41 mg/kg bw per day[d]
	Two-year study of toxicity and carcinogenicity[a]	Toxicity	100 ppm, equal to 3.5 mg/kg bw per day	400 ppm, equal to 14 mg/kg bw per day
		Carcinogenicity	1 600 ppm, equal to 59 mg/kg bw per day[b]	–
	Three-generation study of reproductive toxicity[a]	Parental toxicity	70 ppm, equal to 4.8 mg/kg bw per day	170 ppm, equal to 12 mg/kg bw per day
		Offspring toxicity	170 ppm, equal to 12 mg/kg bw per day[b]	–
		Reproductive toxicity	70 ppm, equal to 4.8 mg/kg bw per day	170 ppm, equal to 12 mg/kg bw per day
	Developmental toxicity studies[c,d]	Maternal toxicity	10 mg/kg bw per day	35 mg/kg bw per day
		Embryo and fetal toxicity	10 mg/kg bw per day	35 mg/kg bw per day
Rabbit	Developmental toxicity studies[c,d]	Maternal toxicity	100 mg/kg bw per day	200 mg/kg bw per day
		Embryo and fetal toxicity	100 mg/kg bw per day	200 mg/kg bw per day
Dog	One-year study of toxicity[a]	Toxicity	300 ppm, equal to 10 mg/kg bw per day	1 000 ppm, equal to 34 mg/kg bw per day

LOAEL: lowest-observed-adverse-effect level; NOAEL: no-observed-adverse-effect level
[a] Dietary administration.
[b] Highest dose tested.
[c] Gavage administration.
[d] Two or more studies combined.

Estimate of acceptable daily intake

0–0.04 mg/kg bw

Estimate of acute reference dose

0.3 mg/kg bw

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

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

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

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid and extensive, 72–79%; 2-fold slower at 24 h at high dose than at low dose
Dermal absorption	No data
Distribution	Widely distributed
Potential for accumulation	No evidence for accumulation
Rate and extent of excretion	Rapid, ~90% excreted within first 24 h, mainly via urine (~75%)
Metabolism in animals	Extensively metabolized, < 2% excreted as parent compound
Toxicologically significant compounds in animals, plants and the environment	Triflumizole and metabolites containing the 4-chloro-2-(trifluoromethyl)phenyl group
Acute toxicity	
Rat, LD_{50}, oral	1 057 mg/kg bw
Rat, LD_{50}, dermal	> 5 000 mg/kg bw
Rat, LC_{50}, inhalation	> 3.6 mg/L (4 h, nose only)
Rabbit, dermal irritation	Not a skin irritant
Rabbit, ocular irritation	Mild eye irritant
Guinea-pig, dermal sensitization	Sensitizer (maximization test)
Short-term studies of toxicity	
Target/critical effect	Liver
Lowest relevant oral NOAEL	15 mg/kg bw per day (rat)
Lowest relevant dermal NOAEL	1 000 mg/kg bw per day, the highest dose tested (rat)
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Liver
Lowest relevant NOAEL	3.5 mg/kg bw per day (rat)
Carcinogenicity	Not carcinogenic
Genotoxicity	
	Not genotoxic
Reproductive toxicity	
Reproduction target/critical effect	Reduced fertility and litter size at parentally toxic doses
Lowest relevant parental NOAEL	4.8 mg/kg bw per day

Lowest relevant offspring NOAEL	12 mg/kg bw per day, the highest dose tested
Lowest relevant reproductive NOAEL	4.8 mg/kg bw per day
Developmental toxicity	
Developmental target/critical effect	Placenta, fetal viability and weight, number of late resorptions at maternally toxic doses
Lowest relevant maternal NOAEL	10 mg/kg bw per day (rat)
Lowest relevant embryo/fetal NOAEL	10 mg/kg bw per day (rat)
Neurotoxicity	
Acute neurotoxicity NOAEL	25 mg/kg bw per day (rat)
Subchronic neurotoxicity	177 mg/kg bw per day, the highest dose tested (rat)[a]
Other toxicological studies	
Immunotoxicity NOAEL	43 mg/kg bw per day (mouse)
Studies on metabolites	FD-1-1, FD-2-1, FD-6-1, FM-2-1, FM-5-1, FM-6-1, FM-8-1, FA-1-5: oral LD_{50}s similar to or higher than that of parent FA-1-1 and FD-7-1: oral LD_{50}s slightly lower than that of parent
Medical data	
	No effects in manufacturing personnel, no cases of poisoning

LC_{50}: median lethal concentration; LD_{50}: median lethal dose; NOAEC: no-observed-adverse-effect concentration; NOAEL: no-observed-adverse-effect level

[a] In the chronic study, possible neurotoxicity (convulsive periods) was observed in female rats from week 30 onwards.

Summary

	Value	Study	Safety factor
ADI	0–0.04 mg/kg bw	Two-year study in rats	100
ARfD	0.3 mg/kg bw	Acute neurotoxicity study in rats	100

ADI: acceptable daily intake; ARfD: acute reference dose

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TRINEXAPAC-ETHYL

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Explanation

Trinexapac-ethyl is the International Organization for Standardization–approved common name for 4-(cyclopropyl-α-hydroxymethylene)-3,5-dioxo-cyclohexanecarboxylic acid ethyl ester (International Union of Pure and Applied Chemistry), with the Chemical Abstracts Service number 95266-40-3. Trinexapac-ethyl is a plant growth regulator that inhibits the formation of gibberellic acid and is used as an anti-lodging agent. The chemical structure of trinexapac-ethyl, including the position of the radiolabel in ^{14}C-labelled trinexapac-ethyl used in rat metabolism studies, is given in Fig. 1.

Fig. 1. Chemical structure of trinexapac-ethyl and [^{14}C]trinexapac-ethyl

Trinexapac-ethyl

[3,5-cyclohexadione-1,2,6-^{14}C]Trinexapac-ethyl

* Denotes the position of the ^{14}C radiolabel

Trinexapac-ethyl 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 integrity of the studies.

Evaluation for acceptable daily intake

1. Biochemical aspects

1.1 Absorption, distribution, metabolism and excretion

Rats

[^{14}C]Trinexapac-ethyl (radiochemical purity > 98%) in ethanol/polyethylene glycol/water (30 : 40 : 30) was administered to CD albino rats (five of each sex per dose) as a single intravenous dose of 0.91 mg/kg body weight (bw), a single gavage dose of 0.97 mg/kg bw, a single gavage dose of 166 mg/kg bw or 14 daily gavage doses of unlabelled trinexapac-ethyl (purity 96.6%) at 0.97 mg/kg bw followed by a single gavage dose of radiolabelled trinexapac-ethyl at 0.91 mg/kg bw. A concurrent control group of one male and one female rat was included with each treatment group. Urine and faeces were collected at various intervals to 168 hours after dosing. Rats were killed at 168 hours for the analysis of tissue radioactivity. Radioactivity was quantified in excreta and tissues by liquid scintillation counting (LSC). Metabolites in excreta were analysed by high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC). The identity of any metabolites was confirmed by mass spectroscopy.

No signs of toxicity were reported.

The results of the excretion/mass balance phase of the study are summarized in Table 1. Mean recovery of radioactivity across the treatment groups was approximately 98% of the administered dose (range for individual animal results: 81–99%). The majority of radioactivity was detected in urine across all dosing regimens, with means ranging from 90% to 97% of the administered dose. Relatively low levels of radioactivity were detected in faeces (means of 0.6–2.0% and 0.9–2.4% of the administered dose over 0–24 and 0–168 hours, respectively). Based on the level of radioactivity in urine, the cage wash and carcass/tissues, the level of gastrointestinal absorption is at least 96% of the administered dose. The comparable levels of radioactivity in urine following oral and intravenous dosing corroborate this high level of gastrointestinal absorption. Following oral dosing, renal excretion of radioactivity was relatively rapid, with the majority (> 90%) excreted within 24 hours.

Radioactivity was generally below the limit of detection in most tissues (heart, spleen, testes, ovaries, uterus, muscle, brain, bone and erythrocytes), with only very low levels varyingly measured in the lungs, kidneys, fat, plasma and carcass in some treatment groups. In fat, low levels of radioactivity were consistently detected across all groups (0.001–0.027 parts per million [ppm]), whereas low levels were detectable in the kidneys of the single low-dose intravenous group (0.001 ppm) and single high-dose oral group (0.016 ppm in males and 0.018 ppm in females). On the basis of these findings, it is concluded that radioactivity did not accumulate in any tissues.

Across all treatment groups, the free acid derivative of trinexapac-ethyl (i.e. trinexapac acid) was identified as the sole metabolite in urine. In faeces, the parent compound was present at approximately 5–22% of the recovered faecal radioactivity (1–2.5% of the administered dose), with the balance comprising trinexapac acid (Capps, 1990).

Table 1. Mass balance in rats following oral dosing with [^{14}C]trinexapac-ethyl

Sample	Mean % of administered radioactivity							
	0.91 mg/kg bw iv		0.97 mg/kg bw po		166 mg/kg bw po		Repeat po	
	Males	Females	Males	Females	Males	Females	Males	Females
Urine								
0–24 h	87	91	91	93	91	95	93	93
0–168 h	90	94	95	95	95	97	95	96
Urine + cage wash								
0–168 h	93	97	97	96	96	98	95	97
Faeces								
0–24 h	2.0	0.8	1.4	0.9	2.0	0.8	1.1	0.6
0–168 h	1.1	1.6	1.6	1.1	2.4	1.0	1.4	0.9
Carcass + tissues								
0–168 h	0.2	0.1	< 0.3	< 0.2	0.1	< 0.1	0.1	< 0.1
Total recovery								
0–24 h	88	92	92	94	93	96	94	94
0–168 h	94	98	99	97	98	99	97	98

iv: intravenous; po: per os (by mouth)
Source: Capps (1990)

In a study by Bissig (1995), [^{14}C]trinexapac-ethyl (radiochemical purity > 96%) in ethanol/polyethylene glycol/water (30 : 40 : 30) was administered by gavage to Tif:RAl f (SPF) rats (four of each sex per dose) as a single gavage dose of 1 or 200 mg/kg bw. Blood was sampled from two rats of each sex per dose at 0.25, 0.5, 1, 2 and 4 hours, with the remaining rats sampled at 4, 8, 12, 12, 24 and 48 hours; standard kinetic parameters were determined. In a tissue distribution experiment, 12 male rats per group were administered a single gavage dose of [^{14}C]trinexapac-ethyl at 1 or 200 mg/kg bw; three rats per dose were killed at 15 minutes, 55 minutes, 2 hours and 6 hours for the analysis of tissue radioactivity. An additional experiment was conducted in four male bile duct–cannulated rats that were gavaged with a single 1 mg/kg bw dose of [^{14}C]trinexapac-ethyl. Bile was collected at 0–1, 1–2, 2–4, 4–8, 8–18, 18–24, 24–42 and 42–48 hours, with urine and faeces collected at 0–24 and 24–48 hours. The gastrointestinal tract and carcass were collected following sacrifice at 48 hours and analysed for radioactivity. Radioactivity was analysed in blood, tissues and bile by LSC. In the bile duct–cannulated rats, metabolites were analysed in bile and urine by TLC and HPLC.

No signs of toxicity were reported.

Kinetic parameters are summarized in Table 2. Radioactivity was rapidly absorbed, with maximum plasma concentrations (C_{max}) reached in 15 minutes at both doses and in both sexes. Equally, radioactivity was rapidly eliminated from plasma, with a mean half-life of 0.4–0.8 hour. The area under the plasma concentration–time curve (AUC) increased in an approximately proportional manner from the low to the high dose, suggesting linear kinetics.

The results of the tissue distribution experiment are summarized in Table 3. Confirming the preceding study by Capps (1990), low levels of radioactivity were detected in tissues. Maximum tissue concentrations were measured at 15 minutes after dosing, consistent with the plasma T_{max}. Radioactivity was rapidly eliminated from tissues; mean first-phase tissue half-lives ranged from 0.2 to 0.5 hour at the low dose and from 0.5 to 0.9 hour at the high dose, whereas the slower second-phase elimination ranged from 1.6 to 3.2 hours at the low dose and from 3.2 to 11.7 hours at the high dose.

Table 2. Kinetic parameters in rats following a single oral dose of [14C]trinexapac-ethyl

Parameter	1 mg/kg bw		200 mg/kg bw	
	Males	Females	Males	Females
C_{max} (ppm)	1.3	0.5	73	85
T_{max} (min)	15	15	15	15
$t_{½}$ (h)	0.4	0.6	0.8	0.8
$AUC_{(0–48)}$ (µg eq·h/g)	1.0	0.9	170	165

AUC: area under the plasma concentration–time curve; C_{max}: maximum plasma concentration; eq: equivalent; $t_{½}$: half-life; T_{max}: time to reach C_{max}
Source: Bissig (1995)

Table 3. Tissue distribution of radioactivity in male rats following a single oral dose of [14C]trinexapac-ethyl[a]

Tissue	1 mg/kg bw			200 mg/kg bw		
	Tissue concentration at 6 h (mg eq/kg bw)	$t_{½}$ (h)		Tissue concentration at 6 h (mg eq/kg bw)	$t_{½}$ (h)	
		Rapid phase	Slow phase		Rapid phase	Slow phase
Blood	0.026	0.2	1.7	7.87	0.7	4.1
Bone	0.019	0.3	3.2	3.02	0.8	11.7
Brain	0.002	0.2	1.9	0.51	0.8	4.3
Fat	0.008	0.2	1.6	1.95	0.7	4.1
Heart	0.012	0.2	1.7	2.95	0.7	3.7
Kidneys	0.265	0.2	1.9	43.37	0.6	6.3
Liver	0.144	0.2	2.3	19.5	0.5	9.3
Lungs	0.024	0.2	1.7	7.86	0.8	3.2
Muscle	0.005	0.2	1.6	1.23	0.7	6.3
Plasma	0.046	0.2	1.7	14.66	0.8	4.2
Spleen	0.006	0.3	1.7	3.53	0.9	–
Testes	0.011	0.5	2.0	1.92	0.9	5.0
Carcass	0.025	0.4	2.4	3.76	1.1	3.9

eq: equivalent; $t_{½}$: half-life
[a] Results expressed as means.
Source: Bissig (1995)

In the four bile duct–cannulated rats, total recovery of radioactivity ranged from 95% to 99% of the administered dose. The mean levels of radioactivity in urine, bile, cage wash and faeces were 79%, 3.3%, 1.1% and 0.7%, respectively, with total (0–48 hours) excretion of 84% of the administered dose. The levels of radioactivity in the gastrointestinal tract and carcass were 11% and 2.1% of the administered dose, respectively. Based on the levels of radioactivity in urine, bile, cage wash, gastrointestinal tract and carcass, gastrointestinal absorption of radioactivity is estimated to be 97%. Trinexapac acid was the main metabolite in urine (69% of the administered dose or 92% of total urinary radioactivity) and was also detected in bile (0.2% of the administered dose or 6% of total biliary radioactivity). Also detected in urine and bile was an undefined conjugate of trinexapac acid (6.3% and 2.9% of the administered dose, respectively, which accounted for 8% and 94% of total radioactivity, respectively) (Bissig, 1995).

The proposed metabolic pathway of trinexapac-ethyl in rats is shown in Fig. 2.

Fig. 2. Proposed metabolic pathway of trinexapac-ethyl in rats

Trinexapac-ethyl
4-(Cyclopropyl-α-hydroxy-methylene)-3,5-dioxo-cyclohexanecarboxylic acid ethyl ester

CH_3CH_2OH

Trinexapac acid
4-(Cyclopropyl-α-hydroxy-methylene)-3,5-dioxo-cyclohexanecarboxylic acid

2. Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

The results of acute toxicity tests with trinexapac-ethyl administered to mice and rats are summarized in Table 4. Clinical signs observed in mice following acute oral dosing included piloerection, hunched posture and dyspnoea, which persisted to 5 days after dosing (Hartmann, 1993). Similar clinical signs were observed in rats following acute oral dosing, in addition to exophthalmia and ruffled fur for up to 10 days after dosing (Hartmann, 1987a); decreased activity, ataxia, diarrhoea, dilated pupils, epistaxis, haematuria, lacrimation, nasal discharge, polyuria, ptosis and salivation were also observed (Kuhn, 1988). Discoloration of the lungs and gastrointestinal tract were noted at necropsy (Kuhn, 1988).

Table 4. Results of studies of acute toxicity of trinexapac-ethyl

Species	Strain	Sex	Route	Purity (%)	Vehicle	LD_{50} (mg/kg bw) or LC_{50} (mg/L)	Reference
Mouse	Tif:MAG f	Male + female	Oral	94.5	0.5% w/v CMC in 0.1% w/v aqueous polysorbate 80	> 2 000	Hartmann (1993)
Rat	Tif:RAlf	Male + female	Oral	96.6	0.1% w/v arachidis oil	2 000–5 000	Hartmann (1987a)
Rat	HSD:(SD) BR	Male + female	Oral	96.9	None	4 613	Kuhn (1988)
Rat	Tif:RAlf	Male + female	Dermal	96.6	None	> 4 000	Hartmann (1987b)
Rat	Tif:RAlf	Male + female	Inhalation MMAD = 2.1 µm	96.6	30% w/v ethanol	> 5.3	Hartmann (1988)

CMC: carboxymethyl cellulose; MMAD: mass median aerodynamic diameter; SD: Sprague-Dawley; w/v: weight per volume

(b) Dermal and ocular irritation

The results of skin and eye irritation tests on trinexapac-ethyl conducted in rabbits are summarized in Table 5. Trinexapac-ethyl was not a skin or eye irritant in rabbits.

Table 5. Results of studies of dermal and ocular irritation of trinexapac-ethyl in rabbits

Strain	Sex	Purity (%)	Application site	Exposure period	Result	Reference
NZW	Male	96.6	20 cm^2, non-abraded skin	4 h, occluded	Non–skin irritant	Schneider (1987a)
NZW	Male	96.6	Left eye, right eye control	Eye unwashed for up to 72 h	Non–eye irritant	Schneider (1987b)

NZW: New Zealand White

(c) Dermal sensitization

Trinexapac-ethyl (purity 96.8%) was analysed for its skin sensitization potential in the guinea-pig maximization test. Twenty male guinea-pigs (Dunkin Hartley strain) were included in the test group, and 10 in the control group. In the induction phase (day 1), animals were injected intradermally on either side of the dorsal midline with 10% weight per volume (w/v) trinexapac-ethyl in arachis oil and Freund's complete adjuvant (1 : 1). Animals were pretreated with sodium lauryl sulfate in petrolatum (day 7) prior to topical induction (day 8) with undiluted trinexapac-ethyl in arachis oil for 48 hours under an occlusive dressing. Epidermal challenge (day 22) involved topical application of undiluted trinexapac-ethyl for 24 hours under an occlusive dressing. At 24 hours after topical challenge, four control and four test animals had slight erythema, with one test animal having well defined erythrema; no reactions were scored at 48 hours after challenge. On the basis of these findings, trinexapac-ethyl was classifiable as a non–skin sensitizer (Ruddock, 2001).

2.2 Short-term studies of toxicity

(a) Oral administration

Rats

Trinexapac-ethyl (purity 95%) in aqueous 1% w/v methyl cellulose was administered by gavage to groups of 10 rats of each sex per group (Tif:Ralf (SPF) hybrids of RII/1 × RII/2) at a dose of 0, 10, 100 or 1000 mg/kg bw per day for 28 days. In the absence of signs of toxicity, the highest dose was increased to 2000 mg/kg bw per day on day 10. Mortality and clinical signs were recorded daily. Body weight, feed consumption and water consumption were recorded weekly. Ophthalmoscopy was performed on all control and high-dose rats pretreatment and on day 25. Blood was sampled at the end of treatment for the analysis of haematology and clinical chemistry parameters. Following termination, rats were necropsied, and organs were weighed and examined histopathologically.

There were no deaths, treatment-related clinical signs or effects on body weight gain or feed consumption. The mean water consumption of high-dose males and females was up to about 1.5-fold higher than in the controls (week 3), with cumulative water consumption to the end of the treatment period approximately 1.2-fold higher than in the controls; no statistical analysis was performed on the water consumption data. Ophthalmoscopy was unremarkable. Prothrombin time was significantly higher ($P < 0.05$) than in the controls in high-dose rats (15.3 versus 14.6 seconds in males and 15.8 versus 15.2 seconds in females); there were no treatment-related effects on any other haematology parameters.

Selected clinical chemistry, organ weight and pathology findings are summarized in Table 6. At the highest dose, plasma potassium (both sexes) and phosphate (males) were significantly higher ($P < 0.05$) than in the controls. The significantly lower ($P < 0.05$) urea levels in low- and mid-dose males were not considered treatment related, as no effect occurred at the highest dose. In contrast,

plasma urea was 2-fold higher than in the controls ($P < 0.05$) in high-dose females. There was no analysis of serum creatinine. Alanine aminotransferase (ALAT) was increased by about 40% ($P < 0.05$) in high-dose females and by about 20% in high-dose males, with the latter not significantly different from the control.

Table 6. Findings in rats following 28 days of gavage administration of trinexapac-ethyl

Parameter	0 mg/kg bw per day		10 mg/kg bw per day		100 mg/kg bw per day		1 000/2 000 mg/kg bw per day	
	Males	Females	Males	Females	Males	Females	Males	Females
Clinical chemistry[a]								
Potassium (mmol/L)	3.60	3.33	3.70	3.48	3.66	3.50	4.15*	3.74*
Phosphate (mmol/L)	2.23	2.03	2.19	1.85	2.16	1.99	2.63*	2.11
Urea (mmol/L)	6.7	5.8	5.7*	5.9	5.3*	6.2	6.3	11.6*
ALAT (U/L)	35.8	28.3	35.1	33.9	37.4	31.6	43.0	39.0*
Organ weights[a]								
Heart								
- Absolute (g)	1.097	0.840	1.126	0.893	1.055	0.857	1.064*	0.990*
- Relative (%)	0.356	0.353	0.359	0.362	0.342	0.353	0.335*	0.396*
Kidneys								
- Absolute (g)	2.334	2.041	2.363	1.954	2.406	2.093	2.693*	2.299*
- Relative (%)	0.757	0.860	0.751	0.792	0.779	0.867	0.849*	0.921
Liver								
- Absolute (g)	11.118	8.766	11.465	11.987*	11.941	11.869*	13.191*	16.133*
- Relative (%)	3.593	3.696	3.643	4.842*	3.859	4.890*	4.150*	6.469*
Histopathology (*n* = 10)								
Heart: Inflammatory cell infiltration								
- Number	6	7	5	4	9	7	10	10
- Weighted grade	0.7	0.7	0.5	0.4	0.9	0.7	1.9	2.1
Liver: Hypertrophy of hepatocytes								
- Number	0	0	0	0	0	0	7	0
- Weighted grade	–	–	–	–	–	–	0.7	–
Liver: Glycogen deposition								
- Number	0	0	0	10	0	10	0	10
- Weighted grade	–	–	–	1.9	–	2.1	–	2.8
Kidneys: PAS-positive droplets								
- Number	0	0	0	0	0	0	9	0
- Weighted grade	–	–	–	–	–	–	1.1	–

ALAT: alanine aminotransferase; PAS: Periodic-Acid Schiff; U, units; *: $P < 0.05$

[a] Results expressed as the mean. Relative organ weights are relative to brain weight.

Source: Basler (1988)

At the highest dose, absolute and relative heart weights were significantly lower ($P < 0.05$) than in the controls in males, but significantly higher ($P < 0.05$) than in the controls in females. This lack of consistency suggests that these differences were incidental findings. At the highest dose, there was an increase in the incidence and grade of inflammatory cell infiltration of the myocardium. The increase in grade was due to the occurrence of moderate to marked multiple foci of inflammatory cells, infiltrating necrotic or degenerating myocardial fibres and interstitial or perivascular spaces of the myocardium in seven males and nine females. Similar inflammatory changes in the heart were not observed in any other studies at higher doses and over longer durations of exposure. On this basis, these effects were not considered treatment related.

Absolute and relative kidney weights were approximately 10% higher than the control values in high-dose males and females, with the differences in males being statistically significant ($P < 0.05$); only absolute kidney weight was significantly different ($P < 0.05$) from the control value in females. Histopathology revealed Periodic-Acid Schiff stain (PAS)–positive droplets in the kidney collecting duct epithelia in nine high-dose males, generally graded as slight. No histopathological abnormalities were observed in the kidneys of high-dose females.

At necropsy, all high-dose females were observed to have enlarged livers. Across all treated groups of females, mean absolute and relative liver weights were significantly higher ($P < 0.05$) than the control values (up to ~80% higher than the control values at the highest dose), but as the increases were coincident with glycogen deposition (the grade of which increased with dose), they were considered an adaptive effect rather than being toxicologically significant. Significantly elevated ($P < 0.05$) absolute and relative liver weights were noted in high-dose males (~19% and ~16% higher than the control values, respectively), with slight centrilobular hepatocellular hypertrophy observed microscopically in seven rats.

The no-observed-adverse-effect level (NOAEL) was 100 mg/kg bw per day for effects on the liver and kidneys at 1000 mg/kg bw per day (Basler, 1988).

Trinexapac-ethyl (96.6% purity) was admixed in the diet at a concentration of 0, 50, 500, 5000 or 20 000 ppm and fed ad libitum to SD (Crl:VAF/Plus™CD®(SD)BR) rats (15 of each sex per dose) for 13 weeks. The achieved doses were 0, 3, 34, 346 and 1350 mg/kg bw per day for males and 0, 4, 38, 395 and 1551 mg/kg bw per day for females at 0, 50, 500, 5000 and 20 000 ppm, respectively. Mortality and clinical signs were recorded daily. Body weight and feed consumption were recorded weekly. Water consumption and urine volume were recorded pretreatment and during weeks 12–13. Ophthalmoscopy was performed pretreatment and during week 13. Baseline haematology, clinical chemistry and urine analysis parameters were analysed in satellite groups of 10 rats of each sex per dose prior to the commencement of dosing; these rats were subsequently discarded. Blood and urine were sampled at the end of the exposure period for the analysis of standard haematology, clinical chemistry or urine analysis parameters. Following termination, rats were necropsied, and organs were weighed and examined histopathologically.

There were no deaths or treatment-related clinical signs. At the highest dose, absolute body weight was significantly lower ($P < 0.001$ or 0.01) than the control values in both sexes throughout the majority of the dosing period (up to ~8%), with mean cumulative body weight gain approximately 7% and 11% lower ($P < 0.05$) than the control values in males and females, respectively. Mean feed consumption was also significantly reduced ($P < 0.001$ or 0.01) in high-dose rats at various times (up to ~12% lower than the control values). There were no intergroup differences in water consumption measured during weeks 12–13. Ophthalmoscopy was unremarkable. Haematology and clinical chemistry parameters were unaffected by treatment. At the highest dose, urinary pH was significantly lower ($P < 0.01$) than the control values (6.7 versus 8.08, respectively, in males and 6.3 versus 7.8, respectively, in females). Urine specific gravity was significantly increased ($P < 0.01$) in high-dose males (1.047 versus 1.034).

There were no treatment-related macroscopic abnormalities. Selected organ weights and histopathological findings are presented in Table 7. At 5000 and 20 000 ppm, the mean relative liver

weight of males was significantly higher ($P < 0.05$) than the control values (~9% and ~12% higher, respectively), but this was not corroborated by any histopathological or clinical chemistry findings and on this basis is not considered toxicologically significant. The relative kidney weight of high-dose males and females was significantly higher ($P < 0.05$) than the control values (~10% and ~12% higher, respectively). At 5000 and 20 000 ppm in males, the incidence (but not grade) of tubular hyaline droplets was significantly higher ($P < 0.05$ or 0.01) than the control values, with the incidence of focal tubular basophilia ($P < 0.001$) and tubular casts also increased; no treatment-related histopathological kidney abnormalities were observed in females. The study authors considered that the histopathological kidney findings in males represented an early spontaneous senile nephropathy, whereas the sponsor attributed them to either renal overload by a metabolite (presumably trinexapac acid) or the binding of a metabolite to α_{2u}-globulin. However, the latter is not supported by observations in the long-term rat study, where there was no evidence of necrosis, hyperplasia, linear mineralization or renal tubule tumour formation in males. Although there might be some uncertainty about the relevance of these kidney effects in male rats to humans, the clear dose–response relationship indicates that at least in male rats, the kidney effects should be considered an adverse effect of treatment and therefore a reasonable basis for the study NOAEL.

Table 7. Observations in rats following 13 weeks of dietary exposure to trinexapac-ethyl

Parameter	0 ppm		50 ppm		500 ppm		5 000 ppm		20 000 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females
Organ weights[a]										
Liver										
- Absolute (g)	13.67	8.05	14.20	8.29	14.28	7.84	14.76	8.09	14.31	8.01
- Relative (%)	2.58	2.76	2.69	2.73	2.72	2.73	2.80*	2.80	2.90**	2.94
Kidneys										
- Absolute (g)	3.82	2.14	3.81	2.22	3.74	2.10	3.93	2.18	3.94	2.26
- Relative (%)	0.73	0.74	0.73	0.73	0.71	0.73	0.75	0.76	0.80*	0.83**
Histopathology: kidney[b] **(*n* = 15)**										
Focal tubular basophilia	3	0	2	0	1	0	7	0	13***	0
Tubular hyaline droplets	5	0	7	0	7	0	11*	0	13**	0
Tubular casts	2	0	2	0	0	0	2	0	6	0

*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$
[a] Results expressed as the mean.
[b] Results expressed as the absolute number of rats with the finding.
Source: Chau, McCormick & Arthur (1989a)

The NOAEL was 500 ppm (equal to 34 mg/kg bw per day), based on histopathological findings in the kidneys (focal tubular basophilia, tubular hyaline droplets and tubular casts) in males at 5000 ppm (equal to 346 mg/kg bw per day) (Chau, McCormick & Arthur, 1989a).

Dogs

In a non-guideline study, trinexapac-ethyl (purity 96.6%) was admixed in the diet at a concentration of 0, 500, 5000, 15 000 (days 1–3), 30 000 (days 4–28) or 50 000 ppm (day 29 onwards) and fed to groups of three Beagle dogs of each sex per group for up to 49 days. The achieved doses were 0, 23, 217, 683, 734 and 965 mg/kg bw per day for both sexes at 0, 500, 5000, 15 000, 30 000 and 50 000 ppm, respectively.

There were no deaths or treatment-related clinical signs. A loss of body weight occurred from days 29 to 49 in dogs consuming the 50 000 ppm diet (1–1.3 kg in males and 1–1.4 kg in females).

Significantly reduced ($P < 0.01$) feed consumption occurred in high-dose males (~50% lower than the control values from days 1 to 29 and ~70% from days 29 to 49), with reductions in females also occurring at 50 000 ppm (~70% lower than the control values from days 29 to 49). There were no ophthalmological effects. Haematology was unremarkable. Serum cholesterol was significantly increased ($P < 0.01$ or 0.05) in both sexes at 50 000 ppm (~1.5-fold increase on day 23 in males and day 45 in both sexes). Urine analysis was unremarkable. The mean thymus weight of high-dose females (3.4 g) was significantly lower ($P < 0.01$) than the control value (14.5 g). It was stated that relative kidney weight was increased in high-dose males. At 50 000 ppm, histopathological kidney findings included albuminous casts (three males), tubular dilatation and tubular degeneration (all dogs). Minimal or diffuse thymic atrophy was observed histopathologically at 50 000 ppm (all dogs). No microscopic abnormalities of the brain were observed.

The NOAEL was 15 000 ppm (equal to 683 mg/kg bw per day), based on a range of effects that occurred at 30 000 ppm (equal to 734 mg/kg bw per day), including body weight loss, lower feed consumption, increased serum cholesterol, increased kidney weight and histopathological findings in the kidney (Spoede, Batastini & Arthur, 1991).

Trinexapac-ethyl (purity 96.9%) was admixed in the diet at a concentration of 0, 50, 1000, 15 000 or 30 000 ppm and 400 g offered on a daily basis to Beagle dogs (four of each sex per dose) for 13 weeks. High-dose dogs were offered diet containing 15 000 ppm for 3 days and thereafter received diet containing 30 000 ppm for 13 weeks. The achieved doses were 0, 2, 35, 516 and 927 mg/kg bw per day for males and 0, 2, 40, 582 and 891 mg/kg bw per day for females at 0, 50, 1000, 15 000 and 30 000 ppm, respectively. Mortality and clinical signs were recorded daily. Body weight and feed consumption were recorded weekly. Physical or auditory examinations and ophthalmoscopy were performed pretreatment and during week 13. Blood and urine were collected pretreatment and during week 13 for the analysis of standard haematology, clinical chemistry or urine analysis parameters. Following termination, dogs were necropsied, and organs were weighed and examined histopathologically.

There were no deaths. Treatment-related clinical signs occurred in two high-dose males and included emaciation (weeks 5–14 or 13–14) and decreased defecation (week 4). At the highest dose, mean feed consumption was significantly lower ($P < 0.01$ or 0.001) than the control values throughout the study (up to 57% lower than the control value in males and 65% lower in females). Consequently, high-dose males and females lost 5% and 1%, respectively, of their starting body weight during the treatment period.

Ophthalmoscopy was unremarkable. There were no treatment-related effects on haematology or urine analysis parameters. Blood glucose was significantly lower ($P < 0.05$) than the control value in high-dose males on day 86 (76.25 versus 91 mg/dL), coincident with the reduction in feed consumption; there were no other treatment-related effects on clinical chemistry parameters. Blood urea nitrogen was approximately 67% higher ($P < 0.01$) than the control value in high-dose males.

There were no treatment-related macroscopic abnormalities. Selected organ weight findings are presented in Table 8. At the highest dose, relative brain (both sexes), relative adrenal (males) and relative liver (males) weights were significantly higher ($P < 0.01$ or 0.05) than the control values; these differences are attributable to the lower mean terminal body weight. In high-dose males, the relative weight of the medial retropharyngeal lymph node was significantly higher ($P < 0.05$) than the control value, whereas the absolute and relative weights of the popliteal lymph node were significantly lower ($P < 0.01$ or 0.05) than the control values at most doses; in the absence of a dose–response relationship, these differences are considered incidental findings. Although not statistically significant, the absolute and relative thymus weights of high-dose males were ~78% and ~60% lower than in the control group. Histopathological examination revealed diffuse thymic atrophy in all high-dose dogs (graded as minimal [one male, three females], moderate [two males, one female] and severe [one male]), compared with none in the control group ($P < 0.01$). The thymic atrophy is most likely a secondary effect of the reduced body weight gain and was not observed in the 52-week dog study.

Table 8. Organ weights in dogs following 13 weeks of dietary exposure to trinexapac-ethyl

Parameter	0 ppm		50 ppm		1 000 ppm		15 000 ppm		30 000 ppm	
	M	F	M	F	M	F	M	F	M	F
Organ weights[a]										
Terminal body weight (g)	8 809	7 491	9 832	8 605	9 402	8 148	10 094	7 010	6 561*	6 668
Brain weight										
- Absolute (g)	77.17	71.16	77.33	77.53	82.58	77.32	80.22	76.27	80.16	75.38
- Relative (%)	0.88	0.95	0.79	0.91	0.89	0.96	0.80	1.09	1.24**	1.14*
Adrenal weight										
- Absolute (g)	1.005	1.140	1.140	1.055	1.077	1.062	1.265	1.030	1.170	1.047
- Relative (%)	0.011	0.015	0.012	0.012	0.012	0.013	0.013	0.015	0.018**	0.016
Liver weight										
- Absolute (g)	300.2	242.1	307.3	238.2	313.1	233	325.3	246.3	266.1	235.8
- Relative (%)	3.43	3.26	3.13	2.78	3.33	2.89	3.24	3.51	4.06**	3.56
Lymph node weight, medial retropharyngeal										
- Absolute (g)	2.500	3.137	3.295	2.900	4.145	3.142	3.845	3.297	3.320	2.615
- Relative (%)	0.028	0.042	0.034	0.034	0.044	0.039	0.038	0.047	0.049*	0.040
Lymph node weight, popliteal										
- Absolute (g)	2.322	1.527	1.607	0.995	1.225**	1.440	1.605	0.870	1.047**	0.787
- Relative (%)	0.027	0.021	0.017*	0.011	0.013**	0.018	0.016*	0.012	0.016**	0.012
Thymus weight										
- Absolute (g)	8.28	6.34	12.93	11.39*	8.05	8.69	7.09	7.58	2.71	5.71
- Relative (%)	0.093	0.086	0.130	0.132	0.086	0.107	0.070	0.106	0.038	0.084

F: females: M: males; *: $P < 0.05$; **: $P < 0.01$
[a] Results expressed as the mean. Relative weights are relative to body weight.
Source: Chau, McCormick & Arthur (1989b)

In the subsequent study by Chau, Kirchner & Arthur (1992), histopathological examination revealed vacuoles in the brain of dogs consuming a diet containing 20 000 ppm trinexapac-ethyl. A re-examination of brain slides prepared in relation to the current study (Chau, McCormick & Arthur, 1989b) by Krinke (1994) indicated that one high-dose male dog had similar vacuoles in the brain.

The NOAEL was 15 000 ppm (equal to 516 mg/kg bw per day), based on clinical signs, reduced body weight gain and reduced feed consumption at 30 000 ppm (equal to 927 mg/kg bw per day) (Chau, McCormick & Arthur, 1989b).

Trinexapac-ethyl (purity > 96.2%) was admixed in the diet at a concentration of 0, 40, 1000, 10 000 or 20 000 ppm, and 400 g was offered on a daily basis to Beagle dogs (four of each sex per dose) for 52 weeks. A maximum dietary concentration of 20 000 ppm was chosen as the highest dose because observations in the 7- and 13-week studies suggested reduced palatability at 30 000 and 50 000 ppm. The achieved doses were 0, 1.6, 32, 366 and 727 mg/kg bw per day for males and 0, 1.4, 40, 357 and 793 mg/kg bw per day for females at 0, 40, 1000, 10 000 and 20 000 ppm, respectively. Observations for mortality and clinical signs were made daily. Body weight and feed consumption were recorded pretreatment, weekly for 13 weeks and monthly thereafter. Physical or auditory

examinations were made pretreatment and during weeks 13, 26, 39 and 52. Ophthalmoscopy was performed pretreatment and during weeks 26 and 52. Blood and urine were sampled pretreatment and during weeks 13, 26 and 53 for the analysis of standard haematology, clinical chemistry or urine analysis parameters. At the end of the exposure period, dogs were killed and necropsied, and organs were weighed and examined histopathologically.

There were no deaths. Emesis was observed sporadically at 20 000 ppm (three males and four females), and mucoid or bloody faeces were seen at 10 000 ppm (three males and four females) and 20 000 ppm (three males and two females). One male each had mucoid or bloody faeces at 0, 40 and 1000 ppm.

There were no intergroup differences in the pattern of body weight gain, and pretreatment differences in absolute body weight persisted throughout the study. Feed consumption was consistent across all groups, and there were no treatment-related ophthalmic abnormalities.

Selected haematology, clinical chemistry, organ weight and pathology findings are summarized in Table 9. At the highest dose, mean red blood cells (both sexes), haematocrit (both sexes) and haemoglobin (females) were significantly lower ($P < 0.05$) than the control values at day 85. Significantly reduced red blood cell count was observed on day 357 at 10 000 ppm (females) and 20 000 ppm (both sexes). Plasma cholesterol was higher than the control value in high-dose males, but not significantly, whereas significantly elevated ($P < 0.01$ or 0.05) cholesterol occurred on day 85 (10 000 and 20 000 ppm) and day 357 in females (20 000 ppm). There were no treatment-related urine analysis findings.

There were no treatment-related macroscopic abnormalities. Mean absolute testes weight was significantly ($P < 0.05$) lower than the control values at and above 1000 ppm, but the decrease was not accompanied by any histopathological abnormalities. Similarly, mean absolute and relative uterus weights were significantly lower ($P < 0.01$ or 0.05) than the control values at and above 1000 ppm. These statistically significant differences in testes and uterine weights were considered incidental findings due to higher than normal control values. There were no intergroup differences in absolute brain weight.

Histopathological examination revealed minimal focal vacuolation of the dorsal medial hippocampus and/or lateral midbrain at 10 000 and 20 000 ppm, with the incidence at 20 000 ppm being significantly higher ($P < 0.05$) than the control value. Vacuoles were confined to a bilateral swelling of oligodendroglial and astrocytic cells and were stated to be larger in size and more closely clumped than vacuoles typically observed as artefacts in control brain sections. The vacuolation was associated with the presence of astrocytes. Cell nuclei but no axons were observed within vacuoles. There was no evidence of myelinopathy or astrocytosis/astrogliosis.

The NOAEL was 1000 ppm (equal to 32 mg/kg bw per day) for cerebral vacuolation at 10 000 ppm (equal to 357 mg/kg bw per day) in the absence of neurodegenerative or inflammatory histopathological changes or neurological signs (Chau, Kirchner & Arthur, 1992).

(b) Dermal application

Rabbits

In a 21-day dermal toxicity study, trinexapac-ethyl (purity 96.6%) in dehydrated alcohol was administered dermally, under semi-occluded conditions, to the backs of five New Zealand White rabbits at a dose of 0, 10, 100 or 1000 mg/kg bw per day. There were no deaths, clinical signs or treatment-related dermal changes. The NOAEL was 1000 mg/kg bw per day, the highest dose tested (Huber, 1989).

2.3 Long-term studies of toxicity and carcinogenicity

Mice

Trinexapac-ethyl (purity 96.2–96.9%) was admixed in the diet at a concentration of 0, 7, 70, 1000, 3500 or 7000 ppm and fed ad libitum to mice (Crl:CD-1(ICR)Br) (70 of each sex per dose) for

Table 9. Observations in dogs following 52 weeks of dietary exposure to trinexapac-ethyl

Parameter	0 ppm		40 ppm		1 000 ppm		10 000 ppm		20 000 ppm	
	M	F	M	F	M	F	M	F	M	F
Haematology[a]										
RBCs (×10^{6})										
- Day 85	6.46	7.2	6.12	6.69	6.66	6.82	6.06	6.05	5.40*	5.91*
- Day 357	7.63	7.97	7.36	7.55	7.62	7.24	7.07	6.56**	6.66*	6.82**
Haematocrit (%)										
- Day 85	42.75	49.50	41.25	47.75	44.25	47.00	41.50	43.50	38.25*	41.50*
Hb (g/dL)										
- Day 85	14.50	17.1	13.83	16.23	14.92	15.83	14.17	15.18	13.08	14.43*
Clinical chemistry[a]										
Cholesterol (mg/dL)										
- Day −18	190	165	171	200	191	170	161	160	203	186
- Day 85	178	144	137	172	159	158	157	178**	220	199*
- Day 176	194	158	153	192	168	164	169	237	254	244
- Day 357	195	185	149	210	170	186	180	202	266	258*
Body/organ weights[a]										
Terminal body weight (g)	10.5	8.52	9.25	9.51	10.38	8.72	9.27	8.84	9.2	7.51
Testes weight										
- Absolute (g)	17.41	–	14.65	–	13.84*	–	12.8*	–	13.07*	–
- Relative (%)	0.17	–	0.15	–	0.14	–	0.14	–	0.14	–
Uterus weight										
- Absolute (g)	–	11.49	–	7.04	–	3.55*	–	2.87**	–	3.26*
- Relative (%)	–	0.13	–	0.07	–	0.04**	–	0.03**	–	0.04*
Brain weight										
- Absolute (g)	83.25	74.31	82.68	78.95	79.68	80.91	81.31	79.58	78.49	73.3
Histopathology										
Focal vacuolation of the brain[b]	0/4	0/4	0/4	0/4	0/4	0/4	1/4	2/4	4/4*	4/4*

*: $P < 0.05$; **: $P < 0.01$

[a] Results expressed as the mean. Relative weights are relative to body weight.

[b] Number of dogs with the finding/total number of dogs per group.

Source: Chau, Kirchner & Arthur (1992)

78 weeks. The achieved doses were 0, 0.9, 9, 131, 451 and 912 mg/kg bw per day for males and 0, 1.1, 11, 154, 539 and 1073 mg/kg bw per day for females at 0, 7, 70, 1000, 3500 and 7000 ppm, respectively. Deaths were recorded twice daily, and clinical signs once daily. Ophthalmoscopy was performed prior to the commencement of dosing and at weeks 77–78. Physical and auditory examinations were performed prior to the commencement of dosing and at weeks 13, 26, 39, 52, 65 and 78. Body weight and feed consumption were recorded weekly for 13 weeks and every 4 weeks

thereafter. Rats were palpated every 4 weeks for the first 9 months and every 2 weeks thereafter. Blood smears were prepared at weeks 52–53 and 79–81 for a differential cell count and an examination of red cell morphology. At the end of the exposure period, surviving rats were killed and necropsied. Organs were weighed and examined histopathologically.

Survival was unaffected by treatment; overall survival was 54%, 61%, 50%, 56%, 71% and 60% for males and 51%, 64%, 70%, 61%, 63% and 57% for females at 0, 7, 70, 1000, 3500 and 7000 ppm, respectively. There were no treatment-related clinical signs or ophthalmic abnormalities. Mean absolute body weight and body weight gain were significantly lower ($P < 0.01$) than in the controls in high-dose females on day 7 (body weight: 22.79 g versus 23.94 g, respectively; body weight gain: 3.62 g versus 7.44 g, respectively), but there was no concomitant reduction in feed consumption. The transient nature of these effects indicates that they are an unsuitable basis on which to establish a chronic NOAEL. There was no treatment-related effect on body weight gain or feed consumption in males. The incidence of palpable masses, haematological findings, organ weights, macroscopic findings, and neoplastic and non-neoplastic lesions were unaffected by treatment.

The NOAEL for chronic toxicity and carcinogenicity was 7000 ppm (equal to 912 mg/kg bw per day), the highest dietary concentration tested (Rudzki, Batastini & Arthur, 1991).

Rats

Trinexapac-ethyl (purity > 92%) was admixed in the diet at a concentration of 0, 10, 100, 3000, 10 000 or 20 000 ppm and fed ad libitum to SD (Crl:VAF/Plus CD(SD)Br) rats (80 or 90 of each sex per dose) for up to 104 weeks. The achieved doses were 0, 0.4, 4, 116, 393 and 806 mg/kg bw per day for males and 0, 0.5, 5, 147, 494 and 1054 mg/kg bw per day for females at 0, 10, 100, 3000, 10 000 and 20 000 ppm, respectively. After 1 year of treatment, 10 rats of each sex per dose were killed, with an additional 10 rats of each sex from the control and high-dose groups subjected to a 4-week recovery period prior to sacrifice. All remaining rats remained on treatment for a 2nd year. Mortalities and clinical signs were recorded daily. Ophthalmoscopy was performed on all rats prior to the commencement of dosing and during week 51, with 10 rats of each sex from the control and high-dose groups examined during weeks 102–103. Physical and auditory examinations were performed pretreatment and during weeks 13, 26, 39, 51, 56/57 (recovery groups), 65, 77/78, 91 and 103. Body weight and feed consumption were recorded weekly to week 13 and every 4 weeks thereafter. Rats were palpated every 4 weeks for the first 9 months and every 2 weeks thereafter. Blood smears were prepared from animals killed in a moribund condition and all surviving rats prior to terminal sacrifice. Blood and urine were collected during weeks 26 or 27, 51 or 52, 56 (recovery groups), 78 and 104 for the analysis of haematology, clinical chemistry or urine analysis parameters. Urine volume and water intake were recorded for 10 rats of each sex per dose during weeks 25, 50 or 51, 55 (recovery groups), 79 and 101. At the end of the exposure period, surviving rats were killed and necropsied. Organs were weighed and examined histopathologically. Survival was unaffected by treatment; overall survival was 40%, 27%, 29%, 27%, 37% and 53% for males and 26%, 43%, 34%, 39%, 43% and 32% for females at 0, 10, 100, 3000, 10 000 and 20 000 ppm, respectively. There were no treatment-related clinical signs. At the highest dose, mean absolute body weight, body weight gain and feed consumption were significantly lower ($P < 0.01$ or 0.05) than the control values, most consistently during the first 6–12 months of treatment. The magnitude of the reduced body weight gain was up to about 7% lower than the control value in males and about 23% lower in females. Whereas male body weight gain remained depressed for the duration of the study, females regained weight to be comparable to the controls by the end of the study.

There were no treatment-related effects on water consumption, the occurrence of physical/auditory abnormalities, ophthalmic abnormalities or haematology parameters. In males on day 180, serum creatinine was significantly higher ($P < 0.05$) than the control value at and above 100 ppm (0.32, 0.44, 0.53, 0.63, 0.52 and 0.53 mg/dL at 0, 10, 100, 300, 10 000 and 20 000 ppm, respectively); in the absence of a dose–response relationship, a similar result in females or a similar result at other times, this was not considered treatment related. Significantly lower ($P < 0.01$) mean urinary pH occurred in both sexes at 10 000 and 20 000 ppm (Table 10), a finding that was reversible following the 4-week recovery period. There were no other treatment-related urinary findings.

Table 10. Mean urinary pH values of rats over 2 years of dietary exposure to trinexapac-ethyl

Day	Mean urinary pH value											
	0 ppm		10 ppm		100 ppm		3 000 ppm		10 000 ppm		20 000 ppm	
	M	F	M	F	M	F	M	F	M	F	M	F
183	8.30	7.80	7.95	7.95	8.25	7.88	8.25	7.55	7.45**	6.70**	6.45**	6.30**
355	7.87	7.65	8.00	7.65	7.90	7.45	7.90	6.80*	7.30	6.45**	6.27**	6.27**
391	7.75	7.15	–	–	–	–	–	–	–	–	7.50	7.27
544	7.60	7.95	7.60	7.15	7.65	7.40	7.05	7.20	6.77**	6.85**	6.50**	6.20**
726	6.94	7.50	7.10	6.70	7.05	6.90	7.10	7.00	6.05**	6.10**	5.90**	5.85**

F: females; M: males; *: $P < 0.05$; **: $P < 0.01$
Source: Giknis, Batastini & Arthur (1992)

There were a number of significant differences in organ weights at the highest dose that were considered to be incidental findings due to their inconsistent occurrence over time and between sexes. There were no treatment-related necropsy findings or palpable masses.

Selected neoplastic and non-neoplastic findings are summarized in Table 11. In high-dose males killed after 104 weeks, the incidence of squamous cell carcinoma of the non-glandular stomach was significantly higher ($P < 0.05$) than in the controls (2/80 versus 0/80). Although this finding was above the historical control incidence of 0%, the absence of a similar result in high-dose males killed at earlier times or in females, accompanying increases in preneoplastic lesions (e.g. hyperplasia or papillomas), similar lesions in the forestomach of mice in the preceding study and evidence of genotoxicity, the finding is considered unlikely to be treatment related. In high-dose females, the incidence of acanthosis in the non-glandular stomach was higher than the control value (13/70 versus 7/70, respectively), but was stated to be within the historical control range and therefore was considered an incidental finding.

In high-dose males, the incidence of thyroid follicular cell adenocarcinomas was significantly higher ($P < 0.05$) than the control value but was close to the historical control range of 0–5%; there was no increase in the incidence of adenomas or the combined incidence of adenomas and carcinomas. In high-dose females, the incidence of bladder papillomas was significantly higher ($P < 0.05$) than the control value (2/80 versus 0/89, respectively; historical control incidence of 0%), with no concomitant increase in the incidence of hyperplasia or carcinomas. In high-dose females, the incidence of galactocele in the mammary gland was increased relative to the control ($P < 0.01$). Also increased at the highest dose was the incidence of bile duct hyperplasia in males ($P < 0.05$). There was a significant increase ($P < 0.05$) in tension lipidosis at and above 3000 ppm in males and at the highest dose in females; in the absence of a dose–response relationship, this was not considered treatment related.

Following 52 weeks of treatment, the incidence and severity of hyaline droplets in the renal tubular epithelium of high-dose males were increased (7/10 versus 0/10 in the controls, $P < 0.05$), with this finding reversed following the 4-week recovery period. No such finding occurred in males killed after 104 weeks or in females killed at 52 or 104 weeks. Also following 52 weeks of treatment, brown pigment was observed in the tubular epithelium of the outer renal cortex of 1/10 females at 10 000 ppm and 2/10 males ($P < 0.05$) and 7/10 females ($P < 0.01$) at 20 000 ppm. Following the 4-week recovery period, this finding remained in 2/10 high-dose females.

The NOAEL for chronic toxicity was 3000 ppm (equal to 116 mg/kg bw per day) for histopathological lesions in the kidneys (hyaline droplets and pigment deposition) at 10 000 ppm (equal to 393 mg/kg bw per day). Trinexapac-ethyl was not carcinogenic under the conditions of this study; the NOAEL for carcinogenicity was therefore 20 000 ppm (equal to 806 mg/kg bw per day), the highest dietary concentration tested (Giknis, Batastini & Arthur, 1992).

Table 11. Histopathological findings in rats following 104 weeks of dietary exposure to trinexapac-ethyl

Parameter	Incidence of finding (no. of rats affected/total no. of rats)											
	0 ppm		10 ppm		100 ppm		3 000 ppm		10 000 ppm		20 000 ppm	
	M	F	M	F	M	F	M	F	M	F	M	F
Forestomach												
Squamous cell carcinoma	0/80	0/89	0/80	0/79	0/80	0/80	0/80	0/80	0/80	0/80	2/80*	0/80
Basal epithelial hyperplasia	1/70	1/70	2/70	1/69	0/70	1/70	0/70	2/70	1/70	3/70	3/70	1/70
Acanthosis	4/70	7/70	10/70	6/69	8/70	1/70	7/70	1/70	7/70	8/70	5/70	13/70*
Thyroid												
Adenomas	4/89	0/90	2/79	1/80	3/80	1/80	5/80	2/80	3/80	1/80	3/80	2/80
Carcinomas	1/89	0/90	0/79	0/80	0/80	0/80	1/80	0/80	1/80	2/80	4/80*	0/80
Total	5/89	0/90	2/79	1/80	3/80	1/80	6/80	2/80	4/80	3/80	7/80	2/80
Bladder												
Epithelial hyperplasia	1/90	2/89	4/80	2/80	0/80	0/80	4/80	1/80	0/80	1/80	2/80	0/80
Papillomas	0/90	0/89	0/80	0/80	0/80	0/80	1/80	0/80	0/80	1/80	0/80	2/80*
Transitional cell carcinomas	1/90	0/89	0/80	0/80	0/80	0/80	0/80	0/80	0/80	0/80	0/80	0/80
Total	2/90	2/89	4/80	2/80	0/80	0/80	5/80	1/80	0/80	2/80	0/80	2/80
Mammary gland												
Galactoceles	–	5/70	–	5/70	–	4/70	–	7/70	–	9/70	–	13/70**
Liver/bile duct												
Bile duct hyperplasia	16/70	11/70	11/70	9/70	13/70	17/70	13/70	8/70	18/70	13/70	35/70**	15/70
Tension lipidosis	0/70	3/70	0/70	0/70	1/70	3/70	4/70*	5/70	5/70*	5/70	2/70*	5/70*

F: females; M: males; *: $P < 0.05$; **: $P < 0.01$
Source: Giknis, Batastini & Arthur (1992)

2.4 Genotoxicity

The results of genotoxicity studies on trinexapac-ethyl are summarized in Table 12.

Table 12. Results of genotoxicity assays on trinexapac-ethyl

End-point	Test object	Concentration or dose	Purity (%)	Results	Reference
In vitro studies					
Gene mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537	Cytotoxicity test: 0.08–5 000 µg/plate (±S9) Mutagenicity test: 20–5 000 µg/plate (±S9) Acetone vehicle	Not specified	Negative	Deparade (1988)
Gene mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537 *Escherichia coli* WP2 uvrA	Cytotoxicity test (TA100 and *E. coli* WP2 uvrA only): 20.6–5 000 µg/plate (±S9) Mutagenicity test: 312.5–5 000 µg/plate (±S9) DMSO vehicle	96.8	Negative	Deparade (2001)

End-point	Test object	Concentration or dose	Purity (%)	Results	Reference
Gene mutation	Mouse lymphoma cells L5178Y/TK	Cytotoxicity test: 0.94–1 930 µg/mL (±S9) Mutagenicity test: 7.54–1 930 µg/mL (±S9) DMSO vehicle	94.5	Negative	Geleick (1993)
Gene mutation	CHO V79 cells	Cytotoxicity test: 46.9–3 000 µg/mL (+S9); 23.4–1 500 µg/mL (−S9) Mutagenicity test: 70–1 400 µg/mL (±S9) DMSO vehicle	96.6	Negative[a]	Dollenmeier (1988)
Cytogenetic test	CHO CCL 61 cells	312.5, 625 or 1 250 µg/mL (±S9) DMSO vehicle	96.8	Negative	Ogorek (2001)
Cytogenetic test	Human lymphocytes	Cytotoxicity test: 0.12–1 000 µg/mL (±S9) Cytogenetic test: 62.5–1 000 µg/mL (+S9) DMSO vehicle	96.6	Negative	Strasser (1989)
Unscheduled DNA synthesis	Rat primary hepatocytes	Cytotoxicity test: 5–5 250 µg/mL Assay 1: 0.8–400 µg/mL (−S9) Assay 2: 4–500 µg/mL (−S9) Cell culture medium vehicle	96.6	Negative	Hertner (1988)
Unscheduled DNA synthesis	Human fibroblasts	Cytotoxicity test: 5.13–5 250 µg/mL Assays 1 and 2: 37.04–4 000 µg/mL (−S9) Cell culture medium vehicle	96.6	Negative	Meyer (1988)
In vivo studies					
Micronucleus	Tif:MAGF mice (5/sex/dose), bone marrow	Study 1: 0 or 3 000 mg/kg bw; killed at 16, 24 or 48 h after dosing Study 2: 0, 750, 1 500 or 3 000 mg/kg bw; killed at 48 h after dosing Arachis oil vehicle	96.6	Negative[b]	Ceresa (1989)
Micronucleus	Tif:MAGF mice (5/sex/dose), bone marrow	0, 1 000, 2 000 or 4 000 mg/kg bw; killed at 16, 24 or 48 h after dosing Arachis oil vehicle	94.5	Negative[c]	Hertner (1992)

CHO: Chinese hamster ovary; DMSO: dimethyl sulfoxide; DNA: deoxyribonucleic acid; S9: 9000 × *g* supernatant fraction of rat liver homogenate

[a] Cytotoxicity at 750–1500 µg/mL.

[b] Toxicity at 3000 mg/kg bw.

[c] Toxicity at 4000 mg/kg bw.

2.5 *Reproductive and developmental toxicity*

(a) Multigeneration studies

Rats

Trinexapac-ethyl (purity 96.2%) was admixed in the diet at a concentration of 0, 10, 1000, 10 000 or 20 000 ppm and fed ad libitum to two parental generations of SD rats (30 of each sex per dose) and their offspring. Estimated doses received by rats throughout premating and gestation are presented in Table 13; mean parental doses across both generations were 0, 0.57, 58.6, 570.5 and 1166 mg/kg bw per day for males and 0, 0.73, 73, 721.5 and 1427 mg/kg bw per day for females at 0, 10, 1000, 10 000 and 20 000 ppm, respectively. The premating exposure period commenced when rats were approximately 7 weeks old and lasted for 13 (F_0) or 12 weeks (F_1). Rats were mated 1 : 1 for up to 3 weeks. Litters were reduced to four pups of each sex on day 4 postpartum. Observations for mortalities and clinical signs were made daily, with body weight and feed consumption recorded weekly. The number of live and dead pups, pup body weight and clinical signs were recorded on days 0, 4, 7, 14 and 21 postpartum. Standard reproduction and litter parameters were recorded or calculated. Necropsies were performed on all parental rats and culled pups. The following tissues were examined histopathologically in parental rats: ovaries, vagina, cervix, uterus, testes, epididymides, seminal vesicles, prostate, pituitary gland and coagulating gland.

Table 13. Doses of trinexapac-ethyl received by parental rats

Phase	Dose (mg/kg bw per day)							
	10 ppm		1 000 ppm		10 000 ppm		20 000 ppm	
	Males	Females	Males	Females	Males	Females	Males	Females
F_0 premating								
- Range	0.44–0.87	0.66–0.89	44.4–85.4	66.4–90.0	445–859	668–844	895–1 577	1 370–1 485
- Mean	0.59	0.75	62.6	74.8	595	737	1 169	1 414
Gestation	–	0.64	–	64.7	–	659	–	1 377
F_1 premating								
- Range	0.44–0.93	0.57–1.01	44.7–92.1	58.2–102	453–913	580–1 026	943–2 002	1 195–2 131
- Mean	0.55	0.71	54.6	71.2	546	706	1 162	1 440
Gestation	–	0.62	–	61.8	–	650.9	–	1 319

Source: Singh, Hazelette & Yau (1991)

There were no treatment-related deaths or clinical signs.

During the premating period, significantly reduced ($P < 0.01$ or 0.05) body weight and body weight gain occurred consistently at and above 10 000 ppm in both parental generations of rats (Table 14). In the F_0 generation, cumulative body weight gain to day 91 of the premating period was significantly lower ($P < 0.01$ or 0.05) than the control values at 10 000 and 20 000 ppm (up to ~10% and ~40% lower in males and females, respectively). This reduction in body weight gain was concomitant with reduced feed consumption. A similar pattern of reduced body weight gain and feed consumption was also evident in the F_1 generation; however, the significantly lower ($P < 0.01$) body weight gain in 1000 ppm F_1 males was not considered treatment related because there was no significant difference in females at the same dose. Further, in a 13-week dietary study in rats (Chau, McCormick & Arthur, 1989a) over a comparable dosing period, no effects on body weight, body

weight gain or feed consumption occurred at a higher dietary concentration of 5000 ppm (equal to 346 mg/kg bw per day in males).

During the gestation of F_0 dams, mean absolute body weight was significantly lower (P < 0.05) than the control value at and above 1000 ppm (7%, 6% and 17% lower than the control value at 1000, 10 000 and 20 000 ppm, respectively). However, there was no significant difference in body weight gain or feed consumption. In F_1 dams, there were no significant intergroup differences in body weight, body weight gain or feed consumption during gestation. During lactation, high-dose dams from both parental generations gained significantly more (P < 0.01) body weight than the controls, in which in fact a slight loss of body weight occurred (Table 14).

Table 14. Effect of trinexapac-ethyl on body weight gain and feed consumption in F_0 and F_1 parental rats[a]

Parameter	0 ppm		10 ppm		1 000 ppm		10 000 ppm		20 000 ppm	
	M	F	M	F	M	F	M	F	M	F
F_0 body weight gain (g)										
Premating: Days 0–91	331	141	320	133	307	124	299**	120*	287**	87**
Gestation: Days 0–20	–	128	–	129	–	119	–	121	–	119
Lactation: Days 0–21	–	−12	–	−12	–	1	–	−1	–	31**
F_0 feed consumption (% of control)										
Premating: Days 0–91	100	100	96	100	94	96	93	70	91	66
Gestation: Days 0–20	–	100	–	99	–	94	–	97	–	91
F_1 body weight gain (g)										
Premating: Days 0–84	368	147	358	137	329**	129	330**	139	305**	119*
Gestation: Days 0–20	–	132	–	126	–	124	–	118	–	118
Lactation: Days 0–21	–	−6	–	−13	–	−6	–	−2	–	21**
F_1 feed consumption (% of control)										
Premating: Days 0–84	100	100	100	99	94	97	94	97	86	89
Gestation: Days 0–20	–	100	–	97	–	95	–	98	–	91

F: females; M: males; *: P < 0.05; **: P < 0.01

[a] Results expressed as the mean.

Source: Singh, Hazelette & Yau (1991)

Reproductive performance and litter parameters were unaffected by treatment in both generations.

Treatment-related effects on pups were confined to the highest dose. Significantly lower ($P <$ 0.05) survival occurred during postpartum days 4–21 in pooled F_1 pups (92.4% versus 97.8% in the controls) and during postpartum days 0–4 in female F_2 pups (92.1% versus 97.6% in the controls). Mean body weight was significantly lower ($P < 0.01$) than the control values throughout gestation in both sexes, with cumulative body weight gain approximately 20% lower than in the controls in F_1 pups and about 25% lower than in the controls in F_2 pups.

There was no treatment-related effect on the incidence of gross or histopathological abnormalities in parental rats. Relative testes weight (F_1 parental males only) and relative ovary weight (F_0 and F_1 females) were significantly higher ($P < 0.01$) than the control values, which were attributable to the significantly lower ($P < 0.01$) terminal body weight of these groups, noting that absolute organ weights were unremarkable (Table 15).

***Table 15. Organ weights in F_0 and F_1 parental rats*[a]**

Parameter	0 ppm	10 ppm	1 000 ppm	10 000 ppm	20 000 ppm
F_0					
Testes weight (g)	3.60	3.63	3.47	3.63	3.51
Terminal body weight (g)	629.83	623.87	609.73	601.30	590.53*
Relative testes weight (%)	0.57	0.59	0.58	0.61	0.60
F_1					
Testes weight (g)	3.79	3.79	3.58	3.95	3.65
Terminal body weight (g)	666.57	667.30	311.53*	631.30	549.53**
Relative testes weight (%)	0.57	0.57	0.60	0.63	0.67**
F_0					
Ovary weight (g)	0.09	0.08	0.08	0.09	0.09
Terminal body weight (g)	349.72	346.77	341.60	334.70	295.35**
Relative ovary weight (%)	0.026	0.022	0.024	0.026	0.032**
F_1					
Ovary weight (g)	0.09	0.08	0.09	0.09	0.10
Terminal body weight (g)	370.23	347.40	332.47**	339.77*	293.90**
Relative ovary weight (%)	0.025	0.024	0.027	0.026	0.033**

*: $P < 0.05$; **: $P < 0.01$

[a] Results expressed as means. Relative organ weights are relative to body weight.

Source: Singh, Hazelette & Yau (1991)

The NOAEL for reproductive toxicity was 20 000 ppm (equal to 1166 mg/kg bw per day), the highest dietary concentration tested. The NOAEL for parental toxicity was 1000 ppm (equal to 58.6 mg/kg bw per day) for reduced body weight gain and feed consumption at 10 000 ppm (equal to 570.5 mg/kg bw per day). The NOAEL for offspring toxicity was 10 000 ppm (equal to 570.5 mg/kg bw per day) for reduced survival and body weight in the F_1 and F_2 generations at 20 000 ppm (equal to 1166 mg/kg bw per day) (Singh, Hazelette & Yau, 1991).

(b) Developmental toxicity

Rats

Trinexapac-ethyl (purity 96.6%) in peanut oil was administered by gavage to groups of 24 pregnant Tif:RAI f (SPF) rats at a dose of 0, 20, 200 or 1000 mg/kg bw per day from day 6 to day 15

of gestation. Dams were observed daily throughout gestation for clinical signs of toxicity, with body weight and feed consumption recorded throughout this period. On day 20 of gestation, surviving dams were killed and fetuses examined for external, visceral and skeletal abnormalities.

There were no treatment-related mortalities, clinical signs or effects on body weight or feed consumption. There were no treatment-related effects on the course of pregnancy, uterine findings, litter parameters or fetal weight. There were no intergroup differences in the overall incidence of fetal malformations and anomalies, the incidence of external or visceral malformations and anomalies or the incidence of skeletal malformations. There was a non-significant, dose-related increase in the incidence of asymmetrically shaped sternebrae (Table 16), with the incidence within one standard deviation of the mean historical control fetal or litter incidence. On this basis, the apparent increase in asymmetrically shaped sternebrae is considered to be an incidental finding. The incidence of delayed ossification of some cervical vertebral centres was significantly higher than the control incidence, but as the differences showed no dose–response relationship and were comparable to the historical control mean, they were not considered treatment related.

Table 16. Incidence of asymmetrically shaped sternebrae in fetuses

Parameter	0 mg/kg bw per day	20 mg/kg bw per day	200 mg/kg bw per day	1 000 mg/kg bw per day	Historical control values
Fetal data					
Fetuses examined	234	248	245	239	2 093
No. affected	2	4	5	8	–
Fetal incidence (%)	0.9	1.6	2.0	3.3	2.28 ± 2.09[a]
Litter data					
Litters examined	22	24	24	24	234
No. affected	2	4	4	7	–
Litter incidence (%)	9	17	17	29.2	15.08 ± 11.57[a]

[a] Mean ± 1 standard deviation.
Source: Schoch (1988)

The NOAEL for maternal toxicity and embryo/fetal toxicity was 1000 mg/kg bw per day, the highest dose tested (Schoch, 1988).

Rabbits

Trinexapac-ethyl (purity 96.6%) in 2% w/v methyl cellulose was administered by gavage to groups of 16 or 17 pregnant New Zealand White rabbits at a dose of 0, 10, 60 or 360 mg/kg bw per day from day 7 to day 19 of gestation. Dams were observed daily throughout gestation for clinical signs of toxicity, with body weight and feed consumption recorded on days 1, 5, 7, 9, 11, 15, 20, 24 and 29 of gestation. On day 29 of gestation, surviving dams were killed and fetuses examined for external, visceral and skeletal abnormalities.

At 360 mg/kg bw per day, two dams died; one of these had convulsions prior to being found dead on day 13, whereas the other aborted its litter and was killed in a moribund condition on day 24. Necropsy of the latter dam revealed macroscopic evidence of irritation of the stomach mucosa (haemorrhagic depressions of the stomach). One dam was killed at 60 mg/kg bw per day for humane reasons due to an intubation error. There were no other deaths or treatment-related clinical signs. Body weight loss occurred across all groups from days 7 to 9, with the largest loss occurring in high-dose dams (42 g compared with a loss of 20 g in the control); this difference was not statistically significant. High-dose dams took longer to recover from the initial body weight loss, regaining weight from day 15 compared with day 11 in all other groups. Mean feed consumption tended to be lower than the control values in high-dose dams ($P < 0.05$ over days 11–14), but was actually already lower than the control value before the commencement of dosing.

At the highest dose, postimplantation losses (24.8%) were significantly higher ($P < 0.05$) than the control value (13.2%), although preimplantation losses at the highest dose (24.3%) were also significantly higher ($P < 0.05$) than the control value (14.3%). The mean number of liver fetuses per dam at the highest dose (5.7) was significantly lower ($P < 0.05$) than the control value (7.7). Mean litter weight at the highest dose (255.7 g) was lower than the control value (332.0 g), but not significantly. Given that these fetal effects occurred only in the presence of frank maternal toxicity, which arose only after 1–2 weeks, they were not considered to be an acute effect.

There was no treatment-related effect on the incidence of external, visceral or skeletal malformations, anomalies or variations.

The NOAEL for maternal toxicity was 60 mg/kg bw for deaths in several dams at 360 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was also 60 mg/kg bw per day, based on increased postimplantation losses and a reduction in the mean number of live fetuses at 360 mg/kg bw per day (Hughes, 1990).

2.6 Special studies

(a) Neurotoxicity

In an acute neurotoxicity study, groups of 10 Crl:CD(SD) rats of each sex received a single gavage dose of trinexapac-ethyl (purity 95.8%) at 0, 500, 1000 or 2000 mg/kg bw. Observations were made twice daily for deaths and clinical signs. Body weight was recorded pretreatment and on days 0, 1, 2, 7, 8, 14 and 15. Feed consumption was recorded during days 0–1, 1–2 and 7–8. A functional observational battery and motor activity assessment were performed pretreatment, at 4 hours after dosing (i.e. the approximate time to peak effect) and at days 7 and 14. On day 15, five rats of each sex per group were killed, their brain weights were recorded and a neurohistopathological examination was performed.

There were no deaths or treatment-related clinical signs. At the highest dose, the mean body weight of males did not increase from day 0 to day 1, whereas the control group gained 5 g; this difference was statistically significant ($P < 0.01$). An examination of individual animal data indicated that three high-dose male rats lost body weight within 24 hours of dosing (−3, −3 and −6 g), two did not gain any weight and the remainder gained 1, 1, 2 and 4 g. In control males, one rat lost 3 g body weight within 24 hours, with the remainder gaining from 2 to 10 g body weight. The mean body weight of high-dose males was approximately 4% lower than the control value on day 1, but not significantly so. In high-dose females, a mean of 4 g body weight was lost from day 0 to day 1, in contrast to a loss of 1 g in the control group. After this initial reduction in body weight gain, there were no intergroup differences in body weight gain. Concomitant with the initial reduction in mean body weight gain was a significant reduction ($P < 0.05$) in mean feed consumption in high-dose males (17 versus 24 g in the control). Feed consumption in high-dose females was comparable to the control values. Although there was clearly a transient, treatment-related reduction in body weight, body weight gain and feed consumption in high-dose males shortly after a single gavage dose, the small magnitude of the reductions without any other accompanying effects is unlikely to represent an adverse effect. There were no treatment-related macroscopic abnormalities, effects on brain dimensions or weight or histopathological abnormalities.

The NOAEL was 2000 mg/kg bw, the highest dose tested. There was no evidence that trinexapac-ethyl was neurotoxic (Beck, 2012a).

In a subchronic neurotoxicity study, trinexapac-ethyl (purity > 95.8%) was admixed in the diet at a concentration of 0, 3750, 7500 or 15 000 ppm and fed ad libitum to groups of 12 Crl:CD(SD) rats of each sex for approximately 13 weeks. The achieved doses were 0, 233, 463 and 948 mg/kg bw per day for males and 0, 294, 588 and 1171 mg/kg bw per day for females at 0, 3750, 7500 and 15 000 ppm, respectively. Observations were made daily for mortalities and clinical signs. Body weight and feed consumption were recorded weekly. Functional observational battery and locomotor

activity assessments were conducted prior to treatment and during weeks 3, 7 and 12. Ophthalmoscopy was performed prior to treatment and during week 11. Five rats of each sex per dose were killed during week 13; their brains were removed and the dimensions recorded. Neurohistopathological examination was performed on selected central and peripheral nervous system tissues from five rats of each sex from the control and high-dose groups. There were no deaths and no treatment-related clinical signs. The mean body weight gain of high-dose females was significantly lower ($P < 0.01$) than the control values from days 0 to 7 (~32% lower than the control values). Thereafter, the mean body weight gain of 15 000 ppm females was generally similar to that of the controls. Mean feed consumption was also significantly reduced ($P < 0.05$) in high-dose females over days 0–7 and 7–14 (by ~11%). The mean body weight of high-dose females was slightly lower than the control values during days 14–91; however, no statistical significance was achieved. The transient nature and small magnitude of these findings in females were considered not to be toxicologically significant. There were no treatment-related effects on body weight gain or feed consumption in any other group, including males. Ophthalmoscopy, the functional observational battery and locomotor activity assessment were unremarkable. Brain weight, brain dimensions and the incidence of neurohistopathological abnormalities showed no relationship with treatment.

The NOAEL was 15 000 ppm (equal to 948 mg/kg bw per day), the highest dietary concentration tested. There was no evidence that trinexapac-ethyl was neurotoxic (Beck, 2012b).

3. Observations in humans

There were no reports submitted on adverse health effects in workers involved in the manufacture or use of trinexapac-ethyl. No cases of human poisonings have been reported.

Comments

Biochemical aspects

In studies conducted in rats using [^{14}C]trinexapac-ethyl, the time to reach the maximum plasma and tissue concentration of radioactivity was 15 minutes following a single gavage dose of 1 or 200 mg/kg bw. Gastrointestinal absorption was at least 96%. The plasma elimination half-life of radioactivity was less than 1 hour. Radioactivity was rapidly eliminated from tissues; mean first-phase tissue half-lives ranged from 0.2 to 0.5 hour at 1 mg/kg bw and from 0.5 to 0.9 hour at 200 mg/kg bw, whereas the slower second-phase elimination ranged from 1.6 to 3.2 hours at 1 mg/kg bw and from 3.2 to 11.7 hours at 200 mg/kg bw. There was no evidence of accumulation of radioactivity in any tissue. Excretion of radioactivity was predominantly via the urine (≥ 90% of the administered dose), with the majority of this occurring within 24 hours of dosing. Low levels of radioactivity were detected in faeces and bile (up to approximately 2.4% and 3.3% of the administered dose, respectively). Trinexapac-ethyl undergoes limited metabolism in the rat, involving predominantly ester hydrolysis of trinexapac-ethyl to trinexapac acid. The predominant urinary metabolite was trinexapac acid (up to 100% of total urinary radioactivity), with low levels of a conjugated derivative of trinexapac acid detected only in the urine of bile duct–cannulated rats (6.3% of the administered dose). In faeces, the parent compound accounted for 5–22% of total faecal radioactivity (1–2.5% of the administered dose), with the balance comprising trinexapac acid. Bile contained mainly the conjugated derivative of trinexapac acid (2.9% of the administered dose), with low levels of the parent compound also detected (0.2% of the administered dose).

Toxicological data

The oral LD_{50} in rats was 2000 mg/kg bw. In rats, the dermal LD_{50} was greater than 4000 mg/kg bw, and the LC_{50} was greater than 5.3 mg/L. Trinexapac-ethyl was neither a skin nor an eye irritant in rabbits. In a guinea-pig maximization test, no skin sensitization occurred.

In repeated-dose toxicity studies in rats and dogs, the main target organ was the kidneys. In rats, increased kidney weight and accompanying histopathological changes (focal tubular basophilia,

tubular hyaline droplets and pigment deposition) occurred. Additional treatment-related effects in the brain were observed in dog studies.

In a 4-week gavage study in rats, which tested doses of 0, 10, 100 and 1000 mg/kg bw per day, the NOAEL was 100 mg/kg bw per day for effects on the liver and kidneys at 1000 mg/kg bw per day.

In a 13-week dietary toxicity study in rats, which tested concentrations of 0, 50, 500, 5000 and 20 000 ppm (equal to 0, 3, 34, 346 and 1350 mg/kg bw per day for males and 0, 4, 38, 395 and 1551 mg/kg bw per day for females, respectively), the NOAEL was 500 ppm (equal to 34 mg/kg bw per day) for histopathological findings in the kidney in males at 5000 ppm (equal to 346 mg/kg bw per day).

In a 7-week, non-guideline study in dogs that tested dietary concentrations of 0, 500, 5000, 15 000, 30 000 and 50 000 ppm (equal to an average of 0, 23, 217, 683, 734 and 965 mg/kg bw per day for both sexes, respectively), the NOAEL was 15 000 ppm (equal to 683 mg/kg bw per day), based on a range of effects that occurred at 30 000 ppm (equal to 734 mg/kg bw per day), including body weight loss, lower feed consumption, increased serum cholesterol, increased kidney weight and histopathological findings in the kidney.

In a 13-week study in dogs, which tested dietary concentrations of 0, 50, 1000, 15 000 and 30 000 ppm (equal to 0, 2, 35, 516 and 927 mg/kg bw per day for males and 0, 2, 40, 582 and 891 mg/kg bw per day for females, respectively), the NOAEL was 15 000 ppm (equal to 516 mg/kg bw per day) for reduced body weight gain and feed consumption at 30 000 ppm (equal to 927 mg/kg bw per day). At 30 000 ppm in males, blood glucose was reduced, whereas focal vacuolation occurred in the brain of one dog.

In a 52-week toxicity study in dogs, which tested dietary concentrations of 0, 40, 1000, 10 000 and 20 000 ppm (equal to 0, 1.6, 32, 366 and 727 mg/kg bw per day for males and 0, 1.4, 40, 357 and 793 mg/kg bw per day for females, respectively), the NOAEL was 1000 ppm (equal to 32 mg/kg bw per day) for cerebral vacuolation at 10 000 ppm (equal to 357 mg/kg bw per day) in the absence of neurodegenerative or inflammatory histopathological changes or neurological signs.

In a 78-week study in mice, which tested dietary concentrations of 0, 7, 70, 1000, 3500 and 7000 ppm (equal to 0, 0.9, 9, 131, 451 and 912 mg/kg bw per day for males and 0, 1.1, 11, 154, 539 and 1073 mg/kg bw per day for females, respectively), the NOAEL for chronic toxicity and carcinogenicity was 7000 ppm (equal to 912 mg/kg bw per day), the highest dietary concentration tested.

In a 104-week study in rats, which tested dietary concentrations of 0, 10, 100, 3000, 10 000 and 20 000 ppm (equal to 0, 0.4, 4, 116, 393 and 806 mg/kg bw per day for males and 0, 0.5, 5, 147, 494 and 1054 mg/kg bw per day for females, respectively), the NOAEL for chronic toxicity was 3000 ppm (equal to 116 mg/kg bw per day) for histopathological lesions in the kidneys at 10 000 ppm (equal to 393 mg/kg bw per day). The NOAEL for carcinogenicity was 20 000 ppm (equal to 806 mg/kg bw per day), the highest dietary concentration tested.

The Meeting concluded that trinexapac-ethyl is not carcinogenic in mice or rats.

Trinexapac-ethyl was tested in an adequate range of in vitro and in vivo genotoxicity tests. No evidence of genotoxicity was found.

The Meeting concluded that trinexapac-ethyl is unlikely to be genotoxic.

Given the lack of genotoxicity and the absence of carcinogenicity in mice and rats, the Meeting concluded that trinexapac-ethyl is unlikely to pose a carcinogenic risk to humans.

In a two-generation reproductive toxicity study in rats, which tested dietary concentrations of 0, 10, 1000, 10 000 and 20 000 ppm (equal to 0, 0.57, 58.6, 570.5 and 1166 mg/kg bw per day for males and 0, 0.73, 73, 721.5 and 1427 mg/kg bw per day for females, respectively), there was no evidence of reproductive toxicity up to 20 000 ppm (equal to 1166 mg/kg bw per day), the highest dietary concentration tested. The NOAEL for parental toxicity was 1000 ppm (equal to 58.6 mg/kg

bw per day) for reduced body weight gain and feed consumption at 10 000 ppm (equal to 570.5 mg/kg bw per day). The NOAEL for offspring toxicity was 10 000 ppm (equal to 570.5 mg/kg bw per day) for reduced survival and body weight in the F_1 and F_2 generations at 20 000 ppm (equal to 1166 mg/kg bw per day).

In a rat developmental toxicity study, which tested doses of 0, 20, 200 and 1000 mg/kg bw per day, the NOAEL for maternal and embryo/fetal toxicity was 1000 mg/kg bw per day, the highest dose tested.

In a rabbit developmental toxicity study, which tested doses of 0, 10, 60 and 360 mg/kg bw per day, the NOAEL for maternal toxicity was 60 mg/kg bw per day for deaths of several dams at 360 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was also 60 mg/kg bw per day for increased postimplantation losses and a reduction in mean number of live fetuses at 360 mg/kg bw per day.

The Meeting concluded that trinexapac-ethyl is not teratogenic in rats or rabbits.

In an acute neurotoxicity study in rats that tested doses of 0, 500, 1000 and 2000 mg/kg bw per day, the NOAEL was 2000 mg/kg bw, the highest dose tested.

In a subchronic neurotoxicity study in rats that tested dietary concentrations of 0, 3750, 7500 and 15 000 ppm (equal to 0, 233, 463 and 948 mg/kg bw per day for males and 0, 294, 588 and 1171 mg/kg bw per day for females, respectively), the NOAEL was 15 000 ppm (equal to 948 mg/kg bw per day), the highest dietary concentration tested.

Toxicological data on metabolites and/or degradates

The Meeting noted the formation of two processing degradates of trinexapac acid, CGA 113745 and CGA 313458, not detected in rat metabolism studies. Based on a structural assessment of these degradates and an estimate of the levels of chronic dietary intake, the Meeting concluded that they are unlikely to pose a dietary risk.

Human data

There were no reports submitted on adverse health effects in workers involved in the manufacture or use of trinexapac-ethyl. No cases of human poisonings have been reported.

The Meeting concluded that the database on trinexapac-ethyl was adequate to characterize the potential hazards to fetuses, infants and children.

Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) of 0–0.3 mg/kg bw per day, expressed as trinexapac acid equivalents[1], based on a NOAEL of 32 mg/kg bw per day for trinexapac-ethyl (equivalent to 29 mg/kg bw per day expressed as trinexapac acid equivalents) for cerebral vacuolation in male and female dogs following 52 weeks of dietary exposure, with the application of a 100-fold safety factor. In the absence of information to the contrary, including mechanistic data, the cerebral vacuolation observed in dogs was considered relevant to humans.

The Meeting concluded that it is not necessary to establish an acute reference dose (ARfD) for trinexapac-ethyl in view of its low acute oral toxicity and the absence of developmental toxicity or any other toxicological effects that would be likely to be elicited by a single dose.

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[1] To cover the possible dietary exposure to a range of salts, esters and conjugates of trinexapac, it is appropriate to express the ADI as trinexapac acid equivalents using a conversion factor of 0.9 based on differences in molecular weight between trinexapac-ethyl and trinexapac acid.

Levels relevant to risk assessment of trinexapac-ethyl

Species	Study	Effect	NOAEL	LOAEL
Mouse	Eighteen-month study of toxicity and carcinogenicity[a]	Toxicity	7 000 ppm, equal to 912 mg/kg bw per day[b]	–
		Carcinogenicity	7 000 ppm, equal to 912 mg/kg bw per day[b]	–
Rat	Thirteen-week study of toxicity[a]	Toxicity	500 ppm, equal to 34 mg/kg bw per day	5 000 ppm, equal to 346 mg/kg bw per day
	Two-year study of toxicity and carcinogenicity[a]	Toxicity	3 000 ppm, equal to 116 mg/kg bw per day	10 000, equal to 393 mg/kg bw per day
		Carcinogenicity	20 000 ppm, equal to 806 mg/kg bw per day[b]	–
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	20 000 ppm, equal to 1 166 mg/kg bw per day[b]	–
		Parental toxicity	1 000 ppm, equal to 59 mg/kg bw per day	10 000 ppm, equal to 571 mg/kg bw per day
		Offspring toxicity	10 000 ppm, equal to 571 mg/kg bw per day	20 000 ppm, equal to 1 166 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	60 mg/kg bw per day	360 mg/kg bw per day
		Embryo and fetal toxicity	60 mg/kg bw per day	360 mg/kg bw per day
Dog	One-year study of toxicity[a]	Toxicity	1 000 ppm, equal to 32 mg/kg bw per day	10 000 ppm, equal to 357 mg/kg bw per day

LOAEL: lowest-observed-adverse-effect level; NOAEL: no-observed-adverse-effect level

[a] Dietary administration.

[b] Highest dose tested.

[c] Gavage administration.

Estimate of acceptable daily intake

0–0.3 mg/kg bw per day

Estimate of acute reference dose

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 trinexapac-ethyl

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid and complete
Distribution	Widespread tissue distribution
Potential for accumulation	No potential for accumulation
Rate and extent of excretion	Rapid and complete
Metabolism in animals	Limited; mainly hydrolysis to trinexapac acid
Toxicologically significant compounds in animals, plants and the environment	Trinexapac-ethyl, trinexapac acid
Acute toxicity	
Rat, LD_{50}, oral	2 000 mg/kg bw
Rat, LD_{50}, dermal	> 4 000 mg/kg bw
Rat, LC_{50}, inhalation	> 5.3 mg/L
Rabbit, dermal irritation	Non-irritating
Rabbit, ocular irritation	Non-irritating
Dermal sensitization	Non-sensitizing (guinea-pig maximization test)
Short-term studies of toxicity	
Target/critical effect	Kidney and brain
Lowest relevant oral NOAEL	32 mg/kg bw per day (dog)
Lowest relevant dermal NOAEL	1 000 mg/kg bw per day, the highest dose tested (rabbit)
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Kidney
Lowest relevant NOAEL	116 mg/kg bw per day (rat)
Carcinogenicity	Not carcinogenic
Genotoxicity	
	Not genotoxic
Reproductive toxicity	
Reproduction target/critical effect	No evidence of reproductive toxicity
Lowest relevant parental NOAEL	59 mg/kg bw per day
Lowest relevant offspring NOAEL	571 mg/kg bw per day
Lowest relevant reproduction NOAEL	1 166 mg/kg bw per day, the highest dose tested
Developmental toxicity	
Developmental target/critical effect	Postimplantation losses at maternally toxic doses (rabbit)
Lowest maternal NOAEL	60 mg/kg bw per day (rabbit)
Lowest embryo/fetal NOAEL	60 mg/kg bw per day (rabbit)
Neurotoxicity	
Acute and subchronic neurotoxicity	Not neurotoxic
Medical data	
	No data

LC_{50}: median lethal concentration; LD_{50}: median lethal dose; NOAEC: no-observed-adverse-effect concentration; NOAEL: no-observed-adverse-effect level

Summary

	Value	Studies	Safety factor
ADI	0–0.3 mg/kg bw	One-year toxicity study in dogs	100
ARfD	Unnecessary	—	—

ADI: acceptable daily intake; ARfD: acute reference dose

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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.
2. Evaluation of the toxicity of pesticide residues in food. Report of a Joint Meeting of the FAO Committee on Pesticides in Agriculture and the WHO Expert Committee on Pesticide Residues. FAO Meeting Report, No. PL/1963/13; WHO/Food Add./23, 1964.
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This volume contains toxicological monographs that were prepared by the 2013 Joint FAO/WHO Meeting on Pesticide Residues (JMPR), which met in Geneva on 17–26 September 2013.

The monographs in this volume summarize the safety data on 14 pesticides that could leave residues in food commodities. These pesticides are benzovindiflupyr, bixafen, chlorfenapyr, cyantraniliprole, diquat, dithianon, fenamidone, fluensulfone, imazapic, imazapyr, isoxaflutole, tolfenpyrad, triflumizole and trinexapac-ethyl. 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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