Pesticide residues in food — 2014

Joint FAO/WHO Meeting on Pesticide Residues

EVALUATIONS 2014

Part II — Toxicological

Food and Agriculture Organization of the United Nations

World Health Organization

Pesticide residues in food – 2014

Toxicological evaluations

Sponsored jointly by FAO and WHO

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

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2014 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

Rome, 16–25 September 2014

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Abbreviations used

ACTH	adrenocorticotrophic hormone
ADI	acceptable daily intake
ADME	absorption, distribution, metabolism and excretion
ae	acid equivalent
a.i.	active ingredient
Alb	albumin
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AMBA	4-(methylsulfonyl)-2-aminobenzoic acid
ANOVA	analysis of variance
AR	androgen receptor
ARfD	acute reference dose
AST	aspartate aminotransferase
AUC_{0-t}	area under the concentration–time curve from time 0 to time *t*
$AUC_{0-\infty}$	area under the concentration–time curve from time 0 to infinity
B	benign
BAM	2,6-dichlorobenzamide
Bil	bilirubin
BrdU	5-bromo-2′-deoxyuridine
BUN	blood urea nitrogen
bw/BW	body weight
CAS	Chemical Abstracts Service
CCPR	Codex Committee on Pesticide Residues
CHL	Chinese hamster lung
CHO	Chinese hamster ovary
Chol	cholesterol
C_{max}	maximum concentration
CPCA	cyclopropane carboxylic acid
CRL	Charles River Laboratories
CYP	cytochrome P450
DMF	*N*,*N*-dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNCB	1-chloro-2,4-dinitrobenzene
dUTP	deoxyuridine-5′-triphosphate
EFSA	European Food Safety Authority
ELISA	enzyme-linked immunosorbent assay
EMA	European Medicines Agency
eq	equivalent
equiv	equivalent
ER	estrogen receptor
EROD	7-ethoxyresorufin *O*-deethylase
F	female
F_0	parental generation
F_1	first filial generation

F_2	second filial generation
F344	Fischer 344
FAO	Food and Agriculture Organization of the United Nations
FCA	Freund's Complete Adjuvant
FSH	follicle stimulating hormone
G6Pase	glucose-6-phosphatase
G6PD	glucose-6-phosphate dehydrogenase
GA	glucuronic acid
GADPH	glyceraldehyde-3-phosphate dehydrogenase
GD	gestation day
GGT	gamma-glutamyltransferase
GI	gastrointestinal
GLP	good laboratory practice
GSD	geometric standard deviation
GSH	glutathione
GST	glutathione *S*-transferase
GST-P	glutathione *S*-transferase placental form
H&E	haematoxylin and eosin
Hb	haemoglobin
HCD	historical control data
Hct	haematocrit
HESI	Health and Environmental Sciences Institute
Hgprt/HGPRT	hypoxanthine–guanine phosphoribosyltransferase
HPAA	4-hydroxyphenylacetate
HPLA	4-hydroxyphenyllactate
HPLC	high-performance liquid chromatography
HPPA	4-hydroxyphenylpyruvate
HPPD	4-hydroxyphenylpyruvate dioxygenase
HPRT	hypoxanthine–guanine phosphoribosyltransferase
HSL	hormone-sensitive lipase
IC_{50}	median inhibitory concentration
IEDI	international estimated daily intake
IESTI	international estimated short-term intake
IET	Institute of Environmental Toxicology (Japan)
IgM	immunoglobulin M
ILSI	International Life Sciences Institute
ISO	International Organization for Standardization
IU	International Units
JMAFF	Japanese Ministry of Agriculture, Forestry and Fisheries
JMPR	Joint FAO/WHO Meeting on Pesticide Residues
LA-11-OH	lauric acid 11-hydroxylase
LC	liquid chromatography
LC_{50}	median lethal concentration
LCA	Leydig cell adenoma
LD_{50}	median lethal dose
LH	luteinizing hormone
LI	5-bromo-2′-deoxyuridine labelling index

LLNA	local lymph node assay
LOAEL	lowest-observed-adverse-effect level
LOD	limit of detection
LOQ	limit of quantification
LSC	liquid scintillation counting
M	male; malignant
MA	mercapturic acid
MARTA	Middle Atlantic Reproduction and Teratology Association
MCH	mean corpuscular haemoglobin
MCHC	mean corpuscular haemoglobin concentration
MCV	mean corpuscular volume
M:E	myeloid:erythroid ratio
MFO	mixed-function oxidase
Mit	erythrocytic mitotic cells
MMAD	mass median aerodynamic diameter
MNBA	2-nitro-4-(methylsulfonyl)-benzoic acid
MQL	minimum quantifiable level
MRT	mean residence time
MS	mass spectrometry
MTD	maximum tolerated dose
NA	not available
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NCEH	neutral cholesteryl ester hydrolase
ND	not determined
NNM	nitrosomorpholine
NOAEC	no-observed-adverse-effect concentration
NOAEL	no-observed-adverse-effect level
NOEL	no-observed-effect level
NR	not reported
NRU	neutral red uptake
NTBC	2-(2-nitro-4-fluoromethylbenzoyl)-1,3-cyclohexanedione
NZW	New Zealand White
OECD	Organisation for Economic Co-operation and Development
OPPTS	Office of Prevention, Pesticides, and Toxic Substances (USEPA)
P	parental generation
P_1	first parental generation
P_2	second parental generation
P	probability
PAS	periodic acid–Schiff
PB	phenobarbitone
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
pK_a	acid dissociation constant
PND	postnatal day
Poly	polychromatic erythrocytes
ppm	parts per million
PROD	7-pentoxyresorufin *O*-depentylase

$Rac_{24\ h}$	^{14}C repetitive accumulation ratio
RBC	red blood cells
RCE	relative cloning efficiency
Retic	reticulocytes
Rf	retardation factor
rT_3	reverse triiodothyronine
S9	9000 × *g* supernatant fraction from rat liver homogenate
SD	standard deviation
SG	glutathione
sRBC	sheep red blood cell
$t_{½}$	half-life
$t_{½\ term}$	terminal half-life
T_3	triiodothyronine
T_4	thyroxine
TAT	tyrosine aminotransferase
TG	Test Guideline
TK	thymidine kinase
TLA	thiolactic acid
TLC	thin-layer chromatography
T_{max}	time to reach the maximum concentration
ToxRefDB	Toxicity Reference Database
TRR	total radioactive residue
TSH	thyroid stimulating hormone
TTC	threshold of toxicological concern
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labelling
U	units; uniformly labelled
UDP-GT	uridine diphosphate-glucuronyltransferase
USA	United States of America
USEPA	United States Environmental Protection Agency
UVA	ultraviolet A
WBC	white blood cells
WHO	World Health Organization
w/v	weight per volume
w/w	weight per weight

Introduction

The toxicological monographs and monograph addenda contained in this volume were prepared by a WHO Core Assessment Group on Pesticide Residues that met with the FAO Panel of Experts on Pesticide Residues in Food and the Environment in a Joint Meeting on Pesticide Residues (JMPR) in Rome, Italy, on 16–25 September 2014.

Eight of the substances evaluated by the WHO Core Assessment Group (aminocyclopyrachlor, cyflumetofen, dichlobenil, flufenoxuron, imazamox, mesotrione, metrafenone and pymetrozine) were evaluated for the first time. Two compounds (myclobutanil and triforine) were re-evaluated within the periodic review programme of the Codex Committee on Pesticide Residues (CCPR). Reports and other documents resulting from previous Joint Meetings on Pesticide Residues are listed in Annex 1.

The report of the Joint Meeting has been published by the FAO as *FAO Plant Production and Protection Paper 221*. 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 2014, 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 2014 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.

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

AMINOCYCLOPYRACHLOR

First draft prepared by
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Explanation

Aminocyclopyrachlor is the International Organization for Standardization (ISO)–approved common name for 6-amino-5-chloro-2-cyclopropylpyrimidine-4-carboxylic acid (International Union of Pure and Applied Chemistry), with Chemical Abstracts Service (CAS) number 858956-08-8. Aminocyclopyrachlor belongs to the pyrimidine carboxylic acid chemical family and is an auxin-mimicking herbicide used for selective control of weeds, invasive species and brush in pasture. The methyl ester (CAS No. 858954-83-3) is also used as a herbicide, and information on this compound was also assessed.

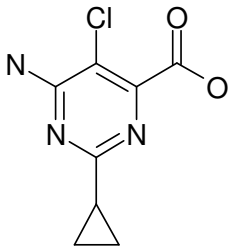
A complete toxicological data package was provided on aminocyclopyrachlor. A subset of toxicological studies was provided on aminocyclopyrachlor-methyl based on the weight of evidence from pharmacokinetic and metabolism studies and the results of bridging studies, which indicated the toxicological equivalence of the two compounds. For comparative purposes, doses of aminocyclopyrachlor-methyl are expressed as aminocyclopyrachlor acid equivalents (ae), calculated using the respective relative molecular masses (227.65 versus 213.62). The chemical structure of aminocyclopyrachlor is given in Fig. 1.

Aminocyclopyrachlor 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 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.

Fig. 1. Chemical structure of aminocyclopyrachlor (DPX-MAT28)

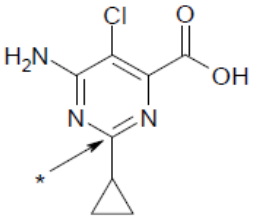


Evaluation for acceptable daily intake

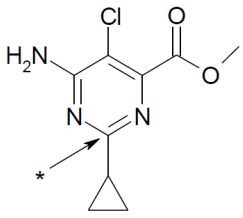
1. Biochemical aspects

Absorption, distribution, metabolism and excretion studies were conducted in rats using [pyrimidine-2-^{14}C]aminocyclopyrachlor or [pyrimidine-2-^{14}C]aminocyclopyrachlor-methyl (Fig. 2).

Fig. 2. Chemical structures of [pyrimidine-2-^{14}C]aminocyclopyrachlor and [pyrimidine-2-^{14}C]aminocyclopyrachlor-methyl



[Pyrimidine-2-^{14}C]aminocyclopyrachlor
* Denotes the position of the radiolabel



[Pyrimidine-2-^{14}C]aminocyclopyrachlor-methyl
* Denotes the position of the radiolabel

1.1 Absorption, distribution and excretion

Rats

Himmelstein (2010a) undertook pilot mass balance and definitive pharmacokinetic and metabolism studies in Crl:CD®(SD) rats dosed with [pyrimidine-2-^{14}C]aminocyclopyrachlor (99.5% radiochemical purity) in 0.5% (weight per volume [w/v]) methyl cellulose. In the pilot mass balance study, one male and one female rat each received a single gavage dose of ^{14}C-labelled aminocyclopyrachlor at 25 mg/kg body weight (bw). At 24-hour intervals, exhaled air (up to 48 hours after dosing), urine and faeces (up to 168 hours after dosing) were collected. Rats were killed at 168 hours, and tissues were sampled. Radioactivity was quantified in plasma, excreta and tissues by liquid scintillation counting (LSC). Total recovery of radioactivity was 69.9% and 107.9% of the administered radioactive dose in the male and female, respectively. In the absence of any detectable radioactivity in exhaled air, no further collection of exhaled air was performed after 48 hours. The majority of radioactivity was recovered equally in urine and faeces within the first 24 hours after dosing (male rat: totals of 36% and 32% of the administered dose in urine and faeces, respectively, up to 168 hours after dosing; female rat: totals of 56% and 52% of the administered dose in urine and faeces, respectively, up to 168 hours after dosing). No radioactivity was detected in

tissues. A low level of radioactivity was detected in the carcass of the male rat (0.039% of the administered dose); no radioactivity was detected in the carcass of the female rat.

In the definitive pharmacokinetic study, rats (four of each sex per dose) received a single gavage dose of ^{14}C-labelled aminocyclopyrachlor at 25 or 500 mg/kg bw. Blood was sampled at various times up to 30 hours after dosing for the analysis of radioactivity in plasma and red blood cells by LSC. Two additional rats (one of each sex) received a single gavage dose of 500 mg/kg bw, and plasma was collected at 2 hours to assist with the development of the analytical method used in the metabolism study. Pharmacokinetic parameters are summarized in Table 1. Radioactivity was rapidly absorbed, with peak plasma concentrations reached at 0.4–1.0 hour after dosing and peak concentrations in red blood cells reached at 0.3–1.0 hour after dosing. The ratio of the maximum concentrations of radioactivity (C_{max}) in red blood cells and plasma ranged from 0.33 to 0.48, indicating limited potential for uptake and binding to red blood cells. Mean plasma elimination half-lives were consistent in males and females (5.6 and 5.7 hours, respectively) and between the low and high doses. There was a proportional increase in the C_{max} and area under the concentration–time curve ($AUC_{0-\infty}$) from the low to the high dose, with the latter suggesting linear first-order kinetic processes for uptake and elimination (Himmelstein, 2010a).

Table 1. Mean pharmacokinetic parameters in plasma and red blood cells of rats following a single oral dose of ^{14}C-labelled aminocyclopyrachlor at 25 or 500 mg/kg bw

	25 mg/kg bw dose				500 mg/kg bw dose			
	Male		Female		Male		Female	
Parameter	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Plasma								
C_{max} (µg eq/g)	3.8	0.9	5.0	1.2	57.3	14.2	61.6	13.0
T_{max} (h)	0.5	0.4	0.4	0.1	0.6	0.3	1.0	0.7
$t_{½}$ (h)	5.6	0.5	5.7	0.4	5.6	0.3	5.7	0.7
$AUC_{0-\infty}$ (µg eq·h/g)	7.0	1.4	9.0	1.9	150.8	28.7	168.4	26.2
Red blood cells[a]								
C_{max} (µg eq/g)	1.3	0.3	2.0	0.7	27.2	6.2	28.7	4.7
T_{max} (h)	0.5	0.4	0.3	0.1	0.6	0.3	1.0	0.7

$AUC_{0-\infty}$: area under the concentration–time curve from time 0 to infinity; bw: body weight; C_{max}: peak concentration; eq: equivalent; SD: standard deviation; $t_{½}$: half-life; T_{max}: time to reach C_{max}

[a] Only C_{max} and T_{max} are presented. Time course data were insufficient to calculate other kinetic parameters.

Source: Himmelstein (2010a)

[Pyrimidine-2-^{14}C]aminocyclopyrachlor-methyl (99.4% radiochemical purity) in 0.5% (w/v) methyl cellulose was administered as a single gavage dose to Crl:CD®(SD) rats under the same experimental conditions used in the preceding study by Himmelstein (2010a). In the pilot mass balance study, total recovery of radioactivity was 89.2% and 82.6% of the administered dose in the male and female, respectively. In the absence of any detectable radioactivity in exhaled air, no further collection of exhaled air was performed after 48 hours. In both rats, the majority of radioactivity (79%) was recovered in urine within 24 hours of dosing. The cumulative amount of radioactivity in urine and faeces at 168 hours after dosing was 80.38% and 6.67%, respectively, in the male and 79.27% and 3.06%, respectively, in the female. The higher proportion of radioactivity excreted in urine suggests a higher level of gastrointestinal absorption than for ^{14}C-labelled aminocyclopyrachlor. At 168 hours after dosing, most tissues contained no detectable radioactivity, with the exception of the gastrointestinal tract (0.009% of the administered dose) and carcass (0.144% of the administered dose). Pharmacokinetic parameters are summarized in Table 2. At the same nominal dose, a single gavage dose of ^{14}C-labelled aminocyclopyrachlor-methyl resulted in a more rapid uptake of radioactivity than for ^{14}C-labelled aminocyclopyrachlor (up to approximately 2-fold), with C_{max} and $AUC_{0-\infty}$ values also noticeably higher (2- to 5-fold and 1.4- to 2.85-fold,

respectively). Plasma elimination half-lives were approximately twice those of ^{14}C-labelled aminocyclopyrachlor. Peak concentrations of radioactivity in red blood cells were 36–49% of those observed in plasma, indicating very limited potential for binding to red blood cells (Himmelstein 2010b).

Table 2. Mean pharmacokinetic parameters in plasma and red blood cells of rats following a single oral dose of ^{14}C-labelled aminocyclopyrachlor-methyl at 25 or 500 mg/kg bw

	25 mg/kg bw dose				500 mg/kg bw dose			
	Male		Female		Male		Female	
Parameter	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Plasma								
C_{max} (µg eq/g)	20.0	7.4	16.7	0.8	126.0	14.9	113.9	27.6
T_{max} (h)	0.3	0.0	0.3	0.0	0.4	0.1	0.5	0.0
$t_{½}$ (h)	13.3	0.6	10.9	1.2	8.7	4.1	11.6	4.1
$AUC_{0-\infty}$ (µg eq·h/g)	14.8	1.4	14.5	1.5	255.8	59.8	241.5	47.8
Red blood cells[a]								
C_{max} (µg eq/g)	7.2	2.8	8.2	1.8	54.7	5.1	48.1	10.1
T_{max} (h)	0.3	0.1	0.3	0.0	0.4	0.1	0.6	0.3

$AUC_{0-\infty}$: area under the concentration–time curve from time 0 to infinity; bw: body weight; C_{max}: peak concentration; eq: equivalent; SD: standard deviation; $t_{½}$: half-life; T_{max}: time to reach C_{max}

[a] Only C_{max} and T_{max} are presented. Time course data were insufficient to calculate other kinetic parameters.

Source: Himmelstein (2010b)

In a series of experiments, Himmelstein (2010c) examined the absorption, distribution, metabolism and elimination of [pyrimidine-2-^{14}C]aminocyclopyrachlor (99.5% radiochemical purity) in 0.5% (w/v) methyl cellulose in Crl:CD®(SD) rats. Radioactivity was quantified by LSC, with metabolites analysed by high-performance liquid chromatography (HPLC) and liquid chromatography with mass spectrometry (LC-MS).

In a mass balance study, rats were administered a single gavage dose of ^{14}C-labelled aminocyclopyrachlor at 0 mg/kg bw (one of each sex), 25 mg/kg bw (four of each sex) or 500 mg/kg bw (four of each sex). Exhaled air was collected to 48 hours after dosing, whereas urine and faeces were collected to 72 hours after dosing. Rats were killed at 72 hours, and tissues were sampled. Radioactivity was quantified in excreta, tissues and carcass, whereas metabolites were analysed in urine and faeces. In a repeated-dose experiment, also designed to analyse the excretion of radioactivity and the formation of metabolites, 24 male and six female rats were administered ^{14}C-labelled aminocyclopyrachlor at 25 mg/kg bw per day for 14 days. Urine and faeces were collected daily from each rat. Three males were killed on days 2, 6, 10, 14 (six rats) and 15 for the analysis of radioactivity in whole blood, plasma, red blood cells, liver, kidney, fat and muscle. Three males were also killed on days 13 and 16, with three females killed on days 13 and 16 for a more comprehensive analysis of radioactivity in tissues and the carcass.

The mass balance of radioactivity is summarized in Table 3. No radioactivity was detected in exhaled air. Recovery of radioactivity was greater than 97%, with the majority detected in urine and faeces, in approximately equal proportions, at the low single dose. At the high single dose, a higher proportion of radioactivity was detected in faeces, suggesting a reduction in gastrointestinal absorption. The majority of radioactivity was excreted within 24 hours of a single oral dose. Other than the skin, kidneys, gastrointestinal tract and its contents, which contained relatively low levels of radioactivity, there was no detectable radioactivity in any other sampled tissues (blood, bone

marrow, brain, fat, heart, lungs, spleen, liver, pituitary, thyroid, thymus, ovaries, testes, pancreas, adrenals, uterus, muscle, bladder and bone) 72 hours after a single 25 or 500 mg/kg bw oral dose of ^{14}C-labelled aminocyclopyrachlor (Table 4). In the repeated-dose experiment, the pattern of tissue distribution of radioactivity was consistent with that occurring after a single dose, with tissue radioactivity rapidly increasing to apparent steady state by day 3. Table 5 summarizes the concentrations of radioactivity in various tissues at 6 and 72 hours after the final repeated dose. Radioactivity was rapidly eliminated from tissues following the cessation of dosing, with no indication of any accumulation.

Table 3. Cumulative mass balance in rats following oral dosing with ^{14}C-labelled aminocyclopyrachlor

	Mean % of administered radioactivity					
	25 mg/kg bw (single dose)		**500 mg/kg bw (single dose)**		**25 mg/kg bw (repeated dose)**	
Sample	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
Urine						
6 h	35.75	45.12	22.14	31.93	–	–
12 h	40.78	50.29	33.73	36.20	–	–
24 h	46.08	55.08	38.08	41.59	38.65	49.06
48 h	47.64	56.21	39.86	43.70	38.45	51.51
72 h	47.74	56.51	40.04	44.08	39.53	51.79
16 days	–	–	–	–	38.85	55.26
Faeces						
6 h	0.18	0.49	0.38	0	–	–
12 h	24.16	23.17	18.58	5.37	–	–
24 h	44.02	38.12	47.73	40.43	42.28	44.44
48 h	47.69	39.29	54.22	50.23	42.68	48.96
72 h	47.70	39.48	54.76	51.08	40.45	50.94
16 days	–	–	–	–	36.57	57.25
Cage wash	2.43[a]	2.87[a]	1.84[a]	2.08[a]	2.62[b]	4.04[b]
Feed residue	0.328[a]	0.316[a]	0.648[a]	0.243[a]	0.253[b]	0.146[b]
Tissues + carcass	0.064[a]	0.222[a]	0.085[a]	0.184[a]	0.030 0[b]	0.026 3[b]
Recovery	98.5[a]	99.4[a]	97.4[a]	97.7[a]	97.01[b]	98.03[b]

bw: body weight

[a] Cumulative radioactivity to 72 hours.
[b] Cumulative radioactivity to 16 days.

Source: Himmelstein (2010c)

The excretion of radioactivity was analysed in groups of bile duct–cannulated rats that were administered a single gavage dose of ^{14}C-labelled aminocyclopyrachlor at 0 mg/kg bw (one of each sex), 25 mg/kg bw (four of each sex) or 500 mg/kg bw (four of each sex). Urine, faeces and bile were collected at 0–6, 6–12, 12–24 and 24–48 hours after dosing. Rats were killed at 48 hours, and radioactivity was analysed in blood, urine, faeces, bile, the gastrointestinal tract and its contents, tissues, carcass and cage wash. At both doses, the majority of absorbed radioactivity was excreted in the urine (22.2–34.5%), with a relatively small proportion recovered in bile (0.13–0.25%). Based on

the concentration of radioactivity in urine, bile and carcass (not including the gastrointestinal tract and its contents), the estimated extent of gastrointestinal absorption was 22.4–34.9%. This estimate of gastrointestinal absorption is lower than an estimate based on the level of radioactivity in urine, tissues and carcass in the mass balance experiment conducted in non-bile duct–cannulated rats (39.9–56.2%) at the same doses. It is noted that in the bile duct–cannulated rats, the majority of radioactivity was eliminated unabsorbed in the faeces (57.9–68.5% of the administered dose), which suggested that under the experimental conditions, gastrointestinal absorption was reduced. The author proposed that this was due to the combination of the intake of the drinking solution (pH 6.3) containing dextrose (276 mmol/L), potassium chloride (6.7 mmol/L) and sodium chloride (154 mmol/L) to help maintain hydration and bile flow and the aminocyclopyrachlor (acid dissociation constant [pK_a] 4.65) dosing preparation (pH 7). On this basis, the estimate of gastrointestinal absorption from the mass balance experiment (accounting for 37–57% of the administered dose by 72 hours) is considered a more reliable estimate of the absorbed dose.

Table 4. Mean radioactivity in selected tissues 72 hours after a single oral dose of ^{14}C-labelled aminocyclopyrachlor

	Mean radioactivity (% of the administered dose)			
	Male		**Female**	
Tissue	**25 mg/kg bw**	**500 mg/kg bw**	**25 mg/kg bw**	**500 mg/kg bw**
Carcass[a]	0.067	0.058	0.200	0.101
Skin	0.043	< LOD	< LOD	< LOD
Kidney	0.000	0.000 2	< LOD	0.000 3
Gastrointestinal tract	0.001	0.001 6	0.001	0.002 9
Gastrointestinal tract contents	0.013	0.040	0.022	0.080
Total	**0.034**	**0.042**	**0.023**	**0.083**

bw: body weight; LOD: limit of detection

[a] Percentage of dose recovered in carcass is not included in the total.

Source: Himmelstein (2010c)

The tissue distribution of radioactivity was further analysed in groups of eight rats of each sex that were administered a single gavage dose of ^{14}C-labelled aminocyclopyrachlor at 25 or 500 mg/kg bw. At 1 and 6 hours after dosing (the approximate time to C_{max} [T_{max}] and T_{max} + 5 hours, respectively), four rats of each sex per group were killed, and radioactivity was analysed in various tissues. Results are summarized in Table 6. The majority of radioactivity was associated with the gastrointestinal tract contents. Other than the gastrointestinal tract and its contents, the concentration of radioactivity in all sampled tissues was low, with the highest concentrations detected in the skin, muscle, whole blood, kidney and liver. There was no indication of accumulation in any tissue. A comparison of the level of radioactivity at 6 hours after dosing in this study with that at 72 hours in the mass balance study (Table 3) indicated relatively rapid elimination of radioactivity from tissues.

Himmelstein (2010d) examined the absorption, distribution, metabolism and elimination of a single gavage dose of 25 mg/kg bw of [pyrimidine-2-^{14}C]aminocyclopyrachlor-methyl (99.2% radiochemical purity) in 0.5% (w/v) methyl cellulose when administered to Crl:CD®(SD) rats. Radioactivity was quantified by LSC, with metabolites analysed by HPLC and LC-MS.

In a mass balance and tissue distribution experiment, four rats of each sex were dosed with ^{14}C-labelled aminocyclopyrachlor-methyl, with a separate male and female serving as controls. Urine and faeces were collected predosing and at 0–6, 6–12, 12–24, 24–48 and 48–72 hours. Exhaled volatiles and carbon dioxide were collected predosing and at 0–6, 6–12, 12–24 and 24–48 hours. Rats were killed 72 hours after dosing, and various tissues were collected for the analysis of

radioactivity. There were no signs of toxicity. The mean recovery of radioactivity was at least 98%, with the majority detected in urine and relatively little detected in faeces and exhaled air (Table 7). The majority of radioactivity was excreted in urine within 24 hours of dosing. At 72 hours after dosing, relatively low levels of radioactivity were detected in the majority of sampled tissues, with the highest levels detected in the carcass, skin, fat and gastrointestinal tract contents (Table 8).

Table 5. Mean radioactivity in selected tissues 6 and 72 hours after the final repeated oral dose of ^{14}C-labelled aminocyclopyrachlor

	Mean radioactivity (% of the administered dose)			
	Male		**Female**	
Tissue	**6 h after last dose**	**72 h after last dose**	**6 h after last dose**	**72 h after last dose**
Carcass[a]	0.137 74	0.026 29	0.133 74	0.022 98
Skin	0.013 69	0.002 70	0.010 21	0.002 39
Whole blood	0.008 06	0.000 20	0.004 32	0.000 20
Bone marrow	NA	0.001 03	NA	NA
Brain	0.000 09	0.000 02	0.000 12	0.000 03
Fat	0.005 90	0.002 45	0.003 03	0.002 21
Heart	0.000 16	0.000 02	0.000 12	NA
Lungs	0.000 30	0.000 03	0.000 34	0.000 03
Spleen	0.000 10	0.000 01	0.000 05	NA
Liver	0.010 36	0.000 31	0.002 80	0.000 20
Kidney	0.005 32	0.000 07	0.003 29	0.000 12
Gastrointestinal tract	0.219 29	0.000 22	0.164 95	0.000 18
Gastrointestinal tract contents	7.142 52	0.002 61	7.105 25	0.002 55
Pituitary	< LOD	0.000 01	< LOD	< LOD
Thyroid	< LOD	0.000 01	< LOD	NA
Thymus	0.000 06	0.000 02	0.000 06	NA
Ovaries	–	–	< LOD	< LOD
Testes	0.001 12	0.000 04	–	–
Pancreas	0.000 30	0.000 02	0.000 13	< LOD
Adrenals	0.000 03	0.000 01	< LOD	< LOD
Uterus	–	–	0.000 09	< LOD
Muscle	0.034 61	0.002 02	0.008 54	< LOD
Bladder	0.003 36	0.000 01	0.000 48	< LOD
Bone	0.001 26	0.000 34	0.000 68	< LOD
Total	**7.446**	**0.010**	**7.304**	**0.008**

LOD: limit of detection; NA: not available

[a] Percentage of dose recovered in carcass is not included in the total.

Source: Himmelstein (2010c)

Table 6. Mean radioactivity in selected tissues 1 and 6 hours after a single oral dose of ^{14}C-labelled aminocyclopyrachlor

	Mean radioactivity (% of the administered dose)							
	Male				Female			
	25 mg/kg bw		500 mg/kg bw		25 mg/kg bw		500 mg/kg bw	
Tissue	**1 h**	**6 h**	**1 h**	**6 h**	**1 h**	**6 h**	**1 h**	**6 h**
Carcass[a]	3.458	5.167	4.154	0.276	3.187	0.354	2.462	0.427
Skin	1.306	0.073	0.780	0.057	1.368	0.060	0.695	0.047
Whole blood	1.020	0.039	0.587	0.029	0.954	0.034	0.492	0.029
Bone marrow	0.072	0.053	0.042	0.007	0.076	0.011	0.039	0.005
Brain	0.004	0.000 8	0.002 4	0.000 7	0.005	0.001	0.002 7	0.000 8
Fat	0.238	0.025	0.375	0.023	0.210	0.019	0.106	0.017
Heart	0.026	0.001 2	0.015	0.000 9	0.021	0.001	0.010 8	0.000 9
Lungs	0.036	0.001 9	0.024	0.001 6	0.038	0.002	0.023 2	0.001 5
Spleen	0.006	0.000 6	0.038	0.000 4	0.006	0.001	0.003 1	0.000 3
Liver	0.750	0.038	0.392	0.030	0.517	0.023	0.264	0.018
Kidney	0.894	0.025	0.562	0.030	1.072	0.051	0.431	0.024
Gastrointestinal tract	13.083	1.363	11.913	1.753	9.544	1.428	10.847	3.928
Gastrointestinal tract contents	58.039	46.766	63.535	67.032	51.504	41.749	64.058	56.035
Pituitary	0.000 4	< LOD	0.002	< LOD	0.001	< LOD	0.000 3	< LOD
Thyroid	0.000 6	< LOD	0.002	0.000 3	0.001	< LOD	0.001 0	< LOD
Thymus	0.006	0.000 4	0.004	0.004	0.007	0	0.003 9	0.003
Ovaries	–	–	–	–	0.006	0	0.002 9	0.003
Testes	0.025	0.003 1	0.031	0.002	–	–	–	–
Pancreas	0.023	0.001 4	0.038	0.003 4	0.020	0.001	0.018 7	0.000 7
Adrenals	0.001	0.000 1	0.019	0.000 1	0.005	0	0.001 1	0.000 1
Uterus	–	–	–	–	0.018	0.008	0.018 2	0.001
Muscle	1.189	0.098	2.008	0.057	0.993	0.067	0.385	0.052
Bladder	0.235	0.030	0.126	0.003	0.124	0.004	0.138	0.003
Bone	0.088	0.008	0.072	0.018	0.104	0.007	0.049	0.006
Total	**77.043**	**48.500**	**80.567**	**69.042**	**66.594**	**43.453**	**77.591**	**60.168**

bw: body weight; LOD: limit of detection

[a] Percentage of dose recovered in carcass is not included in the total.

Source: Himmelstein (2010c)

The excretion of radioactivity was analysed in a group of bile duct–cannulated rats (four of each sex). Urine, faeces and bile were collected at 0–6, 6–12, 12–24 and 24–48 hours after dosing. Rats were killed 48 hours after dosing. Mean total recovery of radioactivity was 91.7% in males and 93.2% in females. The majority of the radioactivity was recovered in the urine (87.2% and 87.1% in

males and females, respectively), with only minor amounts recovered in the carcass plus gastrointestinal tract (0.13% and 0.26% in males and females, respectively) and bile (0.60% and 0.46% in males and females, respectively). By summation, the mean percentage of absorbed radioactivity was 87.9% and 87.8% in male and female rats, respectively. Comparison of the estimated absorption from the bile duct–cannulated rats was consistent with the cumulative excretion in urine of non-bile duct–cannulated animals from the mass balance experiment (Himmelstein, 2010d).

Table 7. Cumulative mass balance in rats following a single oral dose of 25 mg/kg bw of ^{14}C-labelled aminocyclopyrachlor-methyl

	Mean % of administered radioactivity			
	Males		Females	
Sample	**Mean**	**SD**	**Mean**	**SD**
Urine				
6 h	85.87	5.42	79.52	7.34
12 h	90.49	1.72	81.68	7.11
24 h	91.53	1.97	83.44	6.31
48 h	92.30	2.09	84.96	5.25
72 h	92.37	2.07	85.06	5.18
Faeces				
6 h	0.03	0.05	0.07	0.15
12 h	2.41	1.17	2.54	0.75
24 h	4.25	0.79	3.91	0.52
48 h	4.54	0.81	4.33	0.55
72 h	4.59	0.81	4.44	0.55
Exhaled air[a]	0.015	0.002	0.016	0.002
Cage wash[b]	1.30	0.45	5.04	4.07
Feed residue[b]	0.36	0.39	3.35	3.73
Tissues + carcass[b]	0.11	0.03	0.11	0.04
Recovery[b]	98.7	1.7	98.0	1.4

bw: body weight; SD: standard deviation

[a] Radioactivity recovered in the carbon dioxide trap at 0–6 hours after dosing only – no radioactivity was detected in volatile organic or water traps.

[b] Cumulative radioactivity to 72 hours.

Source: Himmelstein (2010d)

1.2 Biotransformation

In the pilot metabolism study by Himmelstein (2010a) described in section 1.1 above, rats received a single gavage dose of ^{14}C-labelled aminocyclopyrachlor at 0 mg/kg bw (one of each sex) or 500 mg/kg bw (three of each sex) and were killed 30 minutes later for the analysis of plasma metabolites by HPLC and LC-MS. Urine and faeces collected until 24 hours after dosing in the pilot mass balance study were also analysed for metabolites. Aminocyclopyrachlor was the only compound detected in plasma, urine and faeces.

Table 8. Mean radioactivity in selected tissues 72 hours after a single oral dose of 25 mg/kg bw of ^{14}C-labelled aminocyclopyrachlor-methyl

	Mean radioactivity (% of administered dose)	
Tissue	**Males**	**Females**
Carcass[a]	0.088 5	0.080 5
Skin	0.021 0	0.017 1
Whole blood	0.002 6	0.002 5
Bone marrow[b]	< LOD	< LOD
Brain	0.000 3	0.000 5
Fat	0.014 9	0.015 3
Heart	0.000 2	0.000 3
Lungs	0.000 4	0.000 5
Spleen	< LOD	< LOD
Liver	0.003 8	0.003 5
Kidney	0.000 8	0.001 2
Gastrointestinal tract	0.001 9	0.002 1
Gastrointestinal tract contents	0.013 1	0.016 6
Pituitary	< LOD	< LOD
Thyroid	< LOD	< LOD
Thymus	0.000 2	< LOD
Ovaries	—	< LOD
Testes	0.000 4	—
Pancreas	0.000 2	0.000 2
Adrenals	NA	< LOD
Uterus	—	< LOD
Muscle	0.015 0	0.014 7
Bladder	0.000 3	< LOD
Bone	0.002 3	0.002 7
Total	**0.076 5**	**0.071 5**

LOD: limit of detection; NA: not available

[a] Percentage of dose recovered in carcass is not included in the total.

Source: Himmelstein (2010d)

In the study in rats by Himmelstein (2010b) described in section 1.1 above, the only metabolite detected in plasma, urine and faeces was the free acid form of aminocyclopyrachlor.

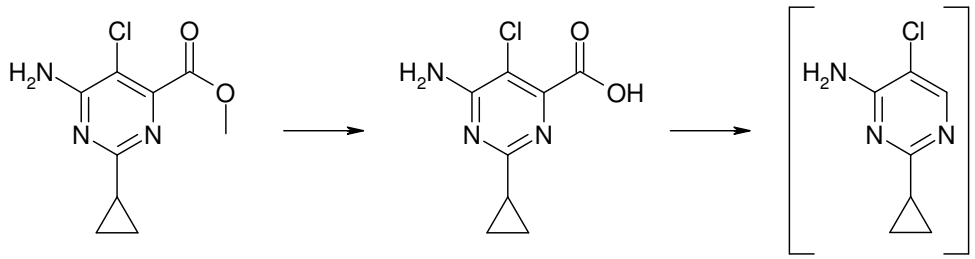
In the study in rats by Himmelstein (2010c) described in section 1.1 above, metabolites were analysed in urine, faeces and bile collected at various times up to 288 hours after the commencement of dosing. Only parent aminocyclopyrachlor was detected. On this basis, aminocyclopyrachlor is not metabolized to any great extent in rats.

In the study in rats by Himmelstein (2010d) described in section 1.1 above, the analysis of urine, faeces and bile for potential metabolites of aminocyclopyrachlor-methyl detected

aminocyclopyrachlor, the ester cleavage product of aminocyclopyrachlor-methyl, as the predominant metabolite. A very low level of aminocyclopyrachlor-methyl was quantifiable in urine (0.10% and 0.08% of the administered dose in males and females, respectively) and bile (0.003% of the administered dose in males) shortly (0–6 hours) after dosing, but at no other times. No additional metabolism of aminocyclopyrachlor-methyl was evident under the conditions of the current study.

The proposed metabolic pathway of aminocyclopyrachlor-methyl in rats is shown in Fig. 3, noting that low levels of the compound designated IN-LXT69 were detected in 90-day rat (Mawn, 2010a; Anand, 2011c) and dog (Mawn, 2008b; Luckett, 2011) studies only.

Fig. 3. Proposed metabolic pathway of aminocyclopyrachlor-methyl in rats



DPX-KJM44
Aminocyclopyrachlor-methyl

DPX-MAT28
Aminocyclopyrachlor

IN-LXT69 *

* Small amounts detected in rats and dogs, only following repeated dietary exposure.

2. Toxicological studies

2.1 Acute toxicity

The results of tests of the acute toxicity of aminocyclopyrachlor and aminocyclopyrachlor-methyl, including skin and eye irritation and skin sensitization studies, are summarized in Table 9. There were no deaths in any of these studies. In the oral dosing study with aminocyclopyrachlor, one female rat had diarrhoea the day after dosing (Carpenter, 2011a), but otherwise no clinical signs were observed in any of the studies. There was no effect on body weight, and there were no gross lesions.

2.2 Short-term studies of toxicity

Mice

In a 90-day toxicity study, aminocyclopyrachlor (90.5% purity) was admixed in the diet and fed ad libitum to groups of 15 Crl:CD1(ICR) mice of each sex at a dietary concentration of 0, 300, 1000, 3000 or 7000 parts per million (ppm). The mean intakes of aminocyclopyrachlor were 0, 47, 154, 459 and 1088 mg/kg bw per day for males and 0, 61, 230, 649 and 1629 mg/kg bw per day for females, respectively. Deaths, clinical signs, body weight and feed consumption were recorded throughout the dosing period. Ophthalmoscopy was performed prior to the commencement of dosing and at the end of the study. Five mice of each sex per group were killed on day 60, and blood was collected for the analysis of aminocyclopyrachlor metabolites. In the remaining mice, blood was sampled at the end of the exposure period for the analysis of standard haematological parameters and total protein. Survivors were killed after approximately 90 days and necropsied, organs were weighed and histopathology was performed.

Plasma metabolite analysis revealed only parent aminocyclopyrachlor, with metabolite IN-LXT69 below the limit of quantification (LOQ) of 10 ng/mL. The mean plasma concentration of

aminocyclopyrachlor was slightly higher in males than in females at all dose levels, with the highest concentrations reaching 2484 and 2313 ng/mL at 7000 ppm in males and females, respectively.

Table 9. Results of studies of the acute toxicity of aminocyclopyrachlor and aminocyclopyrachlor-methyl

Species	Strain	Sex	Route	Purity (%)	Vehicle	LD_{50} (mg/kg bw) or LC_{50} (mg/L)	Reference
Aminocyclopyrachlor							
Rat	Crl:CD(SD)	F	Oral	92.2	Deionized water	> 5 000	Carpenter (2011a)
Rat	Crl:CD(SD)	M + F	Dermal	92.2	Deionized water	> 5 000	Carpenter (2011b)
Rat	Crl:CD(SD)	M + F	Inhalation MMAD = 3.2–3.7 µm	90.5	Air	> 5.4	Anand (2011a)
Rabbit	NZW	M	Skin irritation	90.5	Deionized water	Not irritating	Carpenter (2011c)
Rabbit	NZW	M	Eye irritation	90.5	None	Not irritating	Carpenter (2011d)
Mouse	CBA/JHsd	F	LLNA	90.5	DMF	Not sensitizing	Carpenter (2011e)
Aminocyclopyrachlor-methyl							
Rat	Crl:CD(SD)	F	Oral	96.9	0.5% (w/v) aqueous methyl cellulose	> 5 000	Carpenter (2007a)
Rat	Crl:CD(SD)	M + F	Dermal	96.9	Deionized water	> 5 000	Carpenter (2007b)
Rabbit	NZW	M	Skin irritation	96.9	Deionized water	Not irritating	Carpenter (2007c)
Rabbit	NZW	M	Eye irritation	96.9	None	Not irritating	Carpenter (2007d)
Mouse	CBA/JHsd	F	LLNA	96.9	DMSO	Not sensitizing	Carpenter (2011e)

DMF: *N*,*N*-dimethylformamide; DMSO: dimethyl sulfoxide; F: female; LC_{50}: median lethal concentration; LD_{50}: median lethal dose; LLNA: local lymph node assay; M: male; MMAD: mass median aerodynamic diameter; NZW: New Zealand White; w/v: weight per volume

There was no treatment-related effect on any recorded or analysed parameter. The no-observed-adverse-effect level (NOAEL) was 7000 ppm (equal to 1088 mg/kg bw per day for males and 1629 mg/kg bw per day for females), the highest dietary concentration tested (Mawn, 2008a; Anand, 2011b).

Rats

Aminocyclopyrachlor (90.5% purity) was admixed in the diet and fed ad libitum to groups of Crl:CD(SD) rats (15 of each sex per group) at a dietary concentration of 0, 600, 2000, 6000 or 18 000 ppm for 94 or 95 days (males) or 96 or 97 days (females). The mean intakes of

aminocyclopyrachlor were 0, 35, 114, 349 and 1045 mg/kg bw per day for males and 0, 45, 146, 448 and 1425 mg/kg bw per day for females at 0, 600, 2000, 6000 and 18 000 ppm, respectively. Observations for mortalities and clinical signs were made daily. Body weight and feed consumption were recorded weekly. Ophthalmoscopy was performed prior to the commencement of dosing and at the end of the study. Five rats of each sex per group were terminated on day 56, and blood was collected for the analysis of aminocyclopyrachlor metabolites. A functional observational battery and motor activity assessment were performed prior to the commencement of dosing and during weeks 4, 8 and 13. Blood and urine were sampled at the end of the exposure period for the analysis of haematological, clinical chemistry or urine analysis parameters. Survivors were killed after approximately 90 days and necropsied, organs were weighed and histopathology was performed. The latter also included an analysis of the brain, central nerves and peripheral nerves.

Parent aminocyclopyrachlor was the most abundant analyte detected in plasma, reaching 10 268 ng/mL in males and 13 428 ng/mL in females (LOQ = 10 ng/mL) at the highest dietary concentration of 18 000 ppm. Relatively low concentrations of the metabolite IN-LXT69 were detected at the same dietary concentration (92 ng/mL in males and 80 ng/mL in females; LOQ = 10 ng/mL), but not at lower doses.

There were no deaths and no treatment-related clinical signs. At 18 000 ppm, mean body weight of males was up to 9% lower ($P < 0.05$) than the control values from day 28 to day 91 of treatment. In females, mean body weight was up to 8% ($P < 0.05$) lower than the control values at days 56, 70 and 77. At this same dietary concentration, mean body weight gain from day 0 to day 91 was significantly lower ($P < 0.05$) than the control values (15% and 18% lower than the control values in males and females, respectively). Whereas mean feed consumption was generally comparable across all groups, overall (i.e. days 0–91) mean feed conversion efficiency was 11% and 17% lower ($P < 0.05$) than the control values in high-dose males and females, respectively. There was no treatment-related effect on body weight parameters at lower doses. There were no treatment-related ophthalmological abnormalities and no effects on the functional observational battery or motor activity assessment. Haematological, clinical chemistry and urine analysis parameters were unremarkable. There were no treatment-related macroscopic, organ weight or histopathological findings.

The NOAEL was 6000 ppm (equal to 349 and 448 mg/kg bw per day for males and females, respectively), based on reduced body weight, body weight gain and feed conversion efficiency at 18 000 ppm (equal to 1045 and 1425 mg/kg bw per day for males and females, respectively) (Mawn, 2010a; Anand, 2011c).

Aminocyclopyrachlor-methyl (90.5% purity) was admixed in the diet and fed ad libitum to groups of Crl:CD(SD) rats (10 of each sex per group) at a dietary concentration of 0, 600, 2000, 6000 or 18 000 ppm for 94 or 95 days (males) or 95 or 96 days (females). The mean intakes of aminocyclopyrachlor-methyl were 0, 35, 117, 347 and 1022 mg/kg bw per day for males and 0, 43, 133, 405 and 1219 mg/kg bw per day for females at 0, 600, 2000, 6000 and 18 000 ppm, respectively. These doses are equal to 0, 33, 110, 326 and 961 mg ae/kg bw per day for males and 0, 40, 125, 381 and 1146 mg ae/kg bw per day for females, respectively.

Observations for mortalities and clinical signs were made daily. Body weight and feed consumption were recorded weekly. Ophthalmoscopy was performed prior to the commencement of dosing and at the end of the study. Blood was collected from five rats of each sex per group on days 60 and 87 for the analysis of aminocyclopyrachlor metabolites. A functional observational battery and motor activity assessment were performed prior to the commencement of dosing and during weeks 4, 8 and 13. Blood and urine were sampled at the end of the exposure period for the analysis of haematological, clinical chemistry or urine analysis parameters. Survivors were killed after approximately 90 days (days 94–95 and 95–96 for males and females, respectively) and necropsied, organs were weighed and histopathology was performed. The latter also included an analysis of the brain, central nerves and peripheral nerves.

Metabolite analysis revealed no parent compound above the LOQ of 10 ng/mL. Aminocyclopyrachlor was the main plasma metabolite, reaching maximum concentrations of 20 660 and 29 784 ng/mL at 18 000 ppm in males and females, respectively (LOQ = 10 ng/mL). Plasma concentrations of the metabolite IN-LXT69 at this same dose were 439 and 415 ng/mL in males and females, respectively (LOQ = 10 ng/mL).

There were no deaths and no treatment-related clinical signs. At 18 000 ppm, mean body weight was 5–9% lower than the control value in males and 5–11% lower than the control value in females, the difference being statistically significant ($P < 0.05$) in females from days 42 to 84 except day 63. Also at 18 000 ppm, overall body weight gain was 14% and 24% lower ($P < 0.05$) than the control values in males and females, respectively. Concomitant with the reduction in body weight gain was a reduction in feed consumption, which was 9% and 11% lower than the control values ($P < 0.05$) in males and females, respectively, over days 1–91 of exposure. At 18 000 ppm, overall feed conversion efficiency was also lower than the control values (5% lower in males and 15% lower in females), but not statistically significantly so. There was no treatment-related effect on body weight parameters at lower doses.

There were no treatment-related ophthalmological abnormalities and no effects on the functional observational battery or motor activity assessment. Haematological, clinical chemistry and urine analysis parameters were unremarkable. There were no treatment-related macroscopic, organ weight or histopathological findings.

The NOAEL was 6000 ppm (equal to 326 and 381 mg ae/kg bw per day for males and females, respectively), based on reduced body weight, body weight gain and feed consumption at 18 000 ppm (equal to 961 and 1146 mg ae/kg bw per day for males and females, respectively) (Mawn, 2010b; Anand, 2011d).

Dogs

Aminocyclopyrachlor (90.5% purity) was admixed in the diet at a concentration of 0, 250, 1250, 5000 or 15 000 ppm, and 400 g food was offered daily to groups of Beagle dogs (four of each sex) for 90 days. The mean intakes of aminocyclopyrachlor were 0, 6.5, 33, 126 and 426 mg/kg bw per day for males and 0, 7.0, 38, 124 and 388 mg/kg bw per day for females at 0, 250, 1250, 5000 and 15 000 ppm, respectively. Dogs were observed twice daily for mortality and clinical signs, with a more detailed clinical examination performed weekly. A neurobehavioural assessment was performed weekly, with observations covering changes in activity, gait, posture, strength and response to handling, as well as the presence of clonic or tonic movements, stereotypies (e.g. excessive grooming, repetitive circling) or bizarre behaviour (e.g. self-mutilation). Body weight was recorded weekly, and feed consumption was recorded daily. Ophthalmoscopy was performed prior to the commencement of dosing and at scheduled termination. Blood and urine were sampled prior to the commencement of dosing and during weeks 4, 8 and 12 for the analysis of haematological, clinical chemistry or urine analysis parameters. Additional blood samples were collected during week 9 for the analysis of aminocyclopyrachlor metabolites. All dogs were killed at the end of the study and necropsied, and their organs were weighed. Tissues were collected and examined histopathologically. Samples of liver were analysed for cytochrome P450 enzymes.

The main plasma analyte was aminocyclopyrachlor, with higher concentrations present in female dogs compared with male dogs at the same dietary concentration of 15 000 ppm (82 575 ng/mL versus 63 000 ng/mL, respectively; LOQ = 10 ng/mL). IN-LXT69 was detected in male and female dogs only at 15 000 ppm at concentrations marginally above the LOQ of 10 ng/mL (i.e. 12 ng/mL).

There were no deaths and no treatment-related clinical signs or neurobehavioural findings. Body weight and feed consumption were unaffected by treatment. Ophthalmoscopy was unremarkable. There was no treatment-related effect on haematological, clinical chemistry or urine analysis parameters. There were no macroscopic or microscopic findings that were attributable to treatment. Total hepatic cytochrome P450 levels were unaffected by treatment. However, concentrations of cytochrome P450 enzymes CYP1A1 and CYP2B1/2 were increased 5- and 2-fold,

respectively, in male dogs at 5000 and 15 000 ppm (Table 10). In females, a 2-fold increase in the concentration of CYP2B1/2 occurred at 5000 ppm, with a 2-fold increase in CYP2E1 at 1250 ppm (Table 10).

Table 10. Liver enzyme analysis in dogs exposed to aminocyclopyrachlor via the diet for 90 days

	Mean (± SD) content (net intensity) (nmol/mg microsomal protein)				
Cytochrome	**0 ppm**	**250 ppm**	**1 250 ppm**	**5 000 ppm**	**15 000 ppm**
Males					
Total P450[a]	0.407 ± 0.103	0.409 ± 0.115	0.367 ± 0.047	0.320 ± 0.046	0.405 ± 0.163
CYP1A1	173 ± 78	255 ± 212	Not detected	**869 ± 279***	899 ± 502
CYP1A2	Not detected	Not detected	Not detected	Not detected	Not detected
CYP2B1/2	6 466 ± 195	8 097 ± 303	6 673 ± 195	**14 537 ± 687***	12 333 ± 300
CYP2E1	774 ± 526	890 ± 300	709 ± 215	1121 ± 0	487 ± 5
CYP3A2	14 061 ± 7 742	8 054 ± 4 453	10 164 ± 4 440	8 635 ± 4 759	10 505 ± 6 058
CYP4A1/2/3	110 960 ± 5 614	109 584 ± 8 517	100 190 ± 2 079	102 521 ± 6 806	**99 175 ± 4 382***
Females					
Total P450[a]	0.421 ± 0.060	0.396 ± 0.078	0.378 ± 0.042	0.394 ± 0.049	0.415 ± 0.054
CYP1A1	781 ± 204	835 ± 407	1 086 ± 327	**979 ± 270***	1 033 ± 176
CYP1A2	Not detected	Not detected	Not detected	Not detected	Not detected
CYP2B1/2	13 086 ± 651	7 477 ± 314	6 963 ± 610	**25 237 ± 583***	17 513 ± 865
CYP2E1	481 ± 120	693 ± 92	**900 ± 194***	594 ± 300	523 ± 211
CYP3A2	19 631 ± 9 223	19 028 ± 12 474	20 628 ± 5 434	23 226 ± 3 427	22 411 ± 2 447
CYP4A1/2/3	10 4017 ± 6 971	111 144 ± 1 494	**117 163 ± 5 892***	113 233 ± 5 705	**120 065 ± 1 655***

ppm: parts per million; SD: standard deviation; *: $P < 0.05$ (statistically significant results are in boldface type)

Source: Mawn (2008b); Luckett (2011)

The NOAEL was 15 000 ppm (equal to 426 mg/kg bw per day for males and 388 mg/kg bw per day for females), the highest dietary concentration tested (Mawn, 2008b; Luckett, 2011).

Aminocyclopyrachlor (90.5% purity) was admixed in the diet at a concentration of 0, 1250, 5000, 15 000 or 30 000 ppm, and 400 g of food was offered daily to groups of Beagle dogs (four of each sex) for 1 year. Mean daily intakes were 0, 38, 178, 465 and 1077 mg/kg bw per day for males and 0, 47, 175, 542 and 1073 mg/kg bw per day for females at 0, 1250, 5000, 15 000 and 30 000 ppm, respectively. Dogs were observed twice daily for mortalities and clinical signs. Body weight was recorded weekly. Feed consumption was recorded daily. Ophthalmoscopy was performed prior to the commencement of dosing and prior to termination. Blood and urine were sampled prior to the commencement of dosing and on days 90, 188 and 366 for the analysis of haematological, clinical chemistry or urine analysis parameters. Following termination, dogs were necropsied and their organs weighed. Tissues were collected from the high-dose and control groups for histopathological examination, with potential target tissues examined at lower doses as necessary.

There were no deaths and no treatment-related clinical signs. Body weight and feed consumption were unaffected by treatment. Ophthalmoscopy was unremarkable. There was no

treatment-related effect on any haematological, clinical chemistry or urine analysis parameters. There were no treatment-related macroscopic abnormalities or effects on organ weights. Histopathological examination revealed an increase in hepatic vacuolation, consistent with glycogen accumulation, in treated females (0/4, 1/4, 0/4, 1/4 and 2/4 dogs at 0, 1250, 5000, 15 000 and 30 000 ppm, respectively). The vacuolation was graded as minimal (Grade 1) with the exception of the vacuolation in one high-dose female, which was graded as slight (Grade 2). In the absence of similar findings in males, changes in clinical chemistry parameters indicative of liver toxicity or a dose-related increase in severity, this finding was not considered adverse.

The NOAEL was 30 000 ppm (equal to 1077 and 1073 mg/kg bw per day for males and females, respectively), the highest dietary concentration tested (Han, 2010).

2.3 Long-term studies of toxicity and carcinogenicity

Mice

Aminocyclopyrachlor (90.5% purity) was admixed in the diet at a concentration of 0, 300, 1000, 3000 or 7000 ppm and fed ad libitum to groups of 60 male and 60 female Crlj:CD1 (ICR) mice for 18 months. The mean intakes of aminocyclopyrachlor were 0, 39, 133, 393 and 876 mg/kg bw per day for males and 0, 50, 171, 527 and 1190 mg/kg bw per day for females at 0, 300, 1000, 3000 and 7000 ppm, respectively. Observations for mortalities and clinical signs were made at least once daily. Body weight and feed consumption were recorded weekly to week 13 and every 2 weeks thereafter. Ophthalmological examinations were made prior to the commencement of dosing and prior to scheduled termination. Blood was sampled at 12 and 18 months for haematological analysis. Following termination, mice were necropsied, organs weighed and tissues examined histopathologically.

There was no treatment-related effect on any parameter, and there were no neoplastic or non-neoplastic changes at any dose. On this basis, the NOAEL for chronic toxicity and carcinogenicity was 7000 ppm (equal to 876 mg/kg bw per day for males and 1190 mg/kg bw per day for females), the highest dietary concentration tested (Huh, 2010).

Rats

Aminocyclopyrachlor (90.5% purity) was admixed in the diet at a concentration of 0, 600, 2000, 6000 or 18 000 ppm and fed ad libitum to groups of Crl:CD(SD) rats (80 of each sex per group) for 2 years. Mean daily intakes of aminocyclopyrachlor were 0, 27, 97, 279 and 892 mg/kg bw per day for males and 0, 29, 100, 309 and 957 mg/kg bw per day for females at 0, 600, 2000, 6000 and 18 000 ppm, respectively. Satellite groups of 10 rats of each sex per group were killed after 1 year. Rats were observed at least twice daily for mortalities and clinical signs. Body weight and feed consumption were recorded weekly to week 13 and every 2 weeks thereafter. Ophthalmoscopy was performed prior to the commencement of dosing and after 1 (satellite groups) and 2 years. Blood and urine were collected at approximately 12, 18 and 24 months for the analysis of haematological, clinical chemistry or urine analysis parameters. For the satellite groups, blood and urine were collected at 26 weeks and 12 months. Following scheduled termination after 1 or 2 years, rats were necropsied and their organs weighed. Histopathology was performed on the standard range of tissues.

There were no treatment-related deaths or clinical signs. At the highest dose, mean body weight and body weight gain were significantly lower ($P < 0.01$ or 0.05) than the control values in both sexes (Table 11). Also at the highest dose, feed consumption (females) and feed conversion efficiency (both sexes) were significantly lower than the control values ($P < 0.01$ or 0.05) (Table 11). There were no treatment-related ophthalmological abnormalities or haematology, clinical chemistry, urine analysis, organ weight or macroscopic findings.

Microscopic examination of rats killed after 2 years of exposure revealed an increased incidence of brain tumours in high-dose males (Table 12); no brain tumours or preneoplastic lesions were observed in satellite groups of rats killed after 1 year. Statistical analysis using the Cochran-

Armitage trend test determined that this increase was significant ($P < 0.05$); however, analyses using the Peto and Fisher exact tests detected no significance. A single undifferentiated glioma was observed in one high-dose male, and an oligodendroglioma was observed in one 6000 ppm male. Two granular cell tumours were also observed in 18 000 ppm males. The incidence of combined glial tumours was significantly increased ($P < 0.05$) in high-dose males using either the Cochran-Armitage trend test or Peto analysis, but not by the Fisher exact test. In females, no significant difference in the incidence of brain tumours was found.

Table 11. Effect of 2 years of dietary exposure to aminocyclopyrachlor on body weight and feed parameters in rats

	Mean value (% of control)				
Parameter	**0 ppm**	**600 ppm**	**2 000 ppm**	**6 000 ppm**	**18 000 ppm**
Mean body weight (g)					
Males					
Day 0	216.3	216.2 (100%)	216.3 (100%)	**210.4**** (97%)	214.2 (99%)
Day 91	565.6	571.3 (101%)	567.3 (100%)	555.0 (98%)	**536.0**** (95%)
Day 357	791.8	796.1 (101%)	805.2 (102%)	803.1 (101%)	**737.3**** (93%)
Day 721	877.6	879.3 (100%)	856.2 (98%)	840.9 (96%)	788.5 (90%)
Females					
Day 0	158.7	158.6 (100%)	157.1 (99%)	**155.0*** (98%)	**154.2**** (97%)
Day 91	298.8	302.4 (101%)	303.6 (102%)	300.3 (101%)	**286.6**** (96%)
Day 357	426.5	427.0 (100%)	431.6 (101%)	424.8 (100%)	**394.0*** (92%)
Day 721	535.2	538.7 (101%)	533.3 (100%)	518.8 (97%)	487.3 (91%)
Mean body weight gain (g)					
Males					
Days 0–91	349.2	355.1 (102%)	351.0 (101%)	344.6 (99%)	**321.8**** (92%)
Days 0–357	576.1	579.9 (101%)	589.2 (102%)	592.9 (103%)	**523.3**** (91%)
Days 0–721	664.4	664.9 (100%)	641.3 (97%)	633.0 (95%)	577.0 (87%)
Females					
Days 0–91	140.2	143.8 (103%)	146.5 (104%)	145.3 (104%)	**132.4*** (94%)
Days 0–357	267.9	268.6 (100%)	274.3 (102%)	269.8 (101%)	**239.7*** (89%)
Days 0–721	379.5	382.3 (101%)	374.9 (99%)	360.2 (95%)	332.7 (88%)
Feed consumption (g)					
Males					
Days 0–91	24.3	24.6 (101%)	24.6 (101%)	24.3 (100%)	23.8 (98%)
Days 0–357	24.7	24.7 (100%)	25.0 (101%)	24.5 (99%)	24.3 (98%)
Days 0–721	24.6	24.6 (100%)	25.3 (103%)	23.8 (103%)	24.3 (99%)
Females					
Days 0–91	16.5	16.4 (99%)	16.5 (100%)	16.7 (101%)	**16.1*** (98%)
Days 0–357	16.4	16.2 (99%)	16.3 (99%)	16.5 (101%)	16.2 (99%)
Days 0–721	16.7	16.8 (101%)	16.9 (101%)	17.1 (102%)	16.9 (101%)

Table 11 (continued)

Parameter	Mean value (% of control)				
	0 ppm	600 ppm	2 000 ppm	6 000 ppm	18 000 ppm
Feed conversion efficiency (g body weight gain/g feed consumed)					
Males					
Days 0–91	15.7	15.9 (101%)	15.7 (100%)	15.6 (99%)	**14.8**** (94%)
Days 0–357	6.6	6.6 (100%)	6.6 (100%)	6.8 (103%)	**6.0**** (91%)
Days 0–721	3.8	3.7 (97%)	3.5 (92%)	3.7 (97%)	3.3 (87%)
Females					
Days 0–91	9.3	9.6 (103%)	9.7 (104%)	9.6 (103%)	9.1 (98%)
Days 0–357	4.6	4.6 (100%)	4.7 (102%)	4.6 (100%)	**4.2*** (91%)
Days 0–721	3.1	3.1 (100%)	3.1 (100%)	2.9 (94%)	2.7 (87%)

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (statistically significant results are in boldface type)
Source: Moon (2010)

Age- and sex-matched historical control data from four other studies ($n = 267$) conducted by the performing laboratory between 2006 and 2009 indicated that the mean incidence of astrocytomas was 1.1% (range 0–1.5%), and the mean incidence of total glial neoplasms was 3.0% (range 0–4.4%). The incidence of these neoplasms in the current study was marginally outside of this range. A comparison with a more extensive historical control database from Charles River Laboratories, which supplied the rats for the study, indicated that the incidence of astrocytoma was at the historical control maximum. An analysis of published historical control data by the study author indicated that the control maximum for the incidence of astrocytoma was up to 11%.

The increased incidence of brain tumours in high-dose males was not considered treatment related on the basis of the following considerations:

- Microscopic analysis of brains from both males and females detected no preneoplastic changes, including glial cell proliferation.
- There was no decrease in the latency of brain tumour development in either sex.
- The higher survival to termination in high-dose males (36/70 versus 27/70 in the controls) increases the probability of the development of age-related neoplasms.
- A similar pattern of increase did not occur in females.
- The incidence of astrocytoma was well within the published historical control range.
- A pathology working group convened by the sponsor concluded that the slight difference in the incidence of astrocytomas in high-dose males was not treatment related (Hardisty, 2010).
- Aminocyclopyrachlor was negative in the standard battery of genotoxicity tests.

The NOAEL for chronic toxicity was 6000 ppm (equal to 279 mg/kg bw per day for males and 309 mg/kg bw per day for females), based on reduced body weight, body weight gain, feed consumption and feed conversion efficiency at 18 000 ppm (equal to 892 mg/kg bw per day for males and 957 mg/kg bw per day for females). The NOAEL for carcinogenicity was 18 000 ppm (equal to 892 mg/kg bw per day for males and 957 mg/kg bw per day for females), the highest dietary concentration tested (Moon, 2010).

2.4 Genotoxicity

The results of genotoxicity studies on aminocyclopyrachlor or aminocyclopyrachlor-methyl are summarized Table 13.

Table 12. Incidence of brain tumours in rats after 2 years of dietary exposure to aminocyclopyrachlor

	Number of rats with finding (% incidence)					CRL historical control incidence	
Parameter	**0 ppm**	**600 ppm**	**2 000 ppm**	**6 000 ppm**	**18 000 ppm**	**Mean**	**Range**
Males							
N	*70*	*70*	*70*	*70*	*69*	*2 146*	–
Astrocytoma[M]	0	0	0	1	**3*** (4.35%)	1.21%	0–4.29%
Glioma, undifferentiated[M]	0	0	0	0	1 (1.45%)	0.14%	0–1.92%
Oligodendroglioma[M]	0	0	0	1	0	0.14%	0–2%
Granular cell tumour[B]	0	0	0	0	2 (2.90%)	0.37%	0–2%
Combined glial tumours	0	0	0	2	**4*** (5.8%)	–	–
Females							
N	*70*	*70*	*70*	*70*	*70*	*2 344*	–
Astrocytoma[M]	0	2 (2.9%)	1 (1.4%)	0	2 (2.8%)	0.38%	0–2.31%
Glioma, undifferentiated[M]	0	0	1[a] (1.4%)	0	0	0.04%	0–1.43%
Oligodendroglioma[M]	0	0	0	0	1	0.21%	0–2.00%
Granular cell tumour[B]	0	0	0	1	0	0.21%	0–2.00%
Combined glial tumours	0	2 (2.9%)	2 (2.9%)	0	2 (2.9%)	–	–

B: benign; CRL: Charles River Laboratories; M: malignant; ppm: parts per million; *: $P < 0.05$ (statistically significant results are in boldface type)

[a] Alternatively diagnosed as a pineal carcinoma (non-glial tumour).

Source: Moon (2010)

2.5 Reproductive and developmental toxicity

(a) Single or multigeneration studies

Rats

In a one-generation study, aminocyclopyrachlor-methyl (99.7% purity) was admixed in the diet at a concentration of 0, 600, 5000 or 17 000 ppm and fed ad libitum to Crl:CD®(SD)IGS BR rats (10 rats of each sex per concentration) through premating, mating, gestation and lactation. Observations for mortalities and clinical signs were made daily, with a more detailed clinical assessment undertaken weekly. Body weight and feed consumption were recorded weekly during the premating period and for dams on days 0, 7, 14 and 21 of gestation and lactation. A functional observational battery and a motor activity assessment were undertaken in F_0 rats prior to the commencement of dosing and at day 27 (males) or 28 (female) of the premating exposure period. Blood was sampled from five F_0 rats of each sex per group on day 29 for the analysis of

haematological and clinical chemistry parameters, with blood also sampled on days 60 (males) and 74–90 (females) for the analysis of coagulation parameters. Following weaning, F_1 body weights and feed consumption were recorded weekly until sexual maturity. Litter evaluations were made on postnatal days 0, 4, 7, 14 and 21. The time of vaginal patency or preputial separation was recorded. Following scheduled termination, all parental rats were necropsied, and the following organs were weighed: testes, ovaries (with oviducts), pituitary gland, epididymides, uterus (with cervix), adrenal glands, prostate, liver and seminal vesicles (with coagulating glands and their fluids). These organs, in addition to the thyroid, vagina, pancreas and any gross lesions, were examined histopathologically in five rats of each sex in the control and high-dose groups. In one male and one female F_1 weanling from each litter, the pancreas, thyroid and any gross lesions were examined histopathologically.

Table 13. Results of genotoxicity assays on aminocyclopyrachlor or aminocyclopyrachlor-methyl

End-point	Test object	Concentration or dose and vehicle	Purity (%)	Results	Reference
Aminocyclopyrachlor					
Gene mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537 *Escherichia coli* WP2 uvrA	1.5–5 000 µg/plate (±S9) DMSO vehicle	90.5	Negative	Wagner & VanDyke (2011)
Gene mutation	CHO cells	750–2 150 µg/mL (±S9) DMSO vehicle	90.5	Negative	Clarke (2011)
Cytogenetic test	Human lymphocytes	267–2 136 µg/mL (±S9) DMSO vehicle	90.5	Negative	Gudi & Rao (2011)
In vivo micronucleus	Crl:CD1(ICR) mice (bone marrow)	500, 1 000 or 2 000 mg/kg bw	90.5	Negative	Krsmanovic & Huston (2007)
Aminocyclopyrachlor-methyl					
Gene mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537 *E. coli* WP2 uvrA	100–5 000 µg/plate (±S9) DMSO vehicle	95.3	Negative	Ford (2005)

bw: body weight; CHO: Chinese hamster ovary; DMSO: dimethyl sulfoxide; S9: 9000 × *g* supernatant fraction from rat liver homogenate

The mean dietary intakes of aminocyclopyrachlor-methyl are summarized in Table 14.

There were no treatment-related deaths or clinical signs. Mean body weight, body weight gain, feed consumption and feed conversion efficiency were unaffected by treatment of F_0 parental males during premating exposure. In F_0 females, there were no significant intergroup differences in mean body weight during premating. Mean body weight gain from days 0 to 28 of premating exposure was 35% lower than the control value in high-dose F_0 females, but this difference was not statistically significant. Feed consumption by high-dose F_0 females was significantly lower ($P < 0.05$) than the control values on premating days 0–7 (12% lower), 7–14 (15% lower) and 0–28 (11% lower), but there was no difference in feed conversion efficiency. During gestation, there were no significant intergroup differences in mean body weight, body weight gain, feed consumption or feed conversion efficiency in F_0 dams. During lactation, mean body weight was unaffected by treatment in F_0 dams, whereas there was a significant increase ($P < 0.05$) in mean body weight gain in high-

dose dams (26.4 versus 0.7 g in the controls). There were no significant differences in feed consumption or feed conversion efficiency during lactation.

Table 14. Mean intakes of aminocyclopyrachlor-methyl in a one-generation reproduction study in rats

	Mean intake (mg/kg bw per day)[a]		
Group	**600 ppm**	**5 000 ppm**	**17 000 ppm**
Males			
F_0 premating	38 (36)	304 (285)	1 059 (994)
F_1 (weaning to termination)	60 (56)	491 (461)	1 697 (1 592)
Females			
F_0 premating	44 (41)	352 (330)	1 190 (1 118)
F_0 gestation	52 (49)	346 (325)	1 199 (1 125)
F_0 lactation	77 (72)	681 (629)	2 340 (2 196)
F_1 (weaning to termination)	63 (59)	518 (486)	1 787 (1 677)

bw: body weight; F_0: parental generation; F_1: first filial generation; ppm: parts per million

[a] Doses are expressed as aminocyclopyrachlor-methyl, with the equivalent dose of aminocyclopyrachlor acid equivalents contained in parentheses.

Source: Lewis (2006)

There were no treatment-related effects observed during the functional observational battery in F_0 parental rats, and there was no treatment-related effect on motor activity. Haematological and clinical chemistry parameters were unaffected by treatment.

There was no treatment-related effect on any reproduction or litter parameter or developmental landmarks. At the highest dose, mean pup weight was lower than the control values on days 7, 14 and 21 of lactation (6%, 9% and 17% lower than the control values, respectively), with the reductions on days 14 and 21 being statistically significant ($P < 0.05$).

In F_1 male weanlings at the highest dose, mean body weight was significantly lower ($P < 0.05$) than the control values on post-weaning days 0, 7, 14 and 21 (up to 17% lower than the control values), whereas there was no significant difference in body weight gain. Mean feed consumption was significantly lower ($P < 0.05$) than the control values at the highest dose on post-weaning days 0–7, 14–21, 21–28 and 0–39 (up to 17% lower than the control values), but there were no differences in feed conversion efficiency. Feed consumption was also significantly lower ($P < 0.05$) than the control values at 5000 ppm on post-weaning days 0–7 and 14–21; however, in the absence of any corresponding effects on body weight gain, these differences were not considered adverse. There were no intergroup differences in feed conversion efficiency in F_1 male weanlings.

In high-dose F_1 female weanlings, mean body weight was significantly lower ($P < 0.05$) than the control values on post-weaning days 0 and 7 (15% and 11% lower, respectively), whereas there was no significant difference in body weight gain. At the highest dose, overall feed consumption (postnatal days 0–39) was significantly lower ($P < 0.05$) than the control value (8% lower), but there was no effect on feed conversion efficiency.

There were no treatment-related organ weight, macroscopic or histopathological findings.

The NOAEL for reproductive toxicity was 17 000 ppm (equal to 994 mg ae/kg bw per day for males and 1118 mg ae/kg bw per day for females), the highest dietary concentration tested. The

NOAEL for both parental toxicity and offspring toxicity was 5000 ppm (equal to 285 mg ae/kg bw per day for males and 330 mg ae/kg bw per day for females) for reduced body weight or pup weight, body weight gain and feed consumption at 17 000 ppm (equal to 994 mg ae/kg bw per day for males and 1118 mg ae/kg bw per day for females) (Lewis, 2006).

Aminocyclopyrachlor (90.5%) was admixed in the diet at a concentration of 0, 500, 1500, 5000 or 17 000 ppm and fed ad libitum to two parental generations of Crl:CD(SD) rats (28 of each sex per group) and their offspring. Observations for mortalities and clinical signs were made daily. Body weight and feed consumption were recorded weekly during the premating period and for dams on days 0, 1, 14 and 21 of gestation and lactation. Litter evaluations were made on postnatal days 0, 4, 7, 14 and 21. Standard reproduction parameters were calculated. Sperm parameters for all F_0 and F_1 parental males were evaluated. Samples were collected from all F_0 and F_1 females from 3 weeks prior to mating until the end of the cohabitation period for the evaluation of the estrous cycle. A quantitative evaluation of primordial and growing follicles was conducted on 10 lactating F_1 control and high-dose females. Following scheduled termination, all parental rats were necropsied, and the following organs were weighed: testes, ovaries (with oviducts), liver, right cauda epididymides, uterus (with cervix), kidneys, spleen, seminal vesicles (with coagulating glands), brain, prostate, pituitary, adrenal glands and thyroid. These same organs were also examined histopathologically.

The mean intakes of aminocyclopyrachlor by both parental generations of rats are summarized in Table 15.

Table 15. Mean intakes of aminocyclopyrachlor in a two-generation study of reproduction in rats

	Mean intake (mg/kg bw per day)			
Group	**500 ppm**	**1 500 ppm**	**5 000 ppm**	**17 000 ppm**
Males				
F_0 premating	30	92	299	1 048
F_1 premating	42	126	426	1 522
Females				
F_0 premating	36	110	367	1 243
F_0 gestation	33	100	331	1 158
F_0 lactation	59	175	599	2 215
F_1 premating	46	141	465	1 666
F_1 gestation	32	102	336	1 192
F_1 lactation	64	189	651	2 243

bw: body weight; F_0: parental generation; F_1: first filial generation; ppm: parts per million

Source: Lewis (2011a)

There were no treatment-related deaths or clinical signs.

In F_0 males, mean body weight and body weight gain were significantly lower ($P < 0.05$) than the control values at 5000 and 17 000 ppm. The magnitude of the body weight reduction ranged from 6% to 7% at 5000 ppm and from 6% to 9% at 17 000 ppm and was evident from week 5. At 5000 ppm, cumulative body weight gain to day 70 was 14% lower than the control value. At 17 000 ppm, cumulative body weight gain to days 70 and 119 was 17% and 13% lower than the control values, respectively. Similar differences in body weight parameters occurred in F_1 males. During premating, mean weekly body weight was significantly lower ($P < 0.05$) than the control values at 5000 ppm (from week 9) and 17 000 ppm (every week), ranging from 7% to 9% and from 12% to

15%, respectively. Mean cumulative body weight gain to day 70 or 105 was 7% or 9% lower, respectively, at 5000 ppm and 13% or 15% lower, respectively, at 17 000 ppm. A similar decrease in body weight and body weight gain did not occur in females at 5000 ppm. As the weight of evidence from all other studies on aminocyclopyrachlor or aminocyclopyrachlor-methyl did not indicate any sex differences, the significant changes in body weight and body weight gain seen in males at 5000 ppm in this study were not considered treatment related.

In F_0 females, mean body weight and body weight gain were significantly lower ($P < 0.05$) than the control value only at 17 000 ppm. During premating, mean body weight was 6–7% lower than the control values over the last 3 weeks of exposure, with cumulative body weight gain (days 0–70) 16% lower than the control value. During gestation, mean body weights were up to 8% lower than the control values on days 7, 14 and 21, with cumulative body weight gain (days 0–21 of gestation) 12% lower than the control value. During the lactation period, mean body weights were 5–9% lower than the control values, with only the difference on days 0 and 7 being statistically significant ($P < 0.05$); overall body weight gain during lactation was comparable to the control value. At 17 000 ppm in F_1 females, mean body weight was up to 14% lower than the control values ($P < 0.05$) on days 0, 7 and 14 and thereafter recovered to be within 7% of the control value by the end of the exposure period. Cumulative body weight gain was within 5% of the control value and not statistically significantly different. At 5000 ppm in F_1 maternal rats, mean body weight was up to 6% lower than the control value, but differences were not statistically significant. There was no treatment-related effect on mean body weight or body weight gain during gestation and lactation in F_1 dams.

During the premating period, feed consumption was unaffected by treatment in F_0 males. At 17 000 ppm, feed conversion efficiency was significantly lower ($P < 0.05$) than the control values throughout the exposure period (up to 24% lower than the control value). Statistically significant differences in feed conversion efficiency occurred inconsistently at lower doses and were considered incidental findings. In F_1 males at 17 000 ppm, mean feed consumption was significantly lower than the control values ($P < 0.05$) during the premating exposure period (5–12% lower), and mean feed conversion efficiency was also significantly ($P < 0.05$) reduced (9–25% lower).

In F_0 females, there was no treatment-related effect on feed consumption during premating, gestation or lactation. At 17 000 ppm, mean feed conversion efficiency over days 0–70 of the premating phase was 12% lower ($P < 0.05$) than the control values, 26% lower ($P < 0.05$) during the first week of gestation and unaffected during lactation. There was no treatment-related effect on feed consumption or feed conversion efficiency in F_1 females during premating, gestation or lactation.

There was no treatment-related effect on sperm parameters, estrous cycling or reproduction parameters. There were no treatment-related clinical signs in pups and no effects on litter parameters. At 17 000 ppm, mean pup weight was significantly lower ($P < 0.05$) than the control values on postnatal days 0, 4, 14 and 21 in the F_1 generation (7–15% lower than the control values) and on postnatal day 21 in the F_2 generation (8% lower than the control value). At 5000 ppm in the F_1 generation, mean pup weight was also significantly lower ($P < 0.05$) than the control value (7% lower). Despite these lower body weights, body weight gain of pups was comparable among the treatment and control groups.

There was no treatment-related effect on developmental landmarks. The slight delay in preputial separation in F_1 males at 17 000 ppm (44.0 versus 42.9 days in the control) was not statistically significant and was within the performing laboratory's historical control range (39.9–45.0 days) and therefore was considered secondary to the lower body weight at weaning.

There were no treatment-related gross abnormalities. Organ weight findings are summarized in Table 16. Mean absolute and relative adrenal weights were significantly higher ($P < 0.05$) than the control weights in F_0 males at 5000 and 17 000 ppm; in the absence of any accompanying histopathological findings, similar effects in females or similar effects in F_1 males, these differences were not considered treatment related. At 17 000 ppm, significantly reduced ($P < 0.05$) absolute and relative spleen weights were determined in F_1 and F_2 weanlings and F_1 males; in the absence of any

accompanying histopathology, these reductions in spleen weight were considered a secondary effect of the lower body weight.

Table 16. Mean organ weights in F_0 and F_1 parental rats exposed to aminocyclopyrachlor

Organ	Mean organ weights (g or %)									
	0 ppm		500 ppm		1 500 ppm		5 000 ppm		17 000 ppm	
	M	F	M	F	M	F	M	F	M	F
F_0 adults										
Adrenals										
Absolute (g)	0.051	0.078	0.054	0.072	0.057	0.071	**0.057***	0.073	**0.058***	0.075
Relative (%)	0.009	0.023	0.009	0.021	0.010	0.021	**0.010***	0.022	**0.010***	0.023
F_1 adults										
Liver										
Absolute (g)	21.95	14.60	21.07	14.05	21.67	14.49	20.10	14.57	**18.26***	14.78
Relative (%)	3.403	4.216	3.418	4.065	3.478	4.202	3.409	4.300	3.357	4.404
Spleen										
Absolute (g)	0.979	0.621	0.908	0.606	0.957	0.647	0.900	0.646	**0.825***	0.617
Relative (%)	0.152	0.180	0.148	0.176	0.155	0.188	0.153	0.191	0.152	0.185
F_1 weanlings										
Spleen										
Absolute (g)	0.266	0.264	0.254	0.243	0.238	0.247	0.232	0.232	**0.199***	**0.182***
Relative (%)	0.448	0.471	0.448	0.443	0.421	0.450	0.433	0.444	**0.391***	**0.382***
F_2 weanlings										
Spleen										
Absolute (g)	0.289	0.267	0.253	0.258	0.275	0.272	0.256	0.261	**0.243***	0.226
Relative (%)	0.467	0.459	0.420	0.443	0.450	0.469	0.432	0.469	0.432	0.424

F: female; M: male; ppm: parts per million; *: $P < 0.05$ (statistically significant results are in boldface type)

Source: Lewis (2011a)

At 17 000 ppm in F_1 females, the incidence of slight thyroid follicular cell hypertrophy was increased compared with the controls (8/28 versus 2/28, respectively). There were no other treatment-related histopathological findings, including effects on the spleen.

The NOAEL for reproductive toxicity was 17 000 ppm (equal to 1048 mg/kg bw per day), the highest dietary concentration tested. The NOAEL for parental toxicity was 5000 ppm (equal to

299 mg/kg bw per day), based on reduced body weight, body weight gain and feed conversion efficiency in males at 17 000 ppm (equal to 1048 mg/kg bw per day). The NOAEL for offspring toxicity was 5000 ppm (equal to 299 mg/kg bw per day), based on reduced pup weights at 17 000 ppm (equal to 1048 mg/kg bw per day) (Lewis, 2011a).

(b) Developmental toxicity

Rats

Aminocyclopyrachlor (90.5% purity) in 0.5% (w/v) methyl cellulose was administered by gavage to groups of 25 pregnant Crl:CD(SD) rats at a dose of 0, 30, 100, 300 or 1000 mg/kg bw per day from day 6 to day 20 of gestation. Dams were observed daily for clinical signs of toxicity, with body weight and feed consumption recorded throughout this period. On day 21 of gestation, surviving dams were killed, and the following parameters were recorded or calculated: pregnancy rate, total resorptions, mean number of implantations, live fetuses, dead fetuses, corpora lutea counts and pup sex ratio. Fetuses were examined for external, visceral and skeletal abnormalities.

There was no treatment-related effect on any parameter. On this basis, the NOAEL for maternal toxicity and embryo/fetal toxicity was 1000 mg/kg bw per day, the highest dose tested (Lewis, 2011b).

Rabbits

Aminocyclopyrachlor (90.5% purity) in 0.5% (w/v) methyl cellulose was administered by gavage to groups of 22 pregnant New Zealand White rabbits at a dose of 0, 100, 300, 500 or 1000 mg/kg bw per day from day 7 to day 28 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 29 of gestation, surviving dams were killed, and the following parameters were recorded or calculated: pregnancy rate, total resorptions, mean number of implantations, live fetuses, dead fetuses, corpora lutea counts and pup sex ratio. Fetuses were examined for external, visceral and skeletal abnormalities.

At 1000 mg/kg bw per day, one dam was found dead on day 13 of gestation, with two dams aborting their litters on day 20 or day 26 of gestation. Clinical signs observed in the decedent included increased respiration at the 1 hour post-dosing observation (gestation day 7) and decreased defecation daily from gestation days 9 to 13. This dam lost 394 g of body weight from the commencement of dosing and consumed less than 14 g feed per day for 4 days prior to death. A postmortem examination could not establish the cause of death. A second dam also died at the highest dose, but this was attributable to an intubation error. Clinical signs observed in the two dams that aborted their litters included decreased defecation several days prior to abortion and red material in the cage pan or anogenital area on the day of abortion. Additionally, both dams lost 636–643 g body weight (approximately 17–19%) from the first day of dosing to the day of abortion. Reduced feed consumption was noted from gestation day 13 or 17 ($\leq$ 17 g/day). These abortions may be considered a secondary effect of body weight loss or reduced feed consumption. There were no treatment-related deaths or clinical signs at lower doses.

There were no significant intergroup differences in mean body weight. Although mean body weight gain was lower than the control values at 500 and 1000 mg/kg bw per day, only mean body weight gain of high-dose dams over gestation days 14–17 was significantly lower ($P < 0.01$) than the control value (a mean loss of 9 g versus a gain of 70 g in the controls). Overall body weight gain during the treatment period (days 7–29 of gestation) was 273, 218, 292, 173 and 166 g at 0, 100, 300, 500 and 1000 mg/kg bw per day, respectively, with the decreases at 500 and 1000 mg/kg bw per day (37% and 40% lower than the control values, respectively) not being statistically significant. At 1000 mg/kg bw per day, mean feed consumption was significantly lower ($P < 0.05$) than the control values on gestation days 14–15 and 15–16 (31% lower), with feed consumption over days 14–17 of gestation also being significantly lower ($P < 0.05$; 27%) than the control value. Although feed consumption was lower than the control value at 500 mg/kg bw per day, the difference was not statistically significant.

There were no treatment-related macroscopic findings. In the high-dose dam that was found dead on gestation day 13, red material was noted around the nasal area and accessory spleen, and a white precipitate was observed in the abdominal cavity adjacent to the right kidney; nine normally developing implantations were noted in utero. Necropsy confirmed that an intubation error was the cause of the second death at 1000 mg/kg bw per day. In the two 1000 mg/kg bw per day dams that aborted their litters, one had one early resorption and red material around the urogenital area, dark red contents in the vagina and nine early resorptions in utero. The second dam had one late resorption, red material around the urogenital area, cystic oviducts, thick white contents in the stomach and nine late resorptions (mummified) in utero.

There was no treatment-related effect on litter parameters or the incidence of external, visceral or skeletal abnormalities.

The NOAEL for maternal toxicity was 500 mg/kg bw per day, based on deaths, clinical signs, reduced body weight gain, reduced feed consumption and abortions at 1000 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 1000 mg/kg bw per day, the highest dose tested (Edwards, 2011).

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 aminocyclopyrachlor (purity 90.5%) in 0.5% (w/v) aqueous methyl cellulose at 0, 200, 1000 or 2000 mg/kg bw. Observations were made twice daily for deaths and clinical signs. Body weights were recorded pretreatment and daily following dosing. A functional observational battery and a motor activity assessment were performed pretreatment, at 2 hours after dosing (i.e. the approximate time to peak effect) and at days 8 and 15. On day 15, all survivors were killed, and a gross necropsy of the thoracic, abdominal and pelvic viscera was performed. Five rats of each sex per group were killed, their brain weights were recorded and a neurohistopathological examination was performed.

There were no deaths and no treatment-related clinical signs. Mean body weight and body weight gain were unaffected by treatment. At 2000 mg/kg bw, mean feed consumption was significantly lower ($P < 0.01$ or 0.05) than the control values in both sexes 1–2 days after dosing (17% lower in males and 15% lower in females). In the absence of any other treatment-related effects or evidence of systemic toxicity or neurotoxicity, this effect on feed consumption was not considered adverse. The functional observational battery and motor activity assessment were unremarkable. There were no treatment-related macroscopic findings or neurohistopathological abnormalities.

The NOAEL was 2000 mg/kg bw per day, the highest dose tested (Barnett, 2011).

(b) Immunotoxicity

Mice

In a study designed to evaluate immunotoxic potential, aminocyclopyrachlor (90.5% purity) was admixed in the diet at a concentration of 0, 300, 3000 or 7000 ppm and fed ad libitum to groups of 10 male Crl:CD1(ICR) mice for 28 days. The mean intakes of aminocyclopyrachlor were 0, 45, 425 and 1056 mg/kg bw per day at 0, 300, 3000 and 7000 ppm, respectively. A positive control group of five male mice was administered an intraperitoneal injection of cyclophosphamide monohydrate (25 mg/kg bw) on days 23–28. Deaths, clinical signs, body weight and feed consumption were recorded throughout the dosing period. Prior to termination, the immune system was stimulated by injecting sheep red blood cells (sRBCs) on day 23, with blood samples collected from each mouse on day 28. Serum samples were analysed for sRBC-specific immunoglobulin M (IgM) antibodies to provide a quantitative assessment of humoral immune response. Mice were killed on day 28 and necropsied, and selected organs were weighed (brain, spleen and thymus).

There was no treatment-related effect on any recorded or analysed parameter. The NOAEL was 7000 ppm (equal to 1056 mg/kg bw per day), the highest dietary concentration tested (Hoban, 2011a).

Rats

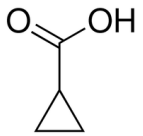
In a study designed similarly to the study conducted by Hoban (2011a) in mice, aminocyclopyrachlor (90.5% purity) was admixed in the diet at a concentration of 0, 600, 6000 or 18 000 ppm and fed ad libitum to groups of 10 male Crl:CD(SD) rats for 28 days. The mean intakes of aminocyclopyrachlor were 0, 42, 407 and 1277 mg/kg bw per day at 0, 600, 6000 and 18 000 ppm, respectively.

There was no treatment-related effect on any recorded or analysed parameter, including immune function. The NOAEL was 18 000 ppm (equal to 1277 mg/kg bw per day), the highest dietary concentration tested (Hoban, 2011b).

(c) Study on the photolytic degradate, cyclopropane carboxylic acid (CPCA)

Cyclopropane carboxylic acid (CPCA) (CAS No. 1759-53-1) is an environmental degradate of aminocyclopyrachlor (Fig. 4), which forms only as an aqueous photolytic metabolite. Low concentrations were detected as a possible extraction artefact in a grass metabolism study. However, dietary exposure to CPCA from rotational crops and animal products is highly unlikely, as CPCA was not detected in aerobic or anaerobic soil metabolism studies, nor was it detected in a goat metabolism study.

Fig. 4. Chemical structure of cyclopropane carboxylic acid (IN-V0977)



In a 90-day study, CPCA (99.7% purity) in deionized water was administered by gavage to Crl:CD(SD) rats (10 of each sex per group) at a dose of 0, 2, 10, 30 or 60 mg/kg bw per day. Observations for mortalities and clinical signs were made daily, with a more detailed clinical assessment undertaken weekly. Body weight and feed consumption were recorded weekly. Ophthalmological and neurobehavioural assessments (consisting of a modified functional observational battery and a motor activity assessment) were performed prior to the start of dosing and near the end of the exposure period. Blood and urine were sampled at the end of the exposure period for the analysis of haematological and clinical chemistry or urine analysis parameters. At the end of the exposure period, survivors were killed (day 92 or 93 in males; day 93 or 94 in females) and necropsied, and their organs were weighed. Histopathology was performed on the standard range of tissues.

There were no treatment-related deaths or clinical signs. There were no adverse effects on body weight, body weight gain, feed consumption or feed conversion efficiency. Although mean body weight at the end of the exposure period and overall body weight gain were higher than the control values in both sexes, these differences were not statistically significant. This increase was coincident with increased feed consumption, which was also not statistically significant. There were no treatment-related ophthalmological or neurobehavioural findings.

Selected haematological, clinical chemistry, urine analysis, organ weight, macroscopic and histopathological findings are summarized in Table 17. Mean neutrophil and monocyte counts were significantly increased ($P < 0.05$) in high-dose males (1.6- and 2.25-fold higher than the control values, respectively), with mean monocyte counts also significantly increased ($P < 0.05$) in high-dose females (2-fold higher than the control value). There were a number of perturbations in clinical chemistry parameters that collectively indicated hepatotoxicity or altered hepatic function, including

Table 17. Effects of exposure to CPCA in a 90-day gavage study in rats[a]

	Males					Females				
Parameter	**0 mg/kg bw per day**	**2 mg/kg bw per day**	**10 mg/kg bw per day**	**30 mg/kg bw per day**	**60 mg/kg bw per day**	**0 mg/kg bw per day**	**2 mg/kg bw per day**	**10 mg/kg bw per day**	**30 mg/kg bw per day**	**60 mg/kg bw per day**
Haematology										
Neutrophils ($\times 10^3/\mu L$)	1.51	1.07	1.07	1.32	**2.46***	0.70	0.68	0.78	0.61	0.62
Monocytes ($\times 10^3/\mu L$)	0.20	0.16	0.15	0.22	**0.45***	0.11	0.10	0.11	0.17	**0.22***
Clinical chemistry										
AST (U/L)	101	120	99	132	**298***	109	115	108	**207***	**150***
ALT (U/L)	35	34	34	39	**62***	44	37	43	47	38
Total bile acids (µmol/L)	9.1	11.4	9.6	9.3	22.1	10.1	9.3	8.6	19.0	**24.9***
Total protein (µmol/L)	7.3	7.0	7.1	**6.8***	**6.7***	7.6	7.5	7.7	7.6	7.5
Globulin (g/dL)	3.5	3.3	3.3	**3.1***	**3.0***	3.2	3.3	3.2	3.2	3.2
Triglycerides (mg/dL)	62	60	65	64	**121***	62	56	62	53	64
BUN (mg/dL)	14	14	14	12	15	17	17	18	**22***	**24***
Creatinine (mg/dL)	0.41	0.38	**0.36***	**0.32***	**0.32***	0.47	0.50	0.45	0.46	0.49
Inorganic phosphorus (mg/dL)	6.8	6.7	6.3	6.3	6.5	4.7	5.2	5.3	**5.7***	**6.5***
Urine analysis										
pH	7.6	7.3	7.2	7.3	**6.8****	6.8	6.8	6.9	**6.4***	**6.2****

Parameter	Males					Females				
	0 mg/kg bw per day	2 mg/kg bw per day	10 mg/kg bw per day	30 mg/kg bw per day	60 mg/kg bw per day	0 mg/kg bw per day	2 mg/kg bw per day	10 mg/kg bw per day	30 mg/kg bw per day	60 mg/kg bw per day
Organ weights										
Liver										
Absolute (g)	14.368	14.087	15.339	15.162	**17.458***	7.004	7.222	7.733	**9.182***	**9.950***
Relative (%)	2.596	2.468	2.613	2.713	**2.996***	2.457	2.499	2.595	**3.104***	**3.262***
Kidney										
Absolute (g)	3.386	3.575	3.773	3.688	**4.104***	1.713	1.854	1.822	**2.048***	**2.089***
Relative (%)	0.612	0.627	0.644	0.663	**0.708***	0.602	0.641	0.611	**0.693***	**0.689***
Macroscopic findings (number of rats/10)										
Liver: discoloration, pale	0	0	0	0	**1**	0	0	0	**6**	**8**
Histopathology (number of rats/10)										
Heart: vacuolation of myocardium										
Total	0	0	0	0	8	0	0	0	**9**	**10**
Minimal	0	0	0	0	3	0	0	0	4	1
Mild	0	0	0	0	5	0	0	0	2	7
Moderate	0	0	0	0	0	0	0	0	3	2
Heart: cardiomyopathy										
Total	4	4	2	**7**	10	1	1	2	**9**	**10**
Minimal	2	2	1	3	0	1	1	2	6	2
Mild	2	2	1	3	4	0	0	0	3	7
Moderate	0	0	0	1	6	0	0	0	0	1
Liver: fatty change, periportal										
Total	3	1	1	2	10	0	0	0	**8**	**9**

Table 17 (continued)

	Males					Females				
Parameter	**0 mg/kg bw per day**	**2 mg/kg bw per day**	**10 mg/kg bw per day**	**30 mg/kg bw per day**	**60 mg/kg bw per day**	**0 mg/kg bw per day**	**2 mg/kg bw per day**	**10 mg/kg bw per day**	**30 mg/kg bw per day**	**60 mg/kg bw per day**
Minimal	2	1	1	1	8	0	0	0	3	3
Mild	1	0	0	1	2	0	0	0	4	5
Moderate	0	0	0	0	0	0	0	0	1	1
Liver: infiltrate, mononuclear cell										
Total	8	7	3	5	6	1	1	1	**6**	**8**
Minimal	8	7	3	5	6	1	1	1	6	6
Mild	0	0	0	0	0	0	0	0	0	2
Thymus: necrosis, lymphoid										
Total	0	0	0	0	5	0	2	1	**6**	**5**
Minimal	0	0	0	0	5	0	2	0	6	5
Severe	0	0	0	0	0	0	0	1	0	0
Pancreas: zymogen, decreased										
Total	0	0	0	**3**	5	0	1	2	**10**	**10**
Minimal	0	0	0	2	4	0	1	2	4	4
Mild	0	0	0	1	1	0	0	0	6	6

ALT: alanine aminotransferase; AST: aspartate aminotransferase; BUN: blood urea nitrogen; U: units; *: $P < 0.05$; **: $P < 0.01$ (statistically significant results are in boldface type)

[a] All results expressed as the mean.

Source: Carpenter (2012)

increased aspartate aminotransferase (AST) (60 mg/kg bw per day males and 30 and 60 mg/kg bw per day females), increased alanine aminotransferase (ALT) (60 mg/kg bw per day males), increased total bile acids (60 mg/kg bw per day females), increased triglycerides (60 mg/kg bw per day males) and decreased globulins and total protein (30 and 60 mg/kg bw per day males). Blood urea nitrogen (BUN) and inorganic phosphorus levels were increased in females at 30 and 60 mg/kg bw per day, concomitant with an increase in kidney weight. However, in the absence of any effect on creatinine or histopathological changes in the kidneys, these increases in BUN and inorganic phosphorus levels were considered to be of uncertain toxicological significance. Urinary pH was significantly reduced ($P < 0.01$ or 0.05) at 30 and 60 mg/kg bw per day in males and at 60 mg/kg bw per day in females, which was attributable to the renal excretion of the acidic test material.

Mean liver weight and relative liver weight were increased in males at 60 mg/kg bw per day (22% and 15% higher than the control values, respectively) and in females at 30 and 60 mg/kg bw per day (31% and 42% higher than the control values, respectively, at 30 mg/kg bw per day; 32% and 42% higher than the control values, respectively, at 60 mg/kg bw per day). These increases in liver weight correlated with pale discoloration observed macroscopically and histopathological abnormalities, including minimal to mild periportal fatty change and minimal to moderate mononuclear infiltrates within the liver. The periportal fatty change was characterized by numerous small clear vacuoles within the cytoplasm of the periportal hepatocytes, with the distribution of the affected lobules locally extensive or diffuse and with females more severely affected than males. The mononuclear cell infiltrates comprised focal to multifocal clusters of mononuclear inflammatory cells.

Mean kidney weight and relative kidney weight were also increased in both sexes at these same doses (21% and 16% higher than the control values, respectively, in males at 60 mg/kg bw per day; 20% and 22% higher than the control values, respectively, in females at 30 mg/kg bw per day; 22% and 14% higher than the control values, respectively, in females at 60 mg/kg bw per day). There were no treatment-related macroscopic or histopathological kidney abnormalities, and therefore these increases in weight are unlikely to be toxicologically significant.

At 60 mg/kg bw per day in males and at 30 and 60 mg/kg bw per day in females, histopathological findings in the heart included minimal to moderate myocardial vacuolation, which consisted of numerous small clear vacuoles within the sarcoplasm of cardiac myocytes. The distribution of the affected myocytes was described as being locally extensive or diffuse. At these same doses, the incidence and severity of cardiomyopathy were also increased; this consisted of one or more areas of myocyte degeneration/necrosis, with a mononuclear inflammatory cell infiltrate. Other treatment-related histopathological findings occurring at these doses included minimal lymphoid necrosis in the thymus (characterized by the presence of a small number of scattered lymphocytes with pyknotic nuclei) and a minimal to mild decrease in zymogen in the pancreas (consisting of reduced eosinophilic staining zymogen within the apical cytoplasm of pancreatic acinar cells).

The NOAEL was 10 mg/kg bw per day, based on increased AST (females only), increased total bile acids (females only), decreased globulin with an associated decrease in total protein (males only) and adverse microscopic findings in the heart, liver (females only) and thymus (females only) at 30 mg/kg bw per day (Carpenter, 2012).

As this NOAEL is lower than that for aminocyclopyrachlor (349 mg/kg bw per day) following 90 days of dietary exposure, and as CPCA was not detected in rat metabolism studies, its toxicological relevance was evaluated using JMPR's metabolite assessment scheme, included in the guidance document for WHO monographers.[1] This scheme uses the threshold of toxicological concern (TTC) and read-across approaches as tools. In the absence of dietary intake data, a read-across approach was taken. CPCA was classified as a Cramer class II substance and had no structural alerts for deoxyribonucleic acid (DNA) or protein binding, carcinogenicity or mutagenicity. Using the

[1] http://www.who.int/entity/foodsafety/areas_work/chemical-risks/jmpr_Guidance_Document_FINAL.pdf

JMPR metabolite decision-tree, CPCA was determined to be a substance that would not be expected to be a safety concern.

3. Observations in humans

There were no reports submitted on the health of workers involved in the manufacture or use of aminocyclopyrachlor or aminocyclopyrachlor-methyl. No cases of human poisoning have been reported.

Comments

Biochemical aspects

In studies conducted using [^{14}C]aminocyclopyrachlor, maximum concentrations of radioactivity in plasma (C_{max}) were reached at 0.4–1 hour after a single gavage dose of 25 or 500 mg/kg bw. Gastrointestinal absorption was estimated to be 37–57%. The plasma elimination half-life was approximately 5.5 hours. More than 90% of the administered dose was excreted within 24 hours of dosing, with equal proportions recovered in urine and faeces at the low dose and a higher proportion of radioactivity detected in faeces at the high dose, suggesting a reduction in gastrointestinal absorption at the high dose. There was no evidence of tissue accumulation. Aminocyclopyrachlor was not metabolized to any great extent and was the only compound identified in plasma, urine and faeces. Low concentrations of an additional plasma metabolite, IN-LXT69, were detected only in 90-day rat and dog studies.

In studies conducted on [^{14}C]aminocyclopyrachlor-methyl using the same nominal doses of 25 or 500 mg/kg bw, radioactivity was more rapidly absorbed than for [^{14}C]aminocyclopyrachlor (up to approximately 2-fold), with C_{max} and $AUC_{0-\infty}$ values also higher (2- to 5-fold and 1.4- to 2.85-fold, respectively). Gastrointestinal absorption was estimated to be 87%. The plasma half-life values were approximately twice those of [^{14}C]aminocyclopyrachlor. The majority of the radioactivity was excreted in urine within 24 hours of dosing. There was no evidence of tissue accumulation. The main metabolite detected in plasma, urine and faeces was the free acid form of aminocyclopyrachlor. Low concentrations of aminocyclopyrachlor-methyl were detected in urine and bile shortly after dosing, but at no other times. Low concentrations of IN-LXT69 were detected in a 90-day rat study at the highest dietary concentration tested.

Toxicological data

The Meeting noted that the kinetic differences between aminocyclopyrachlor-methyl and aminocyclopyrachlor do not appear to translate to any discernible difference in toxicity.

The oral and dermal LD_{50} values in rats for both aminocyclopyrachlor and aminocyclopyrachlor-methyl were greater than 5000 mg/kg bw. In rats, the LC_{50} for aminocyclopyrachlor was greater than 5.4 mg/L. Aminocyclopyrachlor and aminocyclopyrachlor-methyl were not skin or eye irritants in rabbits, nor were they skin sensitizers in mice (local lymph node assay).

In repeated-dose toxicity studies in rats and dogs, the main adverse effects were confined to reduced body weight, body weight gain and feed consumption. No toxicity was observed in mice up to the highest tested dietary concentration.

In a 28-day study in male mice, which tested dietary concentrations of 0, 300, 3000 and 7000 ppm aminocyclopyrachlor (equal to 0, 45, 425 and 1056 mg/kg bw per day, respectively), the NOAEL was 7000 ppm (equal to 1056 mg/kg bw per day), the highest dietary concentration tested.

In a 90-day toxicity study in mice, which tested dietary aminocyclopyrachlor concentrations of 0, 300, 1000, 3000 and 7000 ppm (equal to 0, 47, 154, 459 and 1088 mg/kg bw per day for males and 0, 61, 230, 649 and 1629 mg/kg bw per day for females, respectively), the NOAEL was 7000 ppm (equal to 1088 mg/kg bw per day), the highest dietary concentration tested.

In a 28-day study in male rats, which tested dietary aminocyclopyrachlor concentrations of 0, 600, 6000 and 18 000 ppm (equal to 0, 42, 407 and 1277 mg/kg bw per day, respectively), the NOAEL was 18 000 ppm (equal to 1277 mg/kg bw per day), the highest dietary concentration tested.

In a 3-month toxicity study in rats, which tested dietary aminocyclopyrachlor concentrations of 0, 600, 2000, 6000 and 18 000 ppm (equal to 0, 35, 114, 349 and 1045 mg/kg bw per day for males and 0, 45, 146, 448 and 1425 mg/kg bw per day for females, respectively), the NOAEL was 6000 ppm (equal to 349 mg/kg bw per day), based on reduced body weight, body weight gain and feed conversion efficiency at 18 000 ppm (equal to 1045 mg/kg bw per day).

In another 3-month toxicity study in rats, which tested dietary aminocyclopyrachlor-methyl concentrations of 0, 600, 2000, 6000 and 18 000 ppm (equal to 0, 33, 110, 326 and 961 mg ae/kg bw per day for males and 0, 40, 125, 381 and 1146 mg ae/kg bw per day for females, respectively), the NOAEL was 6000 ppm (equal to 326 mg ae/kg bw per day), based on reduced body weight, body weight gain and feed consumption at 18 000 ppm (equal to 961 mg ae/kg bw per day).

In a 90-day toxicity study in dogs, which tested dietary aminocyclopyrachlor concentrations of 0, 250, 1250, 5000 and 15 000 ppm (equal to 0, 6.5, 33, 126 and 426 mg/kg bw per day for males and 0, 7.0, 38, 124 and 388 mg/kg bw per day for females, respectively), the NOAEL was 15 000 ppm (equal to 388 mg/kg bw per day), the highest dietary concentration tested.

In a 52-week toxicity study in dogs, which tested dietary aminocyclopyrachlor concentrations of 0, 1250, 5000, 15 000 and 30 000 ppm (equal to 0, 38, 178, 465 and 1077 mg/kg bw per day for males and 0, 47, 175, 542 and 1073 mg/kg bw per day for females, respectively), the NOAEL was 30 000 ppm (equal to 1073 mg/kg bw per day), the highest dietary concentration tested.

The overall NOAEL in dogs from the 90-day and 52-week studies was 1073 mg/kg bw per day.

In an 18-month toxicity and carcinogenicity study in mice, which tested dietary aminocyclopyrachlor concentrations of 0, 300, 1000, 3000 and 7000 ppm (equal to 0, 39, 133, 393 and 876 mg/kg bw per day for males and 0, 50, 171, 527 and 1190 mg/kg bw per day for females, respectively), the NOAEL for chronic toxicity was 7000 ppm (equal to 876 mg/kg bw per day), the highest dietary concentration tested. No carcinogenicity was observed in this study.

In a 2-year study in rats, which tested dietary aminocyclopyrachlor concentrations of 0, 600, 2000, 6000 and 18 000 ppm (equal to 0, 27, 97, 279 and 892 mg/kg bw per day for males and 0, 29, 100, 309 and 957 mg/kg bw per day for females, respectively), the NOAEL for chronic toxicity was 6000 ppm (equal to 279 mg/kg bw per day), based on reduced body weight, body weight gain, feed consumption and feed conversion efficiency at 18 000 ppm (equal to 892 mg/kg bw per day). No carcinogenicity was observed in this study.

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

Aminocyclopyrachlor and aminocyclopyrachlor-methyl were tested for genotoxicity in an adequate range of assays, both in vitro and in vivo. No evidence of genotoxicity was found.

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

In a one-generation reproductive toxicity study, which tested dietary aminocyclopyrachlor-methyl concentrations of 0, 600, 5000 and 17 000 ppm (equal to 0, 36, 285 and 994 mg ae/kg bw per day for males and 0, 41, 330 and 1118 mg ae/kg bw per day for females, respectively), the NOAEL for reproductive toxicity was 17 000 ppm (equal to 994 mg ae/kg bw per day), the highest dietary concentration tested. The NOAEL for both parental toxicity and offspring toxicity was 5000 ppm (equal to 285 mg ae/kg bw per day), for reduced body weight or pup weight, body weight gain and feed consumption at 17 000 ppm (equal to 994 mg ae/kg bw per day).

In a two-generation reproductive toxicity study in rats, which tested dietary aminocyclopyrachlor concentrations of 0, 500, 1500, 5000 and 17 000 ppm (equal to 0, 30, 92, 299

and 1048 mg/kg bw per day for males and 0, 36, 110, 367 and 1243 mg/kg bw per day for females, respectively), the NOAEL for reproductive toxicity was 17 000 ppm (equal to 1048 mg/kg bw per day), the highest dietary concentration tested. The NOAEL for parental toxicity was 5000 ppm (equal to 299 mg/kg bw per day), based on reduced body weight, body weight gain and feed conversion efficiency in males at 17 000 ppm (equal to 1048 mg/kg bw per day). The NOAEL for offspring toxicity was 5000 ppm (equal to 299 mg/kg bw per day), based on reduced pup weight at 17 000 ppm (equal to 1048 mg/kg bw per day).

In a developmental toxicity study in rats, which tested aminocyclopyrachlor doses of 0, 30, 100, 300 and 1000 mg/kg bw per day, the NOAEL for both maternal toxicity and embryo/fetal toxicity was 1000 mg/kg bw per day, the highest dose tested.

In a developmental toxicity study in rabbits, which tested aminocyclopyrachlor doses of 0, 100, 300, 500 and 1000 mg/kg bw per day, the NOAEL for maternal toxicity was 500 mg/kg bw per day, based on deaths, clinical signs, reduced body weight gain, reduced feed consumption and abortions at 1000 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 1000 mg/kg bw per day, the highest dose tested.

The Meeting concluded that aminocyclopyrachlor is not teratogenic.

In an acute neurotoxicity study in rats, which tested aminocyclopyrachlor doses of 0, 200, 1000 and 2000 mg/kg bw, the NOAEL was 2000 mg/kg bw, the highest dose tested.

The Meeting concluded that aminocyclopyrachlor is not neurotoxic.

No effects on the immune system were noted in 28-day studies in mice and rats at aminocyclopyrachlor doses up to 7000 ppm (equal to 1056 mg/kg bw per day) in mice and 18 000 ppm (equal to 1277 mg/kg bw per day) in rats.

The Meeting concluded that aminocyclopyrachlor is not immunotoxic.

Toxicological data on metabolites and/or degradates

The Meeting noted the formation of a photolytic degradate of aminocyclopyrachlor, CPCA, which was not detected in rat metabolism studies. Low concentrations of this compound were detected as a possible extraction artefact in a grass metabolism study. Dietary exposure to CPCA from rotational crops and animal products is unlikely, as CPCA was not detected in aerobic or anaerobic soil metabolism studies, nor was it detected in a goat metabolism study.

In a 90-day toxicity study in rats, which tested CPCA doses of 0, 2, 10, 30 and 60 mg/kg bw per day administered by gavage, the NOAEL was 10 mg/kg bw per day, based on increased AST (females only), increased total bile acids (females only), decreased globulin with an associated decrease in total protein (males only) and adverse microscopic findings in the heart, liver (females only) and thymus (females only) at 30 mg/kg bw per day.

As CPCA appeared to be more toxic than aminocyclopyrachlor in rats following repeated oral dosing, and as it was not detected in rat metabolism studies, its toxicological relevance was assessed using JMPR's metabolite assessment scheme included in the guidance document for WHO monographers. On the basis of this assessment, the Meeting concluded that CPCA is unlikely to be a safety concern, even if not an artefact of extraction.

Human data

No information was provided on the health of workers involved in the manufacture or use of aminocyclopyrachlor or aminocyclopyrachlor-methyl. No information on accidental or intentional poisoning in humans is available.

The Meeting concluded that the existing database on aminocyclopyrachlor and aminocyclopyrachlor-methyl 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 per day, expressed as aminocyclopyrachlor acid equivalents, based on a NOAEL of 279 mg/kg bw per day for reduced body weight, body weight gain, feed consumption and feed conversion efficiency at 892 mg/kg bw per day in a 2-year study of toxicity in rats, with the application of a 100-fold safety factor. The ADI is supported by the NOAELs of 299 mg/kg bw per day and 285 mg ae/kg bw per day from the reproductive toxicity studies in rats conducted on aminocyclopyrachlor and aminocyclopyrachlor-methyl, respectively. The ADI is established for the sum of aminocyclopyrachlor and its methyl ester, expressed as acid equivalents.

The Meeting concluded that it is not necessary to establish an acute reference dose (ARfD) for aminocyclopyrachlor in view of its low acute oral toxicity and the absence of developmental toxicity and any other toxicological effects that would be likely to be elicited by a single dose.

Levels relevant to risk assessment based on studies conducted on aminocyclopyrachlor and aminocyclopyrachlor-methyl

Species	Study	Effect	NOAEL	LOAEL
Aminocyclopyrachlor				
Mouse	Eighteen-month study of toxicity and carcinogenicity[a]	Toxicity	7 000 ppm, equal to 876 mg/kg bw per day[b]	–
		Carcinogenicity	7 000 ppm, equal to 876 mg/kg bw per day[b]	–
Rat	Ninety-day study of toxicity[a]	Toxicity	6 000 ppm, equal to 349 mg/kg bw per day	18 000 ppm, equal to 1 045 mg/kg bw per day
	Two-year study of toxicity and carcinogenicity[a]	Toxicity	6 000 ppm, equal to 279 mg/kg bw per day	18 000, equal to 892 mg/kg bw per day
		Carcinogenicity	18 000 ppm, equal to 892 mg/kg bw per day[b]	–
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	17 000 ppm, equal to 1 048 mg/kg bw per day[b]	–
		Parental toxicity	5 000 ppm, equal to 299 mg/kg bw per day	17 000 ppm, equal to 1 048 mg/kg bw per day
		Offspring toxicity	5 000 ppm, equal to 299 mg/kg bw per day	17 000 ppm, equal to 1 048 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	500 mg/kg bw per day	1 000 mg/kg bw per day
		Embryo and fetal toxicity	1 000 mg/kg bw per day[b]	–

Species	Study	Effect	NOAEL	LOAEL
Dog	Ninety-day and 1-year studies of toxicity[a,d]	Toxicity	30 000 ppm, equal to 1 073 mg/kg bw per day[b]	–
Aminocyclopyrachlor-methyl				
Rat	Ninety-day study of toxicity[a,e]	Toxicity	6 000 ppm, equal to 326 mg ae/kg bw per day	18 000 ppm, equal to 961 mg ae/kg bw per day
	One-generation study of reproductive toxicity[a,e]	Reproductive toxicity	17 000 ppm, equal to 994 mg ae/kg bw per day[b]	–
		Parental toxicity	5 000 ppm, equal to 285 mg ae/kg bw per day	17 000 ppm, equal to 994 mg ae/kg bw per day
		Offspring toxicity	5 000 ppm, equal to 285 mg ae/kg bw per day	17 000 ppm, equal to 994 mg ae/kg bw per day

[a] Dietary administration.
[b] Highest dose tested.
[c] Gavage administration.
[d] Two studies combined.
[e] Conducted on aminocyclopyrachlor-methyl; doses expressed as aminocyclopyrachlor acid equivalents (ae).

Estimate of acceptable daily intake (ADI)

0–3 mg/kg bw

Estimate of acute reference dose (ARfD)

Unnecessary

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

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

Critical end-points for setting guidance values for exposure to aminocyclopyrachlor and aminocyclopyrachlor-methyl

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid; 37–57% aminocyclopyrachlor; 87% aminocyclopyrachlor-methyl
Distribution	Rapid tissue distribution
Potential for accumulation	No potential for accumulation
Rate and extent of excretion	Rapid and complete
Metabolism in animals	Limited; hydrolysis of aminocyclopyrachlor-methyl to aminocyclopyrachlor
Toxicologically significant compounds in animals and plants	Aminocyclopyrachlor

Acute toxicity	
Rat, LD_{50}, oral	> 5 000 mg/kg bw
Rat, LD_{50}, dermal	> 5 000 mg/kg bw
Rat, LC_{50}, inhalation	> 5.4 mg/L
Rabbit, dermal irritation	Not irritating
Rabbit, ocular irritation	Not irritating
Mouse, dermal sensitization	Not sensitizing (local lymph node assay)
Short-term studies of toxicity	
Target/critical effect	Reduced body weight, body weight gain and feed consumption
Lowest relevant oral NOAEL	349 mg/kg bw per day (rat) (aminocyclopyrachlor) 326 mg ae/kg bw per day (rat) (aminocyclopyrachlor-methyl)
Lowest relevant dermal NOAEL	No data
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Reduced body weight, body weight gain, feed consumption and feed conversion efficiency
Lowest relevant NOAEL	279 mg/kg bw per day (rat)
Carcinogenicity	Not carcinogenic
Genotoxicity	
	Unlikely to be genotoxic
Reproductive toxicity	
Target/critical effect	No evidence of reproductive toxicity (rat)
Lowest relevant parental NOAEL	299 mg/kg bw per day (two-generation study with aminocyclopyrachlor)
Lowest relevant offspring NOAEL	299 mg/kg bw per day (two-generation study with aminocyclopyrachlor)
Lowest relevant reproductive NOAEL	1 048 mg/kg bw per day, highest dose tested (two-generation study with aminocyclopyrachlor)
Developmental toxicity	
Target/critical effect	No evidence of developmental toxicity (rat and rabbit)
Lowest relevant maternal NOAEL	500 mg/kg bw per day (rabbit)
Lowest relevant embryo/fetal NOAEL	1 000 mg/kg bw per day, highest dose tested (rat and rabbit)
Neurotoxicity	
Acute neurotoxicity NOAEL	2 000 mg/kg bw per day, highest dose tested (rat)
Subchronic neurotoxicity NOAEL	No data
Developmental neurotoxicity NOAEL	No data
Other toxicological studies	
Immunotoxicity NOAEL	1 056 mg/kg bw per day, highest dose tested (mouse)

Medical data
No data

Summary

	Value	Study	Safety factor
ADI	0–3 mg/kg bw	Two-year study of toxicity (rat)	100
ARfD	Unnecessary	–	–

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CYFLUMETOFEN

First draft prepared by
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Explanation

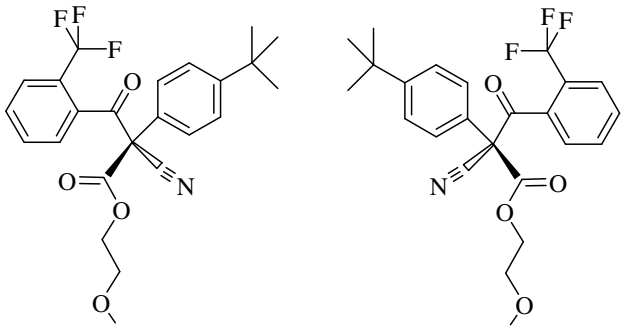
Cyflumetofen is the International Organization for Standardization (ISO)–approved common name for 2-methoxyethyl (*RS*)-2-(4-*tert*-butylphenyl)-2-cyano-3-oxo-3-(α,α,α-trifluoro-*o*-tolyl)propionate (International Union of Pure and Applied Chemistry), with Chemical Abstracts Service (CAS) number 400882-07-7. It is an acaricide and interferes with energy production (inhibition of complex II in mitochondria) on contact with spider mites. Cyflumetofen is a racemic mixture.

Cyflumetofen 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).

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

Fig. 1. Chemical structure of cyflumetofen



S-cyflumetofen *R*-cyflumetofen

Evaluation for acceptable daily intake

1. Biochemical aspects

Cyflumetofen is a racemic mixture, but the specific metabolism or degradation of the individual enantiomers in experimental animals was not investigated. The radiolabelled compounds used in the studies described below were [U-^{14}C-labelled 4-*tert*-butylphenyl ring]cyflumetofen (designated as A-ring-labelled cyflumetofen) and [U-^{14}C-labelled α,α,α-trifluoro-*o*-tolyl ring]cyflumetofen (designated as B-ring-labelled cyflumetofen).

1.1 Absorption, distribution and excretion

(a) Oral route

Mice

To investigate the change in kinetic parameters with increasing doses of cyflumetofen in mice in order to establish the dose range for further toxicological studies (Fabian & Landsiedel, 2011a), a single dose of 98.8% radiochemically pure B-ring-labelled cyflumetofen (specific radioactivity range: 0.04–0.8 MBq/mg) at 50, 250, 500 or 1000 mg/kg body weight (bw) was administered by oral gavage to 30 Crl:CD1 (ICR) male and female mice per dose. Blood was collected from animals in each dose group at 10 sampling times covering 0.5–72 hours post-dosing (three males and three females per time point), means of plasma concentrations were calculated and time course concentrations were analysed.

The pharmacokinetics of cyflumetofen in the plasma of mice differed between males and females (see Table 1): peak concentrations in plasma (C_{max} values) were on average 2.4 times higher in females (range: 34.3–104 µg equivalents [eq]/g) than in males (range: 10.6–65.4 µg eq/g). In both sexes, cyflumetofen was absorbed rapidly, leading to times to reach C_{max} (T_{max} values) of 0.5 hour post-dosing in males and 0.5–1 hour post-dosing in females. Moreover, plasma concentrations in females showed a second C_{max} (range: 6–47 µg eq/g) at 2 hours (dose level: 250 mg/kg bw) or 8 hours post-dosing (all other dose groups), which could indicate enterohepatic recirculation. After T_{max} was reached, concentrations in plasma declined rapidly (initial half-lives ranging from 0.4 to 2.3 hours post-dosing in females and from 0.7 to 2.8 hours post-dosing in males). Terminal half-lives were 1.2–1.8 times higher in males (range: 39.8–60 hours post-dosing) than in females (22–44 hours post-dosing). Finally, 1.4- to 2.0-fold higher internal concentrations, represented by areas under the

concentration–time curve from time 0 to infinity ($AUC_{0-\infty}$), were observed in females (177–1106 µg eq × h/g) than in males (89–717 µg eq × h/g), probably due to enterohepatic recirculation and higher oral absorption in females. In both sexes, $AUC_{0-\infty}$ values increased sublinearly, but continuously, with increasing dose (Fabian & Landsiedel, 2011a).

Table 1. Pharmacokinetic parameters of cyflumetofen in mice after single oral administration

	50 mg/kg bw		250 mg/kg bw		500 mg/kg bw		1 000 mg/kg bw	
Parameter	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
T_{max}[a] (h)	0.5	1; 8	0.5	0.5; 2	0.5	1; 8	0.5	0.5; 8
C_{max}[a] (µg eq/g)	10.61	51.84; 5.96	27.12	34.27; 46.54	43.28	65.32; 27.12	65.4	103.83; 36.87
$t_{½\ init.}$ (h)	2.02	0.42	0.7	2.3	2.82	2.25	1.38	1.02
$t_{½\ term.}$ (h)	39.79	22.04	60.07	41.33	53.16	43.87	54.89	35.22
$AUC_{0-\infty}$ (µg eq × h/g)	89.15	176.74	252.99	365.9	490.41	771.06	716.83	1 106.46

$AUC_{0-\infty}$: area under the concentration–time curve from time 0 to infinity; bw: body weight; C_{max}: peak concentration; eq: equivalent; $t_{½\ init.}$: initial half-life; $t_{½\ term.}$: terminal half-life; T_{max}: time to reach C_{max}

[a] An additional C_{max} and corresponding T_{max} were observed in females.

Source: Fabian & Landsiedel (2011a)

Rats

In a preliminary study to establish test conditions and methods for metabolism studies in rats (Nakamura, 2004a), the excretion pattern, pharmacokinetics and tissue distribution of cyflumetofen were investigated. Single oral doses of 99.2% radiochemically pure A-ring-labelled cyflumetofen or 98.1% radiochemically pure B-ring-labelled cyflumetofen were administered by gavage at 3 or 250 mg/kg bw (3.06 and 0.038 MBq/mg, respectively) to F344/DuCrj-Fischer rats (one rat of each sex per dose and label). For pharmacokinetic analysis, plasma and blood sampling and ^{14}C elimination in urine, faeces and expired air were investigated, covering several time points within 72 hours (24 hours for respiratory excretion) after administration. Tissue ^{14}C concentrations were measured at 72 hours after administration (termination time). Based on the pharmacokinetic analysis from single oral applications, the average (steady-state) concentration of radioactivity, assuming repeated dosing at 24-hour intervals, was estimated for each dose group and used to calculate the ^{14}C repetitive accumulation ratio ($Rac_{24\ h}$), defined as the ratio of the estimated minimum concentration at steady state after repeated dosing to the measured concentration at 24 hours after a single administration. $Rac_{24\ h}$ is a parameter predicting the bioaccumulative potential. Metabolic profiling was performed by high-performance liquid chromatography (HPLC).

No significant radioactivity was found in expired air from any test group. No pronounced differences between radiolabels were observed. At 3 mg/kg bw, the major route of elimination was the urine, ranging from 56.6% to 59.9% of the applied radioactivity in males and from 67.6% to 70.6% of the applied radioactivity in females; excretion in faeces accounted for 35.2–36.5% of the applied radioactivity in males and 23.8–25.3% of the applied radioactivity in females. At 250 mg/kg bw, the major elimination pathway was the faeces, accounting for 72.6–80.8% of the applied radioactivity in males and 68.6–76.4% of the applied radioactivity in females; urinary excretion was lower than at 3 mg/kg bw, amounting to 13.2–19.7% of the applied radioactivity in males and 15.1–25.1% of the applied radioactivity in females. Irrespective of dose level and label, excretion of radioactivity via urine was higher in female rats (about 6–12% of the applied radioactivity) than in male rats, but was lower in both sexes at the high dose than at the low dose. Approximately 92–99% of the applied radioactivity was recovered within 72 hours, and 0.3–2.1% of the applied radioactivity remained in the body. The concentration in plasma declined with biphasic first-order kinetics in all test groups,

with elimination half-lives (β-phase) of 11–20 hours. The T_{max} was in the range of 0.5–1 hour at the low dose and 1–4 hours at the high dose. The C_{max} was 1.3–2.9 mg eq/L at the low dose and 12.5–29.8 mg eq/L at the high dose. The ratio of the $AUC_{0-\infty}$ values between the high- and low-dose groups was 1.6–5 times lower than the dose ratio (approximately 80-fold) for both labels, suggesting saturation of oral absorption at 250 mg/kg bw. $Rac_{24\,h}$ was calculated to be 1.3–1.8, indicating that cumulative absorption was low. The highest total radioactive residue (TRR) concentration was consistently found, aside from the gastrointestinal tract and its contents, in liver (up to 52 times higher than the plasma concentration) and kidney (up to 10 times higher than the plasma concentration), regardless of the label position, dose level or sex. The TRR concentrations from the majority of the other organs or tissues were equal to or less than the plasma concentration in all test groups (Nakamura, 2004a).

To investigate the biliary excretion pattern of cyflumetofen and the radioactive residue levels in rats over 48 hours (Nakamura, 2004b), single doses of [^{14}C]cyflumetofen labelled in the A- or B-ring (99.2% and 98.1% radiochemical purity, respectively) were administered by oral gavage at 3 mg/kg bw (1.88 MBq/mg) or 250 mg/kg bw (0.023 MBq/mg) to bile duct–cannulated F344/DuCrj-Fischer rats (3–4 rats of each sex per dose and label). Urine, bile, faeces and cage wash were collected over 48 hours (termination time), and terminal blood samples were collected, together with the gastrointestinal tract and residual carcass; each sample taken was then analysed for the distribution of orally administered radioactivity. Bile samples were also generated for the metabolite distribution study (see section 1.2), summarized under the study of Ohyama (2004a).

In all test groups, more than 90% of the applied radioactivity was recovered from bile, urine, faeces, cage wash and the carcass. As no pronounced differences between radiolabels were generally observed, ranges and means (if the difference in mean values between radiolabels was < 3% of the applied radioactivity) are given for both radiolabels together. At 3 mg/kg bw, the major routes of elimination of radioactivity recovered over 48 hours post-dosing in both males and females were the urine (means of 30% of the applied radioactivity in males and 41–51% of the applied radioactivity in females) and the bile (means of 37% of the applied radioactivity in males and 24.5% of the applied radioactivity in females). Faecal excretion accounted for about 6–17% of the applied radioactivity in males and 6.5–10% of the applied radioactivity in females. At 250 mg/kg bw, the urinary excretion rate was less than at the low dose (11–15% of the applied radioactivity in males and 16–23.5% of the applied radioactivity in females), whereas the biliary excretion rate was similar to that at the low dose (means of 30.5% of the applied radioactivity in males and 18–21% of the applied radioactivity in females). The major route of elimination at 250 mg/kg bw was faecal excretion (means of 35% of the applied radioactivity in males and 35–41.4% of the applied radioactivity in females), which was higher than at the low dose. Generally, the biliary excretion rate was higher in males than in females, irrespective of the dose level and label position (the difference was about 8–14% of the applied radioactivity), and the urinary excretion rate was higher in females than in males, regardless of the dose level and label position. The applied radioactivity remaining in the body (excluding the gastrointestinal tract and its contents) was about 2.2% in males and 2–6% in females at 3 mg/kg bw and about 1.4% in males and 1.3% in females at 250 mg/kg bw. The estimated absorption rate (up to 48 hours) of cyflumetofen (sum of the radioactivity detected in bile, urine and the body, excluding the gastrointestinal tract and its contents) was a mean of 69% of the applied radioactivity in males and 70.8–78.4% of the applied radioactivity in females at 3 mg/kg bw and a mean of 45% of the applied radioactivity in males and 35.3–45.7% of the applied radioactivity in females at 250 mg/kg bw (Nakamura, 2004b).

To investigate the pharmacokinetics of cyflumetofen (Nakamura, 2004d), single doses of [^{14}C]cyflumetofen labelled in the A- or B-ring (99.2% and 98.1% radiochemical purity, respectively) were administered by oral gavage at 3 mg/kg bw (3.06 MBq/mg) or 250 mg/kg bw (0.039 MBq/mg) to F344/DuCrj-Fischer rats (four rats of each sex per dose and label). For pharmacokinetic analysis, means of plasma concentrations were calculated for every test group for each of nine sampling times spanning 72 hours. Owing to the formation of thrombi starting at 48

hours after dosing, some cannulas were damaged, and some blood samples could not be taken. Therefore, blood samples of only three instead of four animals could be taken in some test groups. $Rac_{24\,h}$ was calculated for each dose to predict the bioaccumulative potential. Additionally, urine and faeces for metabolite profiling were collected and analysed in a separate study (see section 1.2; Ohyama, 2004b).

At 3 mg/kg bw, maximum plasma TRR concentrations were achieved more rapidly (1 hour) than at 250 mg/kg bw (2–4 hours) in male and female rats (Table 2). The TRR concentrations in plasma decreased following biphasic first-order kinetics at about 8 hours after dosing. The slow-phase elimination half-life was shorter (14–17 hours) for the radioactivity from the A-label compared with the radioactivity from the B-label (17–21 hours). The $AUC_{0-\infty}$ did not proportionally increase with dose (the increase was 2- to 5.5-fold less than expected), which is an indication that saturational processes in systemic absorption could occur. There were no pronounced differences in pharmacokinetic parameters between sexes at the low dose; however, at the high dose, the C_{max} (see Table 2) and the $AUC_{0-\infty}$ values were higher in females and the duration of peak concentrations was generally increased in females (1–8 hours after dosing) compared with all other test groups (0.5–2 hours). The $Rac_{24\,h}$ was low in all test groups, indicating that cyflumetofen has no bioaccumulative potential (Table 2) (Nakamura, 2004d).

Table 2. Plasma pharmacokinetic parameters in rats following single oral administration of cyflumetofen at 3 or 250 mg/kg bw

	3 mg/kg bw		250 mg/kg bw	
	Males	**Females**	**Males**	**Females**
A-ring label				
T_{max} (h)	1	1	2	4
C_{max} (µg eq/g)	1.394	0.946	10.01	15.33
Final elimination phase half-life (h)	13.9	14.1	16.7	12.4
$AUC_{0-\infty}$ (µg eq × h/g)	10.388	6.562	159	251.1
$Rac_{24\,h}$	1.4	1.4	1.6	1.4
B-ring label				
T_{max} (h)	1	1	2	2
C_{max} (µg eq/g)	1.059	1.014	10.79	15.37
Final elimination phase half-life (h)	18.2	21.8	21.8	16.9
$AUC_{0-\infty}$ (µg eq × h/g)	10.176	9.196	165.9	327.8
$Rac_{24\,h}$	1.7	1.9	1.9	1.6

$AUC_{0-\infty}$: area under the concentration–time curve from time 0 to infinity; bw: body weight; C_{max}: peak concentration; eq: equivalent; $Rac_{24\,h}$: ^{14}C repetitive accumulation ratio; T_{max}: time to reach C_{max}

Source: Nakamura (2004d)

To investigate the tissue distribution of cyflumetofen (Nakamura, 2004e), single oral doses of 99.2% radiochemically pure A-ring-labelled cyflumetofen or 98.1% radiochemically pure B-ring-labelled cyflumetofen were administered by gavage at 3 or 250 mg/kg bw (1.88 and 0.023 MBq/mg, respectively) to F344/DuCrj-Fischer rats (six rats of each sex per dose and label). To analyse the TRR concentrations in organs and tissues, three rats of each sex per treatment group were terminated near the T_{max}, at either 1 hour for the low-dose group or 2 hours for the high-dose group, and another three rats of each sex per treatment group were terminated at 24 hours after application. To derive tissue

elimination curves and calculate half-lives of ^{14}C concentrations in selected tissues, the mean ^{14}C concentrations in tissues near the T_{max} and at 24 and 72 hours after administration were considered. The results after 72 hours were obtained from a separate study (elimination balance study of Nakamura, 2004c).

Regardless of the label position, dose level or sex, the majority of the applied radioactivity (46–83%, 5.9–31% and ≤ 1.4% after 1–2, 24 and 72 hours, respectively) was observed in the gastrointestinal tract including contents. For all time points, and irrespective of label, sex and dose, the highest TRR concentrations were found in the liver, followed by the kidney. At 3 mg/kg bw, the highest concentrations of TRR in these organs were 7.6–9 mg eq/kg (attributed to 10.4–12% of the applied radioactivity) and 4.7–8 mg eq/kg (attributed to 1.2–1.9% of the applied radioactivity) after 1 hour (T_{max}) in liver and kidney, respectively, regardless of sex or label position. At 250 mg/kg bw, the highest TRR concentrations of 66–117 mg eq/kg (reflecting 1.1–1.8% of the applied radioactivity) and 40–61 mg eq/kg (reflecting 0.12–0.19% of the applied radioactivity) were measured after 2 hours (T_{max}) in liver and kidney, irrespective of sex or label position. At 1–2 hours and 24 hours after application, mean TRR concentrations in kidney were 4 times higher than those in plasma, and those in liver were 12 times higher. TRR concentrations in other tissues were equal to or less than those in plasma at these two time points. However, some other tissues showed higher TRR concentrations compared with plasma concentrations at 72 hours. Additionally, some TRR concentrations in tissues were higher in B-ring-labelled cyflumetofen experiments than in A-ring-labelled cyflumetofen experiments at 72 hours (see also below: Nakamura, 2004c). Regardless of sex, label position or dose group, TRR concentrations in tissues declined with time. At 72 hours post-dosing, the TRR remaining in the carcass and tissues accounted for less than about 2.5% of the applied radioactivity at the low dose and about 1% of the applied radioactivity at the high dose, regardless of label position or sex. At 3 mg/kg bw, the half-life for the elimination of radioactivity from plasma was 9–13 hours, somewhat lower than the half-life for the elimination of radioactivity from bone marrow (14–22 hours) and remarkably lower than the half-life for the elimination of radioactivity from adipose tissue (19–24.5 hours). At 250 mg/kg bw, the half-life for the elimination of radioactivity from plasma was 10–15 hours, slightly lower than the half-lives for the elimination of radioactivity from blood, bone marrow and liver (ranges were 14–18.5, 20–30 and 16–19 hours, respectively), but remarkably lower than the half-life for the elimination of radioactivity from adipose tissue (31–42.5 hours). The elimination pattern of radioactivity from most selected tissues (except liver) appeared to be biphasic at the lower dose, particularly in female rats (Nakamura, 2004e).

To investigate the excretion of radioactivity in urine and faeces and the systemic distribution of radioactivity after 72 hours (Nakamura, 2004c), single oral doses of 99.2% radiochemically pure A-ring-labelled cyflumetofen or 98.1% radiochemically pure B-ring-labelled cyflumetofen were administered by gavage at 3 or 250 mg/kg bw (3.06 and 0.038 MBq/mg, respectively) to F344/DuCrj-Fischer rats (four rats of each sex per dose and label). Samples were also generated to analyse the metabolite distribution in the study of Ohyama (2004b) (see section 1.2), and the TRR results of this study were used to establish tissue elimination half-lives in the study of Nakamura (2004d).

At the end of the study (72 hours post-dosing), overall recovery of radioactivity was at least 95% of the applied radioactivity. The major pathway of elimination of radioactivity was the urine at 3 mg/kg bw and the faeces at 250 mg/kg bw. Irrespective of dose level or label, excretion of radioactivity via urine was higher in female rats than in male rats, but was lower in both sexes at the high dose than at the low dose. Excretion of radioactivity in urine at 3 mg/kg bw was 58.1–59.4% of the applied radioactivity in males and 66.1–66.5% of the applied radioactivity in females, whereas excretion of radioactivity in faeces at 3 mg/kg bw was 32.5–32.9% of the applied radioactivity in males and 25–27.3% of the applied radioactivity in females. At 250 mg/kg bw, excretion of radioactivity in urine was 14.3–16.2% of the applied radioactivity in males and 21.8–25.8% of the applied radioactivity in females, whereas excretion of radioactivity in faeces was 76.8–79.6% of the applied radioactivity in males and 68.3–74.4% of the applied radioactivity in females. No pronounced differences in excretion were observed between radiolabels. The tissues and carcass contained 0.9–2.5% of the applied radioactivity at 3 mg/kg bw and 0.4–0.8% of the applied radioactivity at 250

mg/kg bw for both labels at 72 hours post-dosing. The highest radioactivity concentrations in tissues were measured (aside from the gastrointestinal tract including contents and residual carcass) in the liver (> 6-fold higher than plasma concentrations) and kidney (> 4-fold higher than plasma concentrations), regardless of treatment group or sex, and in the bone marrow (> 5-fold higher than plasma concentrations) in females treated with A-ring-labelled cyflumetofen, regardless of the dose. Other tissues and organs with a ratio of tissue to plasma concentrations above 2 were adipose tissue (predominantly high-dose groups with A-ring label only), adrenal gland (females in low-dose group with A-ring label only) and erythrocytes (females in high-dose group with A-ring label only). Generally, radioactivity concentrations in tissues showed no remarkable sex differences, but (with the exception of liver and bone marrow) radioactivity concentrations in tissues were generally higher in B-ring-labelled than in A-ring-labelled cyflumetofen experiments. Concentrations of radioactivity in tissues measured in animals in the high-dose groups were approximately 53 times higher than those measured in animals in the low-dose groups (Nakamura, 2004c).

To investigate the excretion and tissue distribution of cyflumetofen (Hardwick, 2008), 14 daily repeated oral doses of 98.1% radiochemically pure B-ring-labelled cyflumetofen were administered by gavage at 3 mg/kg bw (1.88 MBq/mg) to female F344/DuCrj-Fischer rats. Groups consisting of four female rats were terminated 24 hours after the fourth and seventh repeated doses and 2, 24 and 72 hours after the 14th dose. Moreover, urine and faeces were collected from an additional group of four females at three (faeces) and five (urine) different time points during the 72 hours after the 14th dose. This group was terminated at 72 hours after the last applied dose as well. From all terminated animals, selected tissues were sampled to analyse TRR concentrations. The urinary and faecal samples were collected to characterize excretion patterns and were pooled across sample collection periods to analyse and quantify metabolites of cyflumetofen (summarized in section 1.2).

Cyflumetofen was widely distributed throughout tissues. After only four daily doses, TRR appeared to reach steady-state concentrations in organs. At all sampling times, TRR concentrations were (with the exception of the gastrointestinal tract and contents) highest in liver, followed by kidney and erythrocytes, and exceeded (except at 2 hours after the last dose in erythrocytes) TRR concentrations in plasma. In all other organs, TRR concentrations were lower than or equal to those in plasma. No remarkable accumulation occurred in any of the tissues or organs investigated. However, the increasing erythrocyte to plasma TRR concentration ratio with respect to the period 24–72 hours after the last dose suggests an association of cyflumetofen or its metabolites with the cellular fraction in blood. The highest TRR concentrations were measured 2 hours after the 14th dose in liver (12.9 mg eq/kg), kidney (7.1 mg eq/kg), erythrocytes (1.15 mg eq/kg) and plasma (1.5 mg eq/kg). Although the TRR concentrations in organs and tissue decreased relatively rapidly until 72 hours post-dosing, TRR concentrations in tissues at 72 hours after the last dose were on average still about 11% of TRR concentrations after 2 hours, with the slowest decline occurring in erythrocytes (25% of TRR concentrations after 2 hours) and skin (30% of TRR concentrations after 2 hours), and TRR concentrations in liver, kidney and erythrocytes were still about 5- to 9-fold higher than TRR concentrations in plasma at that time point. Cumulative elimination of radioactivity at 72 hours after the 14th dose of cyflumetofen administered at 3 mg/kg bw occurred in almost equal proportions in urine (64% of the last dose, equivalent to 4.5% of the total dose) and faeces (59% of the last dose, equivalent to 4.2% of the total dose). Retention of radioactivity in the tissues and carcass was 4.5% (based on the radioactivity in the final dose) or 0.3% (based on the total applied radioactivity). Overall recovery of radioactivity was 132% (based on the radioactivity in the final dose) or 9.1% (based on the total applied radioactivity) at 72 hours post-dosing (Hardwick, 2008).

(b) Dermal route

In vitro

It was not possible to prepare a homogeneous formulation of the representative solution, Scelta 20SC, containing B-ring-labelled cyflumetofen; therefore, B-ring-labelled cyflumetofen was dissolved in acetonitrile. The concentrate, 200 g/L cyflumetofen, and a representative dilution for Scelta 20SC, 0.2 g/L, were applied to four human dermatomed skin discs from two donors and four human skin discs from four donors for 8 hours, respectively, and receptor fluid was analysed for 24 hours. The results show that the actual dermal absorption into the receptor fluid was low: only about 0.6% was absorbed 24 hours after application of a low or high dose. The vast majority remained in the skin as a dermal depot, representing about 20% and 27% for the low and high doses, respectively. As a worst case, the depot in skin can be considered as potentially absorbable material, which sums up to dermal absorptions of cyflumetofen of 21% for the low dose and 28% for the high dose (Corral, 2009).

In vivo

Radiolabelled cyflumetofen formulated in BAS 9210 01 at 200 g/L and 2 and 0.2 g/L cyflumetofen dilutions were applied to the skin of 12 male Wistar rats (per dose) for 8 hours, and urine and faeces were collected and analysed 8, 24 or 120 hours (four animals per time point) after application. At the respective time points, animals were terminated, and residues in carcass, skin and application site were measured. Dermal absorption ranged from 0.26% of the applied dose at 8 hours to 0.96% at 120 hours for the 200 g/L cyflumetofen concentrate. For the 2 and 0.2 g/L cyflumetofen dilutions, 0.63% and 4.93% of the applied dose were found at 8 hours, respectively, reaching a maximum of 2.2% and 8% at 120 hours, respectively. The residue in the application site decreased after the exposure period, and radioactivity was still being excreted at 120 hours, suggesting that a certain part of the depot at the application site becomes systemically available after exposure and probably after the observation period (Fabian & Landsiedel, 2011b).

1.2 Biotransformation

One preliminary (Nakamura, 2004a) and three main metabolism studies (Ohyama, 2004a,b; Hardwick, 2008) were performed. The metabolism of cyflumetofen was investigated by analysing bile, faeces and urine samples retained from the previous studies: single oral administration of 3 or 250 mg/kg bw of A- or B-ring-labelled cyflumetofen to male and female rats (Nakamura, 2004a,b,c) and 14 consecutive oral doses of 3 mg/kg bw per day of B-ring-labelled cyflumetofen to female rats only (Hardwick, 2008).

In a preliminary metabolism study (Nakamura, 2004a), unchanged parent was not detected in urine, and the metabolite profile (peaks not identified) differed between the A- and B-ring labels. In total, four specific peaks from each of the labels were noted. Unchanged parent was found as the main components in faecal extract in all test groups. In addition, three peaks for A-ring label and one for B-ring label were detected. From the metabolite profile, it was assumed that cleavage between the A- and B-rings and subsequent degradation to specific metabolites for each label occur. In addition, most of the metabolites seemed to be excreted via urine.

In the first main metabolism study (Ohyama, 2004b), in which samples of urine and faeces were analysed, cyflumetofen was extensively metabolized in rats. Three groups of metabolites were observed: *tert*-butylphenyl (A-ring) specific, trifluorotolyl (B-ring) specific, and A- and B-ring common metabolites. Most of the main metabolites derived from cleavage between the A-ring and the B-ring moieties, and the levels of the uncleaved metabolites, including both the A- and B-ring moieties, which are predominantly represented by metabolites AB-1, AB-2 and AB-3, were relatively low.

At 3 mg/kg bw in non-cannulated rats, most of the major metabolites were excreted in urine. The major metabolites containing the A-ring only in urine and faeces were A-20 (HPLC fraction: M8; urine: 1–4% of the applied radioactivity; faeces: about 3% of the applied radioactivity), A-21 (M10;

urine: 7–21% of the applied radioactivity; only traces in faeces) and A-18 (M28; urine: 14–34% of the applied radioactivity; only traces in faeces). The major metabolites containing the B-ring only were B-1 (M14; urine: 8–10% of the applied radioactivity; faeces: about 17% of the applied radioactivity), [B-1]-MA (M16; urine: 6–13.5% of the applied radioactivity; only traces in faeces) and [B-1]-TLA (M17; urine: 17–20% of the applied radioactivity; only traces in faeces). Metabolite AB-3 (M27; containing the A- and B-ring moieties) was urine specific and occurred at greater than 100-fold higher levels in females (8–9% of the applied radioactivity) than in males. However, AB-2 (M26), which is derived from AB-3 by further oxidation, was found at a similar range of concentrations between sexes. With the exception of AB-3, the profiles of the metabolites were similar in both sexes, but some differences in quantity were apparent. The amounts of A-21 (M10), A-18 (M28), A-20 (M8) and [B-1]-MA (M16) were 1.1%, 14.7%, 3.9% and 6.2% of the applied radioactivity for males, but 6.7%, 33.9%, 1.0% and 13.5% of the applied radioactivity for females, respectively. Unchanged parent was not detected or occurred in minor amounts in urine and was about 2–4% of the applied radioactivity in faeces for both labels and sexes.

At 250 mg/kg bw, the profiles of the identified metabolites and the quantitative differences between sexes were similar to those at 3 mg/kg bw. However, metabolites at 250 mg/kg bw occurred at lower percentages (about ≤ 9-fold lower) of the applied radioactivity in urine and faeces than at 3 mg/kg bw. In contrast, the fraction of the unchanged parent in faeces was 14- to 27-fold higher in the high-dose groups than in the low-dose groups (≤ 66% versus ≤ 4.3% of the applied radioactivity, respectively), which might be due to saturation of oral absorption processes. As for the low dose, unchanged parent was not detected or occurred in minor amounts in urine at the high dose. Unfiltered/unextractable fractions (from different label positions, sexes and doses) in urine and faeces were all less than or equal to 4%.

In the second main metabolism study, in which bile samples were analysed following a dose of 3 or 250 mg/kg bw in rats, unchanged parent was not detected or occurred in minor amounts in bile, regardless of dose, label or sex. The metabolite profile for bile was significantly different from those for urine and faeces in the non-cannulated animals. In bile, the highest portion of applied radioactivity consisted of metabolites containing both the A- and B-ring moieties. With the exception of AB-2 (Mb30), metabolites above 1% of the applied radioactivity in bile were all subjected to phase 2 biotransformation (conjugation to glucuronic acid [GA] and glutathione [SG]) (Ohyama, 2004a).

GA-conjugated AB-1 and AB-3 were eluted in more than one fraction (AB-1 in Mb32 and Mb33; AB-3 in Mb18 and Mb21), possibly due to keto-enol tautomerism and subsequent different positioning of GA on each of the AB-1 and AB-3 molecules.

The major metabolites identified at levels above 5% of the applied radioactivity in bile were the GA-conjugated AB-1 (Mb32 and Mb33; low dose: 5.7–7.5% of the applied radioactivity; high dose: 7.6–13.3% of the applied radioactivity) and the GA-conjugated AB-3 (Mb18; low dose: 5–6.8% of the applied radioactivity; high dose: 3.5–5.5% of the applied radioactivity). A certain portion of the GA-conjugated AB-3 was also detected in the Mb21 fractions (see above). However, the amount was determined only for males in the low-dose group (A-ring label) and accounted for 1.4% of the applied radioactivity, indicating that total levels of GA-conjugated AB-3 in bile might be underestimated. Furthermore, metabolites occurring at levels above 1% of the applied radioactivity were AB-2 (≤ 3.2% of the applied radioactivity) and cleavage products (containing either A- or B-ring moieties), such as the SG-conjugated B-1 ([B-1]-SG: ≤ 2.6% of the applied radioactivity) and the GA-conjugated A-6 ([A-6]-GA: ≤ 3.7% of the applied radioactivity) and A-22 ([A-22]-GA: ≤ 3.8% of the applied radioactivity). All other metabolites were found at levels below 1% of the applied radioactivity. The percentages of the applied radioactivity in bile of SG-conjugated B-1 in males and of AB-2 in all treatment groups were 4- and 5.7-fold, respectively, lower at the high dose than at the low dose, and GA-conjugated AB-1 showed a higher portion of the applied radioactivity in bile at the high dose than at the low dose (see above). Otherwise, all percentages of the applied radioactivity for the other main metabolites were in a similar range in the high- and low-dose groups.

In the third main metabolism study (Hardwick, 2008), B-ring-labelled cyflumetofen at 3 mg/kg bw was administered daily for 14 days to female rats, and samples of urine and faeces were collected and pooled during the 72 hours after the last dose. The only reference standards used for co-chromatography during HPLC were parent and 2-trifluoro-methyl benzoic acid (B1, M14), and no mass spectrometry was performed on the fractions. According to 25 chromatographic regions derived from urine and up to at least six regions in the faeces, cyflumetofen appears to be extensively metabolized.

Unchanged parent was not detected in the urine of female rats, whereas B-1 was observed at 13% of the final dose. The urine contained nine other major unidentified fractions (0.7–11.7% of the applied radioactivity of the final dose). Unchanged parent was observed at 24.7% of the applied radioactivity of the final dose in faeces, and B1 (M14) was found at 22.2% of the applied radioactivity of the final dose in faeces. Other (unidentified) fractions were each less than 1% of the applied radioactivity of the final dose. As the fraction of unchanged parent was higher in the repeated-dose study than in an equivalent single-dose oral study in faeces (25% versus 2.5% of the applied radioactivity, respectively), saturation of oral absorption might occur following repeated dosing.

In conclusion, the major route of metabolism of cyflumetofen is suggested to be cleavage into A-ring and B-ring moieties (Fig. 2). The A-ring moiety was hydrolysed to A-18 (M28 in urine) via A-l (putative intermediate), followed by hydration of the A-ring to A-21 (M10). As another minor route for A-ring metabolism, A-21 was decarboxylated to form the intermediate A-6, which was glucuronidated or transformed to A-20 (M8). The main metabolic pathway of the B-ring moiety consists of two routes: one is the hydrolytic pathway producing B-1 (M14), and the other is the formation of mercapturic acid conjugate ([B-1]-MA, M16) and thiolactic acid conjugate ([B-1]-TLA, M17) via the conjugation of the B-ring moiety with glutathione ([B-1]-SG; bile-specific metabolite). A minor route of metabolism of cyflumetofen is supposed to be the transformation of metabolites maintaining the A- and B-rings. The transformation pathway of these metabolites starts with cleavage of the carboxylic ester moiety, resulting in the formation of AB-1, followed by oxidation of the *tert*-butyl group to alcohol (AB-3; M27) and carboxylic acid (AB-2; M26). Glucuronidated AB-1 and AB-3 were the main metabolites in bile; however, the precise position of glucuronidation could not be determined (see above), and glucuronidated AB-1 and AB-3 metabolites were not identified in urine and faeces of non-cannulated rats, suggesting that rapid degradation of these metabolites occurs.

2. Toxicological studies

2.1 Acute toxicity

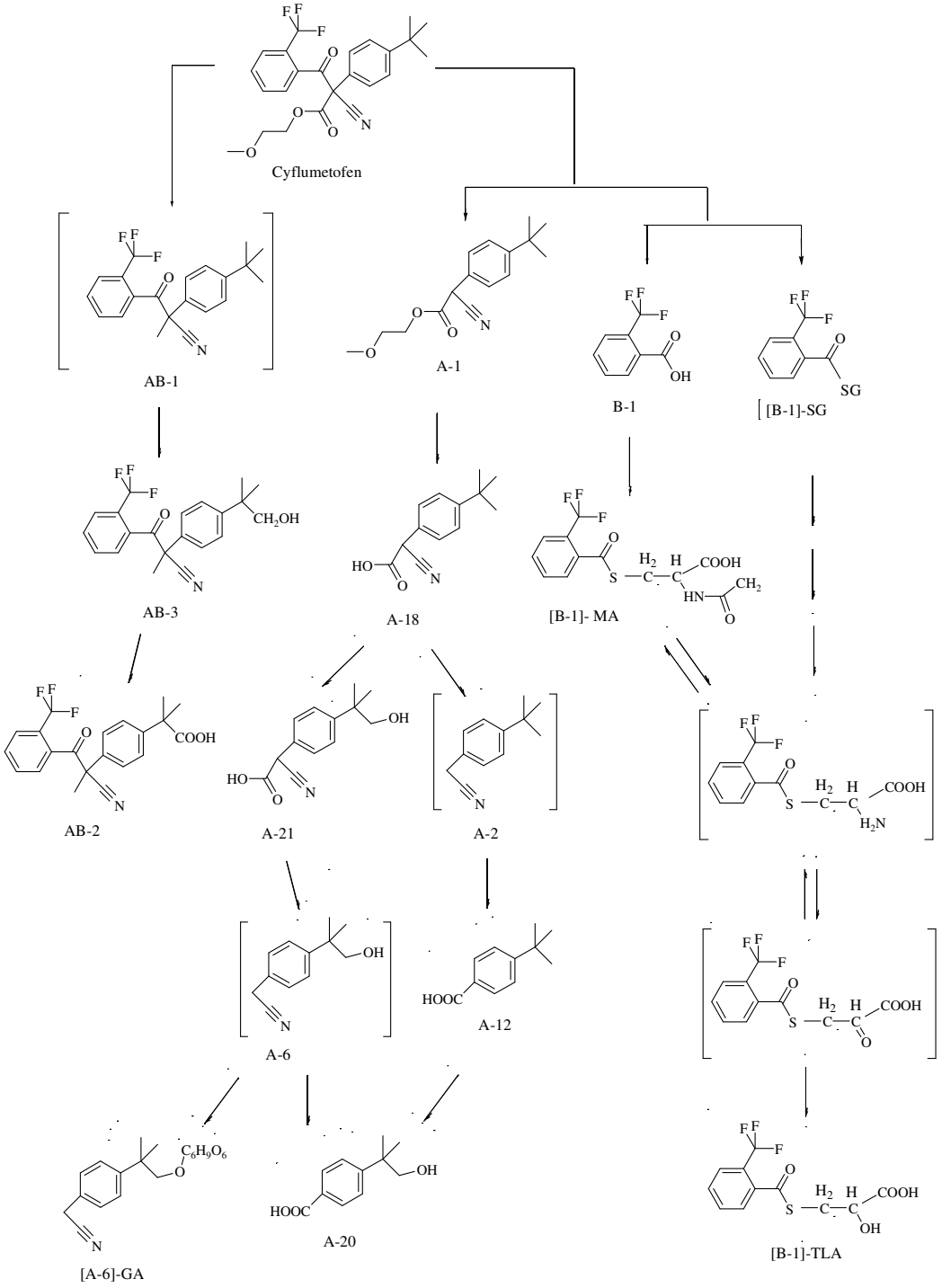
(a) Lethal doses

The acute oral toxicity of cyflumetofen (batch no. 01H1; purity 98.0%) was examined after a single dose administered by gavage to five fasted adult female Han Wistar rats (Table 3). Animals were observed for 14 days. No mortality occurred at 2000 mg/kg bw. Clinical signs were restricted to loose faeces from approximately 5 hours post-dosing in one female. No other apparent abnormalities were observed in any animal at necropsy at the termination of the study. Therefore, the oral median lethal dose (LD_{50}) of cyflumetofen in female rats was found to be greater than 2000 mg/kg bw (Moore, 2003a).

The acute dermal toxicity of cyflumetofen (batch no. 01H1; purity 98.0%) was assessed at 5000 mg/kg bw in five male and five female Han Wistar rats. There were no mortalities, no systemic clinical signs and no local dermal signs of toxicity. The median dermal LD_{50} of cyflumetofen in male and female rats was therefore greater than 5000 mg/kg bw (Moore, 2003b).

The acute inhalation toxicity of cyflumetofen (batch no. 01H1; purity 98.0%) after a 4-hour nose-only exposure was assessed at an actual concentration of 2.65 mg/L (maximum attainable concentration) in five male and five female Han Wistar rats, which were observed for 14 days post-exposure. During treatment, exaggerated breathing was evident in rats 15 minutes after the start of exposure. During the observation period, exaggerated breathing and brown staining around the snout/jaws were observed in rats immediately after exposure, and the exaggerated breathing persisted

Fig. 2. Metabolism of cyflumetofen in the rat



GA: glucuronic acid conjugate; SG: glutathione conjugate; MA: mercapturic acid conjugate; TLA: thiolactic acid conjugate; [ ]: possible intermediate

Table 3. Acute toxicity of cyflumetofen

Species	Strain	Sex	Route	LD_{50} (mg/kg bw)	LC_{50} (mg/L)	Purity (%)	Reference
Rat	Han Wistar	Female	Oral	> 2 000	–	98	Moore (2003a)
Rat	Han Wistar	Male, female	Dermal	> 5 000	–	98	Moore (2003b)
Rat	Han Wistar	Male, female	Respiratory	–	> 2.65	98	Bowden (2003)

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

to day 1 of the observation period. All rats were of normal appearance and behaviour from day 2 of the observation period to termination of the study. A slightly reduced mean body weight gain was apparent for males and females during the first week. During the second week, body weight gain was considered normal. The median respiratory lethal concentration (LC_{50}) of cyflumetofen in male and female rats was found to be greater than 2.65 mg/L. The test substance was of low toxicity via inhalation (Bowden, 2003).

(b) Dermal irritation

The primary dermal irritation potential of 0.5 g cyflumetofen (batch no. 01H1; purity 98.0%) was assessed in three male albino New Zealand White rabbits. Animals were treated for 4 hours and observed for 4 days after exposure. No dermal reactions were observed after exposure, and no sign of toxicity or ill-health was detected during the observation period (Rees, 2003a).

(c) Ocular irritation

The primary eye irritation potential of 0.1 g cyflumetofen (batch no. 01H1; purity 98.0%) per animal was tested in four (one in a preliminary study and three in the main study) female albino New Zealand White rabbits by instillation into the right eye of the animals. In the preliminary study, after a slight initial pain response after instillation, injection of the conjunctival blood vessel was observed during the first week after instillation. In the main study, instillation caused no or slight pain responses. Injection of the conjunctival blood vessel in two animals or, in a single case, a crimson red conjunctival appearance was evident during the first week after instillation, persisting in one case until day 15. Additionally, very slight discharge in two animals was seen 1 hour after instillation. Treated eyes of all animals were normal on day 22 (Rees, 2003b). Cyflumetofen was considered to be a slight eye irritant.

(d) Dermal sensitization

The skin sensitizing potential (Hooiveld, 2003) of cyflumetofen (batch no. 01H1; purity 98.0%) was assessed using the maximization assay with 15 female guinea-pigs (five animals were used as concurrent controls and 10 animals for the treatment group). The highest irritating dose for intradermal injection was 1% cyflumetofen (slight erythema); however, for topical application, 50% cyflumetofen did not reveal any irritation reactions in a preliminary irritation test. As no irritation was evident during topical induction, the application site was pretreated with 10% sodium dodecyl sulfate at 24 hours prior to topical induction in the main study. The same concentration (50% cyflumetofen) was used for the challenge application (as the highest non-irritating dose). At challenge, well defined to moderate erythema in 100% of females (10/10) was noted after 24 and 48 hours. In one and two females, scabbing was seen after 24 and 48 hours, respectively. Additionally, scaliness was observed in all females at 48 hours. Overall, cyflumetofen was considered to be a skin sensitizer. No mortality, symptoms of systemic toxicity or changes in body weight were observed.

2.2 Short-term studies of toxicity

(a) Oral administration

Mice

Cyflumetofen (batch no. 01D1; purity 97.67%) was administered for 28 days in the diet to groups of six male and six female 5-week-old ICR (Crj:CD-1) mice. The concentrations in feed were 0, 100, 500, 1000 and 5000 parts per million (ppm) (equal to 0, 13.1, 67.2, 135 and 663 mg/kg bw per day for males and 0, 14.5, 74.9, 150 and 763 mg/kg bw per day for females, respectively) (Yoshida, 2004d). Clinical observations of all animals were recorded daily. Body weights were measured at initiation and weekly thereafter, and feed consumption was measured on 4 consecutive days every week. Haematological analysis and clinical chemistry were performed on all animals after 4 weeks of treatment. At study termination, organ weights and macroscopy were investigated in all dose groups. Histological analysis of adrenals was performed in all animals at study termination.

No mortality and no treatment-related effects on clinical signs, body weight or feed consumption were noted. Haematological analysis revealed a statistically significantly reduced platelet count in females at 5000 ppm. However, a clear dose–response relationship was not apparent. Clinical chemistry showed a non-statistically significant increase in blood urea nitrogen (+14%) at 5000 ppm in females. Absolute and relative thyroid weights in males and uterine weights in females were non-statistically significantly reduced in all treatment groups; however, a clear dose–response pattern was absent. Absolute and relative adrenal weights were statistically significantly increased by 24% and 15% in males and by 21% and 19% in females, respectively, at 5000 ppm. Macroscopy showed no abnormalities. Histopathology showed diffuse vacuolation of adrenocortical cells in 1/6 males and 5/6 females at 5000 ppm, compared with 1/6 males and 0/6 females in the control group. Diffuse hypertrophy of adrenocortical cells was observed in 4/6 males and 6/6 females at 5000 ppm, compared with 0/6 males and 1/6 females in the control group. Additionally, 1/6 females exhibited subcapsular cell hyperplasia in the adrenals (versus 0/6 in controls and all other dose groups), which was, however, not observed in other short-term mouse studies.

Based on adrenocortical vacuolation in females and adrenocortical hypertrophy and increased adrenal weights in both sexes at 5000 ppm (equal to 663 mg/kg bw per day), the NOAEL was 1000 ppm (equal to 135 mg/kg bw per day) (Yoshida, 2004d).

Cyflumetofen (batch no. 01D1; purity 97.67%) was administered for 90 days in the diet to groups of 10 male and 10 female 5-week-old ICR (Crj:CD-1) mice. The concentrations in feed were 0, 300, 1000, 3000 and 10 000 ppm (equal to 0, 35.4, 117, 348 and 1200 mg/kg bw per day for males and 0, 45.0, 150, 447 and 1509 mg/kg bw per day for females, respectively) (Yoshida, 2004f). Clinical observations of all animals were recorded daily. Body weights and feed consumption were measured at initiation and weekly thereafter (feed consumption was measured on 4 consecutive days). Ophthalmology and urine analysis were not performed. Haematological analysis and clinical chemistry were performed on all animals after 13 weeks of treatment. At study termination, organ weights and macroscopy were investigated in all dose groups. Histological analysis of adrenals, ovaries and organs and tissues that revealed gross lesions at macroscopy was performed in all animals at study termination; the remaining organs and tissues were investigated only in the control and highest-dose groups at study termination.

No treatment-related effects on mortality, clinical signs, body weight, feed consumption or haematology were noted. Clinical chemistry analysis demonstrated statistically significantly reduced aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities at 1000, 3000 or 10 000 ppm (at this concentration, statistically significant for AST only) in males. As the mean AST and ALT activities in concurrent controls were, however, 2-fold higher than the historical control group means and no clear dose–response relationship could be deduced from the data, a relationship with treatment is unlikely. Absolute and relative adrenal weights were statistically significantly increased by 32% and 27% in males and by 11% and 23% (not statistically significant) in females at

10 000 ppm. Macroscopy revealed enlargement of the adrenals in 9/10 males at 10 000 ppm (compared with 0/10 in controls and all other treatment groups). Histopathology showed diffuse vacuolation of adrenocortical cells at 3000 ppm (2/10) and 10 000 ppm (7/10) in females (all other groups: 0/10) and diffuse hypertrophy of adrenocortical cells in 1/10 males at 3000 and 10 000 ppm (all other groups: 0/10).

Based on vacuolation and hypertrophy of the adrenal cortex in females and males, respectively, at 3000 ppm (equal to 348 mg/kg bw per day), the NOAEL was 1000 ppm (equal to 117 mg/kg bw per day) (Yoshida, 2004f).

Rats

Cyflumetofen (batch no. HN0101004; purity 98%) was administered for 14 days in the diet to groups of six male and six female 5-week-old Fischer (F344/DuCrj) rats. The concentrations in feed were 0, 1000 and 10 000 ppm (equal to 0, 101 and 981 mg/kg bw per day for males and 0, 105 and 1000 mg/kg bw per day for females, respectively) (Sakai, 2001). Clinical observations, body weights and feed consumption of all animals were recorded daily. No haematological analysis was done. Urine collection for analysis was performed overnight after 2 weeks of treatment. At study termination, clinical chemistry and organ weights were investigated in all dose groups. Histological analysis in all other organs was performed only in the control and high-dose groups.

No treatment-related effect on mortality was noted. No changes in clinical signs, body weight or feed consumption were noted at 1000 ppm. In females at 10 000 ppm, soiled perineal region, a statistically significant (≤ 10%) decrease in body weights (throughout the treatment period) and a statistically significant decrease in feed consumption (at the early and late treatment periods) were observed; in males at the same dose, a statistically significant decrease in feed consumption (in the absence of obvious body weight effects) was restricted to the first 2 days of treatment. Additionally, a statistically significant decrease in feed efficiency was noted in both sexes at the first day of treatment. There were no changes in urine analysis in any dose group of either sex.

Clinical chemistry revealed statistically significant decreases in alkaline phosphatase, AST, ALT and creatine phosphokinase activities and a significantly higher albumin/globulin ratio in males at 1000 and 10 000 ppm. Additionally, a statistically significant increase in total protein and albumin levels and a decrease in total cholesterol, triglyceride and total bilirubin levels were noted in males at 10 000 ppm. Females at 10 000 ppm showed a statistically significant decrease in creatine phosphokinase, creatine and globulin and a statistically significant increase in albumin and albumin/globulin ratio. In females at 1000 ppm, a statistically significant decrease in triglyceride and total bilirubin levels was observed, and a decreasing trend (although not statistically significant) was also detected in females at 10 000 ppm. In males, organ weight analysis revealed a statistically significant increase in relative liver and adrenal weights at 1000 and 10 000 ppm, which was paralleled by statistically significantly higher absolute liver and adrenal weights at 10 000 ppm. Furthermore, in males, a statistically significant decrease in absolute and relative thymus weights and a statistically significant increase in relative testis (right side only) and kidney weights were noted. In females, a statistically significant increase in relative and absolute adrenal weights at 1000 and 10 000 ppm and a statistically significant increase in relative kidney and liver weights were observed at 10 000 ppm. Moreover, in females, absolute spleen weight was statistically significantly decreased at 10 000 ppm. At macroscopy, hypertrophy of the adrenals was observed in all males and females at 10 000 ppm and in 5/6 females at 1000 ppm (versus 0/6 in controls).

Histopathological examination showed, in all males and females at 1000 and 10 000 ppm, diffuse vacuolation of cortical cells in adrenals and diffuse hypertrophy of hepatocytes in the liver (versus none in controls). Vacuolation of interstitial gland cells was observed in all females at 1000 and 10 000 ppm, and vacuolation of corpora lutea in the ovary was additionally noted in all females at 10 000 ppm only (compared with none in controls).

Based on changes in clinical chemistry, an increase in liver and adrenal weights, hypertrophy of the adrenals in females and histopathological alterations in the adrenal cortex in both sexes and in

the ovary at the lowest dose of 1000 ppm (equal to 101 mg/kg bw per day), no NOAEL was derived from this study (Sakai, 2001).

Cyflumetofen (batch no. 01C1; purity 99.3%) was administered for 28 days in the diet to groups of six male and six female 5-week-old Fischer (F344/DuCrj) rats. The concentrations in feed were 0, 100, 500, 1000 and 5000 ppm (equal to 0, 7.5, 37.6, 75.1 and 384 mg/kg bw per day for males and 0, 8.05, 40.8, 79.8 and 409 mg/kg bw per day for females, respectively) (Yoshida, 2004e). Clinical observations of all animals were made daily, whereas body weights and feed consumption of all animals were measured weekly. Urine collection for analysis was performed after 4 weeks of treatment. At study termination, haematology, clinical chemistry, organ weights and macroscopy were investigated in all dose groups. Histological analysis of selected organs (liver, kidneys, pituitary, adrenals, testes, ovaries) was performed in all animals at study termination. In order to characterize vacuolations observed in the adrenals of both sexes and in the ovaries of females, some animals of the control and highest-dose groups were stained with Oil Red O.

There were no deaths or treatment-related changes in general clinical observations, body weights, feed consumption or haematology in any dose groups. In females, urine analysis revealed a statistically significant increase in ketones at 5000 ppm. Clinical chemistry showed statistically significant increases in blood urea nitrogen (+15% compared with controls) and potassium (+7% compared with controls) at 5000 ppm in males, statistically significant decreases in total cholesterol and triglycerides at 1000 and 5000 ppm in males and in total cholesterol at 5000 ppm in females, and a statistically significant decrease in sodium (−5% compared with controls) at 5000 ppm in females. Additionally, a slight, statistically significant decrease in alkaline phosphatase activity was noted in males at 5000 ppm. Absolute and relative liver weights were statistically significantly increased at 1000 ppm in males (+10% and +8% compared with controls, respectively) and at 5000 ppm in both sexes (males: +29% and +31% compared with controls, respectively; females: +16% compared with controls for both parameters). Absolute and relative kidney weights were statistically significantly increased at 5000 ppm in both sexes (≤ 14% increase compared with controls for both sexes and parameters). Relative adrenal weight was elevated by 13% and 15% at 1000 ppm in males and females, respectively (statistically significant in females only). Both absolute and relative adrenal weights were statistically significantly increased at 5000 ppm in males (+36% and +33% compared with controls, respectively) and females (+100% compared with controls for both parameters). Macroscopy exhibited enlarged and discoloured adrenals in all animals in both sexes at 5000 ppm (versus none in controls). Histopathology revealed diffuse hypertrophy of hepatocytes in all males at 1000 ppm and in all animals of both sexes at 5000 ppm (compared with 0/6 in controls). Diffuse vacuolation of adrenocortical cells was observed in all animals of both sexes at 1000 and 5000 ppm and was paralleled by diffuse hypertrophy in all females at 1000 and 5000 ppm (compared with 0/6 in controls). Vacuolation of interstitial cells in the ovaries was observed in 2/6 females at 1000 ppm and 6/6 females at 5000 ppm compared with 0/6 in controls. The vacuolation in the adrenals of rats of both sexes and in the ovaries of female rats was demonstrated to be lipid deposition.

Based on decreased serum cholesterol and triglyceride concentrations and increased absolute and relative liver weights in males, increased relative adrenal weights in both sexes and histopathology in the liver (males), adrenals (both sexes) and ovaries at 1000 ppm (equal to 75.1 mg/kg bw per day), the NOAEL was 500 ppm (equal to 37.6 mg/kg bw per day) (Yoshida, 2004e).

Cyflumetofen (batch number and purity not provided) was administered for 26 days in the diet to groups of five male and five female CRL:WI(Han) rats. The concentrations in feed were 0, 500, 1500, 4000 and 12 000 ppm (equal to 0, 43, 128, 339 and 1028 mg/kg bw per day for males and 0, 46, 132, 351 and 1039 mg/kg bw per day for females, respectively) (Buesen, 2010). No details on clinical observations were reported. Feed consumption and body weight were determined on study days 0 (body weight only), 3, 7, 14, 21 and 26 for all animals. Clinical pathology (urine analysis, clinical chemistry, haematology) and ophthalmology were not performed. At study termination, organ

weights and histopathology of adrenal, ovary and liver were measured or investigated in all animals. Macroscopy was performed on all animals; however, details were not reported. This range-finding study was conducted to determine the susceptibility of Wistar rats to cyflumetofen toxicity.

Body weight was statistically significantly decreased in males (maximum of −9% on day 26) and in females (maximum of −10% on day 26) at 12 000 ppm. Moreover, body weight change was statistically significantly decreased in males during the treatment period (maximum −27% on day 3) and in females (maximum −59% on day 3), reaching statistical significance towards the end of the study at 12 000 ppm. Body weight gain was also statistically significantly decreased in males on day 3 (−22%) at 4000 ppm. Feed consumption was decreased in females (statistically significantly at the beginning and end of the study) and males at 12 000 ppm during the treatment period. Statistically significant increases in absolute and relative adrenal weights were observed starting from 500 ppm in females and from 4000 ppm in males, reaching maximal increases relative to controls in females (absolute: +82%; relative: +101%) and males (absolute: +69%; relative: +87%) at 12 000 ppm. Additionally, statistically significant increases in absolute and relative liver weights in males were noted starting from 4000 ppm onwards and in females at 12 000 ppm. In females, statistically significant increases in absolute and relative ovary weights at 4000 ppm (absolute: +29%; relative: +34%) and 12 000 ppm (not statistically significant) were observed. Enlarged and/or discoloured adrenals were seen at 4000 and 12 000 ppm in both sexes. Additionally, discoloration of the ovaries was noted in all females, and a focus (not specified in detail) in the liver was detected in one female at 12 000 ppm.

Centrilobular, hepatocellular hypertrophy in the liver of all males and in one female at 4000 ppm and in all males and three females at 12 000 ppm was noted. Adrenocortical cell vacuolation in males (3/5) and females (3/5) at 1500 ppm and in all animals at 4000 and 12 000 ppm was identified. Finally, vacuolation of luteal cells in the ovary was detected at 4000 ppm (4/5) and 12 000 ppm (5/5) in females.

Based on the increased incidence of adrenal cell vacuolation in both sexes and an increase in absolute and relative adrenal weights in females at 1500 ppm (equal to 128 mg/kg bw per day), the NOAEL was 500 ppm (equal to 43 mg/kg bw per day) (Buesen, 2010).

Cyflumetofen (batch no. 01D1; purity 97.67%) was administered for 90 days in the diet to groups of 10 male and 10 female 6-week-old Fischer (F344/DuCrj) rats. The concentrations in feed were 0, 100, 300, 1000 and 3000 ppm (equal to 0, 5.4, 16.5, 54.5 and 167 mg/kg bw per day for males and 0, 6.28, 19.0, 62.8 and 193 mg/kg bw per day for females, respectively) (Yoshida, 2004g). Detailed clinical observations, body weights and feed consumption were recorded for all animals prior to initiation of treatment and weekly thereafter. A functional observational battery analysis was performed on all animals at week 11. Ophthalmology, urine analysis, haematology and clinical chemistry were performed at 13 weeks. Macroscopic and organ weight investigations were done in all test groups at termination. Histological analysis of selected organs (liver, kidneys, adrenals, ovaries and gross lesions identified during macroscopy) was performed for all animals, and the remaining tissues and organs were investigated in the control and highest-dose groups only at study termination. Additionally, electron microscopic analysis of the left adrenal (both sexes) and left ovary (females) from a single animal per dose group was performed.

No treatment-related effects on mortality, body weight, feed consumption, functional observations or ophthalmoscopy were noted. Clinical observations revealed a statistically significant decrease in rearing in males: 6/10 at 100 ppm (not statistically significant), 3/10 at 300 ppm, 2/10 at 1000 ppm and 0/10 at 3000 ppm, compared with 9/10 in controls at 1 week of treatment. However, as the decreased incidences were observed only in the first week of treatment and not thereafter, this was not considered to be an adverse effect. Haematology showed a statistically significantly elongated prothrombin time in males at 3000 ppm (+17%) and a statistically significantly decreased total leukocyte count in females receiving 1000 or 3000 ppm. Clinical chemistry exhibited a slight, statistically significant reduction in globulin and a slight, statistically significant increase in albumin/globulin ratio at 1000 and 3000 ppm in females. Absolute liver weight was statistically

significantly increased at 3000 ppm in males (+8%), and relative liver weight was statistically significantly increased at 1000 ppm in males (+4%) and at 3000 ppm in both sexes (+12% in males; +6% in females). Relative kidney weight was slightly, but statistically significantly, increased at 3000 ppm in both sexes. Relative adrenal weight was statistically significantly increased at 1000 ppm (+8%) and 3000 ppm (+42%) in females and was paralleled by a statistically significant increase in absolute adrenal weight (+42%) compared with controls at 3000 ppm in the same sex. Macroscopy and histopathology confirmed the toxicological effects on the adrenals; enlarged and discoloured adrenals were observed at 3000 ppm in all females (compared with 0/10 in all other groups), and diffuse hypertrophy of adrenocortical cells was noted at 1000 and 3000 ppm in all females (compared with 0/10 in all other groups). Mild to moderate diffuse vacuolation of adrenocortical cells was observed in males at 1000 ppm (6/10) and 3000 ppm (10/10), compared with 0/10 in all other groups. Additionally, mild to moderate vacuolation of interstitial gland cells in the ovaries was seen at 1000 ppm (1/10) and 3000 ppm (8/10), compared with 0/10 in all other groups.

Based on increased relative adrenal weights in females, vacuolation and hypertrophy of the adrenal cortex in males and females, respectively, and vacuolation of ovarian interstitial cells at 1000 ppm (equal to 54.5 mg/kg bw per day), the NOAEL was 300 ppm (equal to 16.5 mg/kg bw per day) (Yoshida, 2004g).

Dogs

Cyflumetofen (batch no. 01H1; purity 98.4%) was administered for 28 days in gelatine capsules to groups of three male and three female 6-month-old Beagle dogs at a dose level of 0, 100, 300 or 1000 mg/kg bw per day (Nagashima, 2003a). Detailed clinical observations were performed at initiation of dosing and 3 times a day thereafter, body weights were measured before initiation of treatment and weekly thereafter and feed consumption of all animals was measured prior to initiation of treatment and daily thereafter. Ophthalmology was investigated before initiation of dosing and at week 3, and urine analysis, haematology and clinical chemistry were performed after 4 weeks of treatment. Organ weights, macroscopy and histopathology were performed in all groups at termination.

No treatment-related effects on mortality, body weight, feed consumption, ophthalmology, haematology, urine analysis or macroscopy were observed. Clinical observations revealed faeces with whitish appearance (probably test substance) in all animals at 1000 mg/kg bw per day during the treatment period and occasionally in two males (weeks 3 and 4) and two females (week 2) at 300 mg/kg bw per day. Clinical chemistry showed a slight increase in potassium and calcium levels at 300 and 1000 mg/kg bw per day in males, with a doubtful dose–response correlation. Increases in absolute and relative adrenal weights were noted in one male and in one female (absolute weight only was increased in a second female) at 300 mg/kg bw per day and in two animals of each sex at 1000 mg/kg bw per day. Macroscopy indicated dark red foci (designated as capillary dilatation) on the right atrioventricular valve of the heart in one female at 1000 mg/kg bw per day. Histopathology revealed fine vacuoles in adrenocortical cells (zona fasciculata and zona reticularis) in two males and three females at 300 mg/kg bw per day and in all animals at 1000 mg/kg bw per day. Fine vacuoles were also observed in the zona glomerulosa in one male at 300 mg/kg bw per day and in two animals of each sex at 1000 mg/kg bw per day.

Based on increased absolute and relative adrenal weights, fine vacuolation of adrenocortical cells in both sexes and dark red foci on the right atrioventricular valve of the heart in one female at 300 mg/kg bw per day, the NOAEL was 100 mg/kg bw per day (Nagashima, 2003a).

Cyflumetofen (batch no. 01H1; purity 98.4%) was administered for 90 days (males) and 91 days (females) in gelatine capsules to groups of four male and four female 6-month-old Beagle dogs at a dose level of 0, 30, 300 or 1000 mg/kg bw per day (Nagashima, 2003b). Detailed clinical observations were performed before initiation of dosing and 3 times a day thereafter, body weights were measured before initiation of treatment and weekly thereafter and feed consumption of all

animals was measured prior to initiation of treatment and daily thereafter. Ophthalmology was investigated before initiation of dosing and at weeks 7 and 12, and urine analysis, haematology and clinical chemistry were performed prior to treatment and at weeks 7 and 13 of treatment. Organ weights, macroscopy and histopathology were performed in all groups at termination of the study.

No treatment-related effects on mortality, feed consumption, ophthalmology, clinical chemistry or urine analysis were noted. Clinical observations indicated faeces with whitish appearance (probably test substance) 1–7 times per week in all animals at 1000 mg/kg bw per day during the treatment period and occasionally in one male and all females at 300 mg/kg bw per day. Body weight gain, from day 1 to day 90, was reduced by 64% and 36% of control values at 1000 mg/kg bw per day in males and females, respectively. Urine analysis exhibited occult blood (accompanied by the observation of erythrocytes in urinary sediment) in 1/4 females at 300 mg/kg bw per day, 1/4 females at 1000 mg/kg bw per day at week 7 and 1/4 females at the highest dose at week 13. It was assumed that the blood finding in urine was attributed to estrous bleeding, which was observed around the same time. Haematology showed a statistically significant increase in monocyte ratios relative to the control group in males at 30 mg/kg bw per day (+61%), 300 mg/kg bw per day (+57%; not statistically significant at this dose) or 1000 mg/kg bw per day (+67%) at week 13. However, as values of treatment groups were similar to pretest values on an individual level and as a clear dose–response correlation was not apparent, findings might be considered to be incidental. A statistically significant increase in blood urea nitrogen (+42%) relative to the control group was observed at 1000 mg/kg bw per day in females, and a statistically significant increase in γ-globulin was noted at 30 mg/kg bw per day (+22%) and 1000 mg/kg bw per day (+22%) in males. As the latter (γ-globulin increase) did not show a clear dose–response relationship and as the values were already considerably elevated at pretest compared with controls, a relationship with treatment was questionable. Macroscopy indicated dark red foci (designated as capillary dilatation) on the right atrioventricular valve of the heart, dark red foci in the mucosa of the urinary bladder and enlargement of the adrenals in one male at 1000 mg/kg bw per day. Absolute and relative adrenal weights were increased (right/left: +26%/+37% and +30%/+40%, respectively) relative to controls in males at 1000 mg/kg bw per day. Additionally, absolute and relative pituitary weights were statistically significantly increased at 300 mg/kg bw per day (+40% for both) and 1000 mg/kg bw per day (+28% and +40%, respectively) relative to controls in females. A non-statistically significant increase in absolute and relative testis weights (pronounced in the right testis: about 130% of control values for both parameters) was also observed at 1000 mg/kg bw per day. High incidences of fine (all three zonae) and large (zona fasciculata only) vacuoles of adrenocortical cells (categorized as slight to mild) were observed in females (fine vacuoles: 2/4; large vacuoles: 3/4) and males (fine vacuoles: 4/4; large vacuoles: 3/4) at 1000 mg/kg bw per day. Large vacuoles (slight in the degree of severity) within the zona fasciculata were also noted in lower-dose groups (30 and 300 mg/kg bw per day): 1/4, 2/4, 2/4 and 3/4 in females and 0/4, 1/4, 0/4 and 3/4 in males at 0, 30, 300 and 1000 mg/kg bw per day, respectively.

Based on reduced body weight gain in both sexes, increased absolute and relative adrenal and testis weights in males and pituitary weights in females, high incidences of vacuolation of the adrenal cortex in both sexes and dark red foci on the right atrioventricular valve of the heart in one male at 1000 mg/kg bw per day, the NOAEL was 300 mg/kg bw per day (Nagashima, 2003b).

Cyflumetofen (batch no. 01H1; purity 98.4%) was administered for 1 year in gelatine capsules to groups of four male and four female 6-month-old Beagle dogs at a dose level of 0, 30, 300 or 1000 mg/kg bw per day (Nagashima, 2008). Detailed clinical observations were performed before initiation of dosing and 3 times a day thereafter. Body weights were measured before initiation of treatment, then weekly until week 14 and every fortnight thereafter. Feed consumption of all animals was measured prior to initiation of treatment and daily thereafter. Ophthalmology was investigated before initiation of dosing and at weeks 26 and 52, and urine analysis, haematology and clinical chemistry were performed before initiation of the study and at weeks 26 and 52 of treatment. Organ weights, macroscopy and histopathology were performed in all groups at termination of the study.

No treatment-related effects on mortality, body weight or feed consumption were noted. Clinical observations indicated faeces with white-yellowish appearance (probably test substance) 1–7 times per week in all animals during the treatment period at 1000 mg/kg bw per day and sporadically in all animals during the treatment period at 300 mg/kg bw per day. Additionally, soft faeces were frequently observed from week 2 to week 4 in one male at 1000 mg/kg bw per day. Urine analysis showed occult blood in one female at 300 and 1000 mg/kg bw per day and erythrocytes in urinary sediment at 1000 mg/kg bw per day at week 26, which are considered to be due to estrous bleeding. Haematology indicated a statistically significant transient increase (+66%) in leukocyte counts in females at 1000 mg/kg bw per day at week 26. Clinical chemistry showed a decrease in triglyceride concentrations in males (−40% and −31% relative to controls at weeks 26 and 52, respectively) and females (−43% relative to controls at week 52 only) at 1000 mg/kg bw per day. Absolute and relative adrenal weights were increased relative to controls in males (right/left: +40%/+43% and +47%/+51%, respectively) and females (right/left: +68%/+64% and +68%/+66%, respectively) at 1000 mg/kg bw per day. Additionally, absolute and relative pituitary weights were increased at 1000 mg/kg bw per day (+22% and +21%, respectively) relative to controls in females. Absolute and relative liver weights were decreased by 19% (statistically significantly) and 15%, respectively, in males, and absolute and relative prostate weights were reduced by 40% (statistically significantly) and 37%, respectively, at 1000 mg/kg bw per day. Enlargement of the adrenals was observed in one female at 300 mg/kg bw per day and in one male and all females at 1000 mg/kg bw per day. Additionally, dark red foci on the right atrioventricular valve of the heart of one male were observed at 30 and 1000 mg/kg bw per day. Histopathology revealed slight swelling of interstitial cells in the testes in one male at 1000 mg/kg bw per day. Fine and/or large vacuolation of adrenocortical cells, degeneration of cortical cells, interstitial fibrosis and infiltration of brown pigment-laden macrophages or cell infiltration (mainly lymphocytes) in the adrenals were observed in both sexes at 300 and 1000 mg/kg bw per day (see Table 4). Fine and large vacuolations (designated as slight and occasionally mild) in adrenocortical cells were also noted in both sexes at 30 mg/kg bw per day and in females of the control group (large vacuolations categorized as slight only). However, vacuolations were of a lower degree of severity and not accompanied by degenerative processes, compared with the 300 and 1000 mg/kg bw per day dose groups. Whereas fine vacuolations were observed in all zonae of the adrenals, large vacuolations and degenerative reactions were confined to the zona fasciculata and zona reticularis (not to the zona glomerulosa).

Based on increased incidences of vacuolations accompanied by degenerative processes (e.g. interstitial fibrosis and infiltration of brown pigment–laden macrophages) at 300 mg/kg bw per day, the NOAEL was 30 mg/kg bw per day (Nagashima, 2008).

(b) Dermal application

Cyflumetofen (batch no. 01H1; purity 98.4%) was administered for 28 days by the dermal route (6 hours/day, 5 days/week, semi-occlusive dressing) to groups of 10 male and 10 female 9-week-old CRL:WI(Han) rats at a dose level of 0, 100, 300 or 1000 mg/kg bw per day (Buesen et al., 2010b). Detailed clinical observations of all animals were performed and feed consumption of all animals was measured prior to initiation of treatment and weekly thereafter. Body weight was measured prior to and at the start of treatment and twice weekly thereafter. A functional observational battery analysis was performed on all animals at the end of the study. Ophthalmology was performed at the end of the study on the control and highest-dose groups, and urine analysis, haematology and clinical chemistry of all animals were performed at the end of treatment. Macroscopy and organ weight investigations were done in all animal groups at termination. Histological analysis of selected organs was performed in the control and highest-dose groups at study termination.

No treatment-related effects on mortality, clinical signs, body weight, feed consumption, functional observational battery or clinical pathology were noted. In addition, no local dermal irritation was observed. The only (minor) finding in organ pathology was confined to a slight, statistically significant increase in absolute kidney weight in males at 300 mg/kg bw per day (+10%)

and 1000 mg/kg bw per day (+8%) relative to controls and a slight, statistically significant increase in relative liver weight in males at 1000 mg/kg bw per day (+8%) relative to controls.

As no adverse systemic (or local) treatment-related effects were observed, the NOAEL was 1000 mg/kg bw per day, the highest dose tested, for male and female rats (Buesen et al., 2010b).

Table 4. Histopathology of the adrenals in the 1-year dog study (four dogs of each sex per dose)

	0 mg/kg bw per day		**30 mg/kg bw per day**		**300 mg/kg bw per day**		**1 000 mg/kg bw per day**	
	M	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Focal cell infiltration (total)	–	–	–	–	1	2	1	3
Slight	–	–	–	–	1	1	1	3
Mild	–	–	–	–	–	1	–	–
Interstitial fibrosis (total)	–	–	–	–	–	2	1	3
Slight	–	–	–	–	–	2	1	3
Fine vacuolation in cortical cells (total)	–	–	1	2	4	4	3	4
Slight	–	–	–	1	1	2	–	–
Mild	–	–	1	1	3	2	1	–
Moderate	–	–	–	–	–	–	2	4
Large vacuolation in cortical cells (total)	–	4	1	3	2	4	4	4
Slight	–	4	1	2	2	1	2	1
Mild	–	–	–	1	–	3	2	2
Moderate	–	–	–	–	–	–	–	1
Degeneration in cortical cells[a] (total)	–	–	–	–	1	2	2	1
Slight	–	–	–	–	1	2	1	–
Mild	–	–	–	–	–	–	1	1
Pigment-laden macrophages (slight)	–	–	–	–	1	–	1	–

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

[a] Enlarged cortical cells filled with cytoplasmic vacuoles, brown pigment in cytoplasm and karyorrhexis-like figure of the cortical cells.

Source: Nagashima (2008)

2.3 Long-term studies of toxicity and carcinogenicity

Mice

Cyflumetofen (batch no. 01D1; purity 97.67%) was administered for 78 weeks in the diet to groups of 52 male and 52 female 5-week-old ICR (Crj:CD-1) mice. The concentrations in feed were 0, 150, 500, 1500 and 5000 ppm (equal to 0, 15.5, 54.3, 156 and 537 mg/kg bw per day for males and 0, 14.3, 48.1, 144 and 483 mg/kg bw per day for females, respectively) (Yoshida, 2004a). All animals were checked for mortality and subjected to general clinical observations (at least twice a day) and body weight and feed consumption measurements (at initiation of treatment, once weekly from week 1 to week 13 and once every 4 weeks from week 16 onwards). Detailed clinical observations were recorded at least weekly during treatment. Blood sampling was performed at week 52, on surviving animals at week 78 (haematological analysis was not performed at week 52, as no pronounced effects were observed at week 78) and on all animals killed in extremis during treatment. No urine analysis or clinical chemistry investigations were done. Selected organ weight analysis (10 animals of each sex per dose) and macroscopy were performed in all dose groups (including all animals killed or found dead). Histopathology was analysed in the control and highest-dose groups at terminal kill after 78 weeks or in all animals (regardless of the dose group) killed in extremis during treatment. Histopathological analysis was additionally performed on the adrenal glands and on all gross lesions in the 150, 500 and 1500 ppm groups.

No effects on mortality, clinical signs, body weight or feed consumption were noted. Haematology revealed a statistically significant decrease in large unstained cells in males (−34% relative to controls) at 5000 ppm. Large unstained cells are normally considered to include immature cells or tumour cells; therefore, reductions in cell count are considered to be of unclear toxicological relevance. A non-statistically significant increase in absolute adrenal weights by 20% relative to the control group was noted. Macroscopy in males killed in extremis or found dead showed a statistically significant increase in ascites of the abdominal cavity (4/15 versus 0/15) at 5000 ppm. Histopathology indicated a statistically significant increase in the incidence of diffuse vacuolation of cortical cells in the adrenals at 5000 ppm in males (8/52 versus 1/52 in controls) and females (26/52 versus 7/52 in controls). The incidences of diffuse/focal hypertrophy of adrenals were similar to those in the control group. There were no treatment-related neoplastic changes.

Cyflumetofen showed no carcinogenic potential. Based on an increase in adrenal weights (most predominant in females) and vacuolation in the adrenal cortex in both sexes at 5000 ppm (equal to 483 mg/kg bw per day), the NOAEL for systemic toxicity was 1500 ppm (equal to 144 mg/kg bw per day) (Yoshida, 2004a).

Cyflumetofen (batch no. 09/0510-3 (009025); purity 97.82%) was administered for 78 weeks in the diet to groups of 52 male and 52 female 5-week-old ICR (Crj:CD-1) mice. The concentrations in feed were 0 and 10 000 ppm (equal to 0 and 1143 or 1132 mg/kg bw per day for males and females, respectively) (Yoshida, 2013). All animals were checked for mortality and subjected to general clinical observations (at least twice a day) and body weight and feed consumption measurements (at initiation of treatment, once weekly from week 1 to week 13 and once every 4 weeks from week 16 onward). Detailed clinical observations were recorded at least weekly during treatment. Blood sampling was performed at week 52, on surviving animals at week 78 (haematological analysis was not performed at week 52, as no pronounced effects were observed at week 78) and on all animals killed in extremis during treatment. No urine analysis or clinical chemistry investigations were done. Selected organ weight analysis, macroscopy and haematology were performed in all animals (including all animals killed or found dead) at study termination.

The following treatment-related effects were observed at 10 000 ppm:

- Mortality was statistically significantly higher in treated females than in control females at weeks 59 and 64–78 (3/52 and 17/52 for females in the 0 and 10 000 ppm groups, respectively, at week 78). However, the increased mortality rates observed for females were

within the historical control range (range: 9/52–23/52; mean: 13/52; number of studies at the Institute of Environmental Toxicology [IET] laboratory in Ibaraki, Japan: 6).

- Clinical signs were confined to a statistically significant increase in the incidences of decreased spontaneous motor activity, bradypnoea, pale-coloured skin and pale-coloured eye/eyelid in females.
- Haematologically, males showed a statistically significant increase in total leukocyte count (+35% relative to the control group).
- Females exhibited statistically significant increases in absolute and relative weights of the adrenals (+34% and 42% relative to controls, respectively) and statistically significant increases in the incidences of soiled fur in the external genital region (5/52 versus 0/52 in controls), enlargement of the spleen (10/52 versus 3/52 in controls) and spot(s) in the liver (5/52 versus 0/52 in controls).

No treatment-related neoplastic lesions were observed.

Non-neoplastic histopathology showed a statistically significant increase in the incidences of diffuse vacuolation of cortical cells in males (21/52 versus 0/52 in controls) and females (41/52 versus 2/52 in controls) and of deposition of brown pigment in the corticomedullary junction of the adrenal gland in males (14/52 versus 6/52 in controls) and females (44/52 versus 27/52 in controls). No treatment-related increase in diffuse/focal hypertrophy of adrenals was observed.

Additionally, a statistically significant increase in the incidence of extramedullary haematopoiesis in the spleen was noted in females (14/52 versus 5/52 in controls).

In conclusion, there was no evidence of carcinogenic potential at 10 000 ppm. Cyflumetofen-induced treatment-related findings were restricted to clinical signs in females and effects on the adrenals (e.g. increased adrenal weights, diffuse vacuolation of cortical cells and brown pigment depositions in both sexes) and the spleen (enlargement and haematopoiesis in females) (Yoshida, 2013).

Rats

Cyflumetofen (batch no. 01D1; purity 97.67%) was administered for 52 weeks in the diet to groups of 50 male and 50 female 5- or 6-week-old Fischer (F344/DuCrj) rats. The concentrations in feed were 0, 50, 150, 500 and 1500 ppm (equal to 0, 1.9, 5.6, 18.8 and 56.8 mg/kg bw per day for males and 0, 2.3, 6.9, 23.3 and 69.2 mg/kg bw per day for females, respectively) (Yoshida, 2004b,c). A main group of 20 animals of each sex per dose was terminated after 52 weeks, and 10 animals of each sex per dose from a satellite group of 30 animals of each sex per dose were terminated after 4, 13 and 26 weeks of administration. All animals in the main and satellite groups were checked for mortality (twice a day) and subjected to general clinical observations (daily) and body weight measurements (at initiation of treatment, once weekly from week 1 to week 13 and once every 4 weeks from week 16 onwards). Detailed clinical observations were recorded in the main study before study initiation and weekly during treatment, and feed consumption of the main group was measured once weekly from week 1 to week 13 and once every 4 weeks from week 16 onwards. The animals in the main group were subjected to functional observations at 49 weeks of treatment (10 animals of each sex per group), and ophthalmology was performed before study initiation and in week 52 in all animals in the control and 1500 ppm groups. Haematology, clinical chemistry, urine analysis, organ weight measurements, macroscopy and histopathology were performed at termination on 10 animals of each sex per dose of the satellite group after 4, 13 and 26 weeks and on 10 animals of each sex per dose (except for macroscopy and histopathology: 20 animals of each sex per dose) of the main group in week 52. Histopathology was performed on selected organs (liver, kidneys, adrenals, ovaries and all gross lesions) of animals in all groups and on the remaining organs of all animals of the control and highest-dose groups. (Organ pathology generally also included those animals found dead or killed during the treatment period.)

No effects on mortality, body weight, feed consumption, ophthalmoscopy or macroscopy were noted. Haematological examinations showed a slight, statistically significant decrease in platelet count in males at 1500 ppm after 4 and 26 weeks of treatment and in females after 13 weeks.

Additionally, a slight, statistically significant decrease in fibrinogen concentration was detected in males at 1500 ppm after 13 weeks and in females from 150 ppm onwards after 52 weeks (however, a clear dose–response relationship was not apparent). Clinical chemistry revealed statistically significant decreases in blood urea nitrogen at 50 ppm and higher in males (a maximum 11% reduction relative to controls), in total protein and albumin concentrations in females from 150 ppm onwards (a maximum 7% reduction relative to controls) and in globulin concentrations at 500 ppm and higher (a maximum 7% reduction relative to controls) in females after 13 weeks, without a clear correlation with dose. Additionally, total cholesterol and triglyceride concentrations were statistically significantly decreased from 150 ppm onwards (except for the decrease in triglyceride concentration at 1500 ppm, which was not statistically significant), with highest reductions of 12% (at 150 ppm) and 33% (at 500 ppm) relative to controls, respectively, in females after 13 weeks. The only statistically significant, pronounced change in electrolytes in several dose groups was a transient decrease in calcium levels from 150 ppm and higher in females (–9% at most) after 13 weeks (no clear dose–response relationship was apparent, however). Slight, statistically significant increases in absolute and relative liver weights in males at 1500 ppm after 4 weeks and in relative liver weight also at 13 and 26 weeks were observed. Relative kidney weight was slightly but statistically significantly increased in males after 13 weeks and in females after 52 weeks at 1500 ppm. In addition, females in this group showed statistically significant increases in absolute and relative adrenal weights at almost all time points (with maximal increases of 15% and 17%, respectively). Finally, a statistically significant decrease in absolute weight of epididymides was observed in males at 1500 ppm after 26 weeks. Non-neoplastic histopathological findings in males were restricted to an increased incidence of hepatocellular diffuse hypertrophy in the liver after 4 weeks, high incidences of diffuse vacuolation (incidence range over all time points: 42–100%) of adrenocortical cells at all time points at 1500 ppm and diffuse hypertrophy of adrenocortical cells in 1/19 males after 52 weeks at the same dose. Additionally, all females examined at 1500 ppm revealed diffuse hypertrophy of adrenocortical cells at all time points.

Vacuolation of interstitial gland cells in the ovaries was observed in 10–30% of females at 1500 ppm at each time point except after 4 weeks.

Based on an increase in absolute and relative adrenal weights in females, an increase in liver weights in males, a reduction in total cholesterol and triglyceride concentrations in both sexes, adrenocortical vacuolations in males and adrenocortical hypertrophy (pronounced in females), and vacuolations in ovary cells at 1500 ppm (equal to 56.8 mg/kg bw per day), the NOAEL for systemic toxicity was 500 ppm (equal to 18.8 mg/kg bw per day) (Yoshida, 2004b,c).

Cyflumetofen (batch no. 09/0510-3 (009025); purity 97.82%) was administered for 52 weeks in the main group (20 animals of each sex per dose) and for up to 26 weeks in the satellite group (30 animals of each sex per dose) in the diet to 5-week-old Fischer (F344/DuCrj) rats. The concentrations in feed were 0 or 6000 ppm (equal to 0 and 250 or 319 mg/kg bw per day for males and females, respectively) (Yoshida, 2012). At each dose, a main group of 20 animals of each sex was terminated after 52 weeks, and satellite groups of 10 animals each were subjected to interim kill after 4, 13 and 26 weeks of treatment. All animals were checked daily for mortality and general clinical conditions, and body weights were recorded at initiation of treatment, once weekly from week 1 to week 13 and once every 4 weeks from week 16 onwards. Detailed clinical observations were checked in the main group once prior to the initiation of treatment and weekly thereafter, and feed consumption of the main group was measured once weekly from week 1 to week 13 and once every 4 weeks from week 16 onwards. The animals in the main group were subjected to functional observations at 49 weeks of treatment (10 animals of each sex per group), and ophthalmology was performed before study initiation and in week 52 in all control animals and animals in the 6000 ppm group. Haematology, clinical chemistry, urine analysis, organ weights, macroscopy and histopathology were performed at termination from 10 animals of each sex per dose of the satellite group after 4, 13 and 26 weeks and from 10 animals of each sex per dose (except for macroscopy and histopathology: 20 animals of each

sex per dose) of the main group after 52 weeks. Histopathology was performed on all test animals. (Organ pathology generally included those found dead or killed during the treatment period.)

No treatment-related effects on feed consumption, mortality or ophthalmoscopy were noted.

The following effects were observed at 6000 ppm:

- General clinical signs included a statistically significant increase in soiled fur (external genital region) in 8/20 females of the main group.
- Body weight was statistically significantly decreased in females during the majority of the study period in the main group (maximally −6% towards the end of treatment).
- Haematology revealed a statistically significant transient decrease in platelet count (maximally −9% relative to controls in females) and fibrinogen concentration (maximally −12% relative to controls in females) and a significant elongation in prothrombin time (maximally +37% relative to controls in males) in both sexes after 4 weeks. Males also showed statistically significant decreases in platelet count and fibrinogen concentration after 13 weeks and a statistically significant elongation in prothrombin time after 13 and 52 weeks (with the tendency to recover with time) as well as a statistically significant increase in activated partial thromboplastin time after 4 (+11%), 13 (+4%) and 52 (+4%) weeks. Reticulocyte counts were statistically significantly decreased in males (−8%) after 52 weeks and in females (−15%) after 26 weeks. Finally, a statistically significant increase in lymphocyte counts was observed in females after 26 and 52 weeks (+23% relative to controls).
- Clinical chemistry findings included significant decreases in total cholesterol and triglyceride levels after 4 weeks (maximal reduction occurring in males: −39% and −9%, respectively) and after 26 weeks (females only), and triglyceride levels were additionally reduced at 52 weeks in males. Males also showed slight, statistically significant increases in albumin and total protein levels and albumin/globulin ratio after 13, 26 or 52 weeks. Finally, blood urea nitrogen was statistically significantly increased in both sexes at several time points (maximal value of +14% relative to control group in females at 52 weeks).
- Macroscopy revealed significant increases in the incidence of white-coloured adrenal glands in both sexes and in the incidence of enlarged adrenal glands in females at all time points.
- Statistically significant increases in absolute and relative adrenal weights were observed in both sexes (maximally +73% relative to control group in females) at each time point. Both sexes showed a statistically significant increase in absolute (males only) and relative liver weights (maximally +13% and +15% in males, respectively, relative to control group) and statistically significant increases in absolute (males only) and relative kidney and thyroid weights (at each time point for kidney only). Finally, statistically significant decreases in absolute (−17%) and relative uterine weights (−12%) were observed after 52 weeks.
- Histopathologically, all males at each time point (except 19/20 males at 52 weeks) showed diffuse vacuolation of adrenocortical cells, whereas all females exhibited diffuse hypertrophy of adrenocortical cells at each examination period. Males also showed significant increases in the incidences of focal atrophy of acinar cells in the pancreas (14/20 versus 4/20 in controls) and hyperplasia of Leydig cells (19/20 versus 6/20 in controls) after 52 weeks, whereas females indicated high increases in the incidences of vacuolation of interstitial gland cells in the ovary at all time periods.

In conclusion, the treated animals showed pronounced effects on body weight, clinical signs and several organs, including adrenals, liver, pancreas, testis and ovary, at 6000 ppm (equal to 250 mg/kg bw per day) (Yoshida, 2012).

Cyflumetofen (batch no. 01D1; purity 97.67%) was administered in the diet to groups of 50 male and 50 female 5- or 6-week-old Fischer (F344/DuCrj) rats for 104 weeks. The concentrations in feed were 0, 150, 500 and 1500 ppm (equal to 0, 4.92, 16.5 and 49.5 mg/kg bw per day for males and 0, 6.14, 20.3 and 61.9 mg/kg bw per day for females, respectively) (Yoshida, 2004b). All animals

were checked for mortality and subjected to general clinical observations (at least twice a day) and body weight and feed consumption measurements (at or before initiation of treatment, once weekly from week 1 to week 13 and once every 4 weeks from week 16 onwards). Detailed clinical observations were recorded prior to and weekly during treatment. Haematology was performed at weeks 53, 78 and 104 and on all animals killed in extremis. Selected organ weight analysis (10 animals of each sex per dose) and macroscopy were performed on animals from all dose groups. Histopathology was performed in the control and highest-dose groups at terminal kill after 104 weeks or in all animals (regardless of the dose group) killed in extremis during treatment. Histopathological analysis was additionally performed on selected organs (liver, kidneys, adrenals, ovaries, uterine horn and all gross lesions) in the 150 and 500 ppm dose groups.

Clinical chemistry, urine analysis, ophthalmology and functional observational battery examination were performed in a concurrent chronic rat study (Yoshida, 2004b).

No effects on mortality, clinical signs, body weight or feed consumption were noted. Haematologically, eosinophil count was statistically significantly decreased in males at 1500 ppm (−17%).

Because an adrenal tumour (complex phaeochromocytoma) was observed in one male of the control group (leading to a decrease in adrenal weights in all treatment groups), reanalysis of adrenal weights was conducted after excluding this animal; this resulted in statistically significant increases in absolute and relative adrenal weights in males at 500 ppm and higher (maximally +21% and +23% at 500 ppm, respectively) and females at 1500 ppm (+9% and +18%, respectively). Macroscopy showed a statistically significant increase in the incidence of mass(es) in the testis in all treated groups killed in extremis or found dead (see Table 5), which corresponded partly to histopathological lesions, such as testicular interstitial cell tumours (Leydig cell adenoma [LCA]), and were associated with increased incidences of atrophies in testis and epididymis. The six males with LCA died between week 84 and week 104 (two control males with LCA died at week 85 and week 88). At study termination, however, the incidences of masses or LCA in males in the treated group were similar to those of the control group, resulting in overall non-statistically significant increases of 14% and 6% for incidences of masses in the testis and LCA, respectively, at 1500 ppm. Moreover, the incidence of Leydig cell hyperplasia was statistically significantly decreased at the highest dose. Increases in the incidence of epididymal atrophy were apparent at 500 ppm (+18%; statistically significant) and 1500 ppm (+14%; not statistically significant), and statistically significant decreases in seminiferous tubule atrophy were observed at the top dose. Furthermore, an increase in luminal dilatation of the gland in the uterine horn was observed in all treatment groups at 0 (14%), 150 (17%), 500 (22%) and 1500 ppm (31%), with statistical significance at the highest dose. However, as this lesion was not elevated relative to controls in a second carcinogenicity study at 6000 ppm, the increase in incidence with dose can be considered incidental. In addition, the incidence of diffuse hypertrophy of cortical cells in the adrenals was statistically significantly increased in both sexes of the 1500 ppm group.

In this study, cyflumetofen showed increased incidences of testicular masses and LCA in animals that died or were terminated ad interim in all treatment groups towards the end of the study. The overall incidences of LCA were, however, similar to those of the control group at study termination (incidence of surviving and killed/dead animals together). The NOAEL for systemic toxicity was 500 ppm (equal to 16.5 mg/kg bw per day), based on increased adrenal weights accompanied by histopathological changes in the adrenals at 1500 ppm (equal to 49.5 mg/kg bw per day in males), and the NOAEL for carcinogenicity was 1500 ppm (equal to 49.5 mg/kg bw per day), the highest dose tested (Yoshida, 2004b).

Cyflumetofen (batch no. 09/0510-3 (009025); purity 97.82%) was administered in the feed to 5-week-old Fischer (F344/DuCrj) rats (50 of each sex per dose) at a dose of 0 or 6000 ppm (equal to 0 and 220 or 287 mg/kg bw per day for males and females, respectively) for 104 weeks (Takahashi, 2013). All animals were checked for mortality and subjected to general clinical observations (at least twice a day) and body weight and feed consumption measurements (at or before initiation of

treatment, once weekly from week 1 to week 13 and once every 4 weeks from week 16 onwards). Detailed clinical observations were recorded prior to and weekly during treatment. Haematology was performed at weeks 53, 78 and 104 and on all animals killed in extremis during treatment. Selected organ weight analysis, macroscopy and histopathology were performed in all animals after 104 weeks or in all animals killed in extremis during treatment. Clinical chemistry, urine analysis, ophthalmology and functional observational battery examination were performed in a concurrent carcinogenicity study in rats.

Table 5. Selected macroscopic/histopathological findings in testis and associated tissues in male rats in a carcinogenicity study

	0 ppm	150 ppm	500 ppm	1 500 ppm
Animals killed in extremis or found dead				
Testis: Mass(es)	0/8	5/10* (50%)	4/7* (57%)	6/9** (66%)
Testis: Atrophy	1/8	6/10	4/7	5/9
Epididymis: Atrophy	0/8	5/10*	4/7*	2/9
Testis: Leydig cell adenoma	2/8 (25%)	5/10 (50%)	4/7 (57%)	6/9 (66%)
All animals examined (terminal kills + ad interim deaths/kills)				
Testis: Mass(es)	38/50 (76%)	41/50 (82%)	39/49 (79%)	45/50 (90%)
Testis: Atrophy	21/50	26/50	29/49	14/50
Epididymis: Atrophy	36/50	40/50	44/49*	43/50
Seminiferous tubule: Atrophy	23/50	13/49	20/48	10/50**
Testis: Hyperplasia, interstitial cell	23/50	12/49	16/48	10/50**
Testis: Leydig cell adenoma	43/50 (86%)	42/49 (86%)	43/48 (89%)	46/50 (92%)
Historical control data for Leydig cell adenoma (IET laboratory)	**Mean**	**Range**		
1998–2003 (7 studies)	78%	68–86%		
2005–2009 (5 studies)	76.4	62–84%		

IET: Institute of Environmental Toxicology (Japan); ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Fisher's exact probability test)

Source: Yoshida (2004b)

No treatment-related effects on mortality or feed consumption were noted.

The following effects were observed at 6000 ppm:

- General clinical signs included statistically significant increases in the incidences of tactile hair loss and soiled fur (mainly observed in the external genital region) in females.
- Body weights were statistically significantly decreased in weeks 76–104 in the males (7% reduction at week 104) and consistently throughout treatment in the females (maximally 3% reduction at week 104).
- Haematological findings included statistically significant decreases in lymphocytes (−18%), neutrophils (−20%), monocytes (−19%) and eosinophils (−14%) in males after 104 weeks.
- Slight, statistically significant increases in absolute and relative weights of the kidneys and liver (non-statistically significant changes in absolute liver weight in males) were observed in both sexes. After exclusion of one male at 6000 ppm and two males in the control group because of spontaneous adrenal tumours, absolute and relative adrenal weights were statistically significantly increased in males (+34% and 43% relative to controls, respectively) and females (+68% and +73% relative to controls, respectively). Statistically significant

increases in absolute and relative weights of ovaries (+29% and +35%, respectively) were noted in females, and statistically significant increases in absolute and relative testis weights (201% and 212% of control values, respectively) were observed in males.

- Males showed statistically significant increases in the incidences of mass(es) in the testis (above the historical control range), which correlated well histopathologically with the increased incidence of LCA (see Table 6) and the increased testis weights mentioned above. The incidence of LCA at 6000 ppm was clearly above the historical control range from the IET laboratory (2005–2012) and above the mean of the historical control data from the same laboratory for 1993–2005. The increase in LCA tumours was accompanied by significant decreases in the incidences of atrophy, softening and hyperplasia in testis and by an increase in atrophy in the epididymis and seminiferous tubule. Additionally, a non-statistically significant increase in C-cell thyroid adenoma (within the historical control range of the IET laboratory) and C-cell carcinoma (outside the historical control range of the IET laboratory) was observed in males (see Table 7). The incidence of the C-cell carcinoma in the control males was, however, at the upper historical control range limit. Further histopathological lesions included a statistically significant increase in focal atrophy of the pancreatic acinar cells in males. Additionally, a statistically significant increase in diffuse adrenocortical cell hypertrophy and a statistically significant increase in focal and diffuse (females only) adrenocortical cell vacuolation were observed in both sexes. In contrast to the preceding carcinogenicity study (Yoshida, 2004b), no treatment-related increase in luminal dilatation of the glands in the uterine horn was noted.

In conclusion, cyflumetofen induced changes in body weight, clinical signs and pathology (primary effects on the adrenal gland and testis) at 6000 ppm (equal to 220 mg/kg bw per day in males). Neoplastic findings included a statistically significantly increased incidence of LCA and a non-statistically significant increase in the incidence of C-cell carcinoma of the thyroid gland in males. Both lesions were above the incidences of the historical control range of recent years for Fischer rats (Takahashi, 2013).

2.4 Genotoxicity

(a) In vitro studies

Cyflumetofen was tested for genotoxicity in five in vitro assays. The results of the in vitro genotoxicity tests are summarized in Table 8. With the exception of the mouse lymphoma thymidine kinase (TK) gene mutation assay, which is summarized in more detail below, all of the other in vitro tests were negative.

In the mouse lymphoma gene mutation assay (Verspeek-Rip, 2007), cyflumetofen concentrations from 1 to 333 µg/mL used in a range-finding test revealed high cytotoxicity at 100 µg/mL, with reduction of relative suspension growth by 97% and 31% in the absence and presence of S9 mix, respectively. At 333 µg/mL, little cell survival was observed. In the mutagenicity test, the following concentrations were chosen for the gene mutagenicity assay: 0, 20, 30, 40, 50, 60, 70, 80 and 90 µg/mL for treatment without S9 mix and 0, 10, 20, 40, 60, 80, 100, 120 and 140 µg/mL for treatment with S9 mix. Mutation frequency was approximately 2-, 2.7- and 3.3-fold increased relative to controls at 70, 80 and 90 µg/mL, respectively, in the experiment without S9 mix and at a mean of 2.5- and 6.2-fold relative to controls at 120 and 140 µg/mL, respectively, in the experiment with S9 mix. Moreover, cyflumetofen revealed an up to 4.5- and 1.6-fold increase (in the absence of S9 mix) and an up to 6.9- and 2.8-fold increase (in the presence of S9 mix) in the mutation frequency of small and large colonies, respectively, which might suggest that cyflumetofen could mainly induce chromosomal aberration; this was, however, not confirmed by the in vitro chromosomal aberration studies in hamsters and the micronucleus assay in mice (see Table 8). The increase in mutation frequency was accompanied by cytotoxicity, reflected by a dose-related 64–91% reduction in relative total growth of cells relative to controls at 70 µg/mL and above in the experiment without S9 mix and a dose-related 56% and 90% reduction in relative total growth of cells relative to controls at 120 and 140 µg/mL in the experiment with S9 mix. Moreover, precipitation of cyflumetofen was observed at

and above 90 µg/mL (without S9 mix) and 100 µg/mL (with S9 mix). A statistical evaluation was not performed in this study.

Table 6. Selected macroscopic/histopathological findings in testis and associated tissues in male rats in a carcinogenicity study

	0 ppm	6 000 ppm	IET laboratory: historical control data	
Animals killed in extremis or found dead				
Number of animals examined	9	7		
Testis: Mass(es)	3 (33%)	7* (100%)		
Testis: Atrophy	5	0*		
Testis: Softening	3	0		
Epididymis: Atrophy	6	6		
Seminiferous tubule: Atrophy	5	0*		
Testis: Hyperplasia, interstitial cell	4	0		
Leydig cell adenoma	5 (55%)	7 (100%)		
All animals examined (terminal kills + ad interim deaths/kills)			Historical control data (2005–2012); *n* = 7; testis masses	
Number of animals examined	50	50	Mean	Range
Testis: Mass(es)	34 (68%)	48** (96%)	65%	56–76%
Testis: Atrophy	12	1**		
Testis: Softening	19	2**		
Epididymis: Atrophy	40	48*		
Seminiferous tubule: Atrophy	27	5**	Historical control data (2008–2012); *n* = 5; Leydig cell adenoma	
Testis: Hyperplasia, interstitial cell	19	3**	Mean	Range
Leydig cell adenoma	38 (76%)	48** (96%)	69%	56–82%

IET: Institute of Environmental Toxicology (Japan); ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Fisher's exact probability test)

Source: Takahashi (2013)

Table 7. Incidences of C-cell tumours in the thyroid of rats in a carcinogenicity study

	Males		Females		IET laboratory: Historical control data (2008–2012), males, *n* = 5	
	0 ppm	6 000 ppm	0 ppm	6 000 ppm		
	n = 50	*n* = 49	*n* = 49	*n* = 50	Mean	Range
C-cell adenoma	11 (22%)	15 (30%)	9 (18%)	5 (10%)	34%	2–44%
C-cell adenocarcinoma	9 (18%)	15 (30%)	2 (4%)	4 (8%)	12.40%	6–18%

IET: Institute of Environmental Toxicology (Japan); ppm: parts per million

Source: Takahashi (2013)

Table 8. Results of genotoxicity studies with cyflumetofen

End-point	Test system	Concentration	Batch no.; purity	Result	Reference
Reverse mutation (Ames)	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 *Escherichia coli* WP2 uvrA	20.6–5 000 µg/plate (±S9)	01D1; 97.67%	Negative[a]	Matsumoto (2001)
Mouse lymphoma TK	L5178Y cell line	10–140 µg/mL (±S9)[b]	01H1; 98.4%	Positive[b]	Verspeek-Rip (2007)
Chromosomal aberration	Chinese hamster lung cells	6.25–200 µg/mL (±S9)[c]	01D1; 97.67%	Negative	Matsumoto (2003a)
Chromosomal aberration	V79 Chinese hamster cells	0.31–320 µg/mL (±S9)[d]	01H1; 98.4%	Negative	Schulz (2011)
Micronucleated bone marrow cells	Male ICR (Crj: CD-1) mice	500, 1 000, 2 000 mg/kg bw in 5 males per dose	01D1; 97.67%	Negative	Matsumoto (2003b)
In vivo DNA repair assay (UDS)	Male Wistar (Han) rats	1 000 and 2 000 mg/kg bw in 3 males per dose	01H1; 98.4%	Negative	Buskens (2007)

bw: body weight; DNA: deoxyribonucleic acid; S9: 9000 × *g* supernatant fraction from rat liver homogenate; TK: thymidine kinase; UDS: uscheduled DNA synthesis

[a] Precipitation of test substance without S9.
[b] More details are provided in the text.
[c] Additionally to a 6-hour exposure ±S9, a continuous treatment test was performed for 24 and 48 hours −S9.
[d] Besides four tests with 4-hour exposure times ±S9, two tests with 18 hours of exposure −S9 were performed.

In conclusion, cyflumetofen was mutagenic in the mouse lypmphoma L5178Y in vitro study. However, mutagenicity was observed only at cytotoxic and precipitating concentrations.

(b) In vivo studies

Cyflumetofen was tested for genotoxicity in two in vivo assays. The results of the in vivo genotoxicity tests are presented in Table 8. Neither the micronucleus assay in mice nor the deoxyribonucleic acid (DNA) repair assay in rats (unscheduled DNA synthesis [UDS]) revealed evidence of a genotoxic potential of cyflumetofen.

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

Rats

In a range-finding one-generation reproductive toxicity study, cyflumetofen (batch no. 01D1; purity 97.67%) was administered in the diet to groups of eight male and eight female at least 7-week-old Wistar Hannover (BrlHan:WIST@Jcl[GALAS]) rats (Takahashi, 2002). The concentrations in feed were 0, 100, 300, 1000 and 3000 ppm. Feed was administered during a 3-week premating period and the subsequent mating period (F_0) until weaning of the F_1 pups to evaluate the potential effects on parental rats and their offspring. Overall group mean intakes of test substance in the treated groups throughout the premating phase and mating period were as follows (in the respective order of parental males and females): 5.89 and 10.59 mg/kg bw per day in the 100 ppm group; 17.4 and 31.1 mg/kg bw

per day in the 300 ppm group; 57.1 and 107.8 mg/kg bw per day in the 1000 ppm group; and 177 and 306 mg/kg bw per day in the 3000 ppm group. Clinical observations, body weight development and feed consumption were recorded regularly (weekly or at least once a day in the case of general clinical observations). In all parental animals, selected organs were weighed, and adrenals were examined microscopically. Reproductive performance was assessed based on successful mating (litter with at least one pup alive), duration of gestation, precoital interval and number of implantation sites. F_1 pups were investigated for clinical conditions and survival (at least daily), body weight development (at least weekly during lactation) and sex ratio. Pups were killed at postnatal days (PNDs) 25–27, the brain, adrenals, spleen, thymus and uterus (females only) were weighed in one male and one female per litter in each dose group, and the adrenals were examined microscopically.

The following treatment-related effects were observed in parental animals: At necropsy, a statistically significant increase in white-coloured adrenals in males (3/8 versus 0/8 in controls) and a statistically significant increase in enlarged and white-coloured adrenals in females (5/8 versus 0/8 in controls) were observed at 3000 ppm. Organ weight measurements revealed an increase in absolute (+7%) and relative kidney weights (+13%, statistically significant relative to controls) in males at 3000 ppm. Moreover, females showed a statistically significant increase in absolute (+27%) and relative (+25%) adrenal weights, relative to controls. Histopathology indicated a statistically significant increase in vacuolation of adrenocortical cells in males (3/8 versus 0/8 in controls) at 1000 ppm (3/8 versus 0/8 in controls) and 3000 ppm (7/8 versus 0/8 in controls). Additionally, statistically significant increases in hypertrophy of adrenocortical cells were observed at 1000 ppm (6/8) and 3000 ppm (8/8) in females (versus 1/8 in controls).

The following treatment-related effects (relative to controls) were observed in F_1 pups: A statistically significant decrease in body weights of male (−7%) and female (−8%) pups on lactation day 21 at 3000 ppm was noted. Organ weight analysis revealed statistically significantly elevated absolute and relative adrenal weights in male (+33% and +43%, respectively) and female (+28% and +36%, respectively) pups at 3000 ppm. Histopathology revealed a statistically significant increase in incidences of hypertrophy of adrenocortical cells in males (6/8 versus 0/0 in controls) and females (5/8 versus 0/8 in controls) at 1000 ppm, and all pups showed this finding at 3000 ppm.

The NOAEL for parental toxicity was 300 ppm (equal to 17.4 mg/kg bw per day), based on increased incidences of vacuolation (males) and hypertrophy (females) of adrenocortical cells at 1000 ppm (equal to 57.1 mg/kg bw per day). The NOAEL for offspring toxicity was also 300 ppm (equal to 17.4 mg/kg bw per day), based on increased incidences of hypertrophy of adrenocortical cells in both sexes at 1000 ppm (equal to 57.1 mg/kg bw per day). The NOAEL for reproductive toxicity was 3000 ppm (equal to 177 mg/kg bw per day), the highest dose tested (Takahashi, 2002).

Cyflumetofen (batch no. 01D1; purity 97.67%) was administered in the diet to groups of 24 male and 24 female 5-week-old Wistar Hannover (BrlHan:WIST@Jcl[GALAS]) rats in a two-generation reproductive toxicity study (Takahashi, 2004). The concentrations in feed were 0, 150, 500 and 1500 ppm. Feed was administered from a 10-week premating period (F_0) over two generations until weaning of the F_2 pups. After a 10-week premating phase under exposure, F_0 animals were mated and allowed to rear F_1 litters. Twenty-four male and 24 female F_1 weanlings (one or two per litter) 22–25 days of age were selected from each group to become F_1 parents and were mated after a 10-week premating period to produce the F_2 generation. The doses achieved are depicted in Table 9. Clinical observations, body weight development and feed consumption were recorded regularly (weekly or at least once a day in the case of general clinical observations) in parental animals. Reproductive performance was assessed based on successful mating (litter with at least one pup alive), duration of gestation, precoital interval, sperm parameters and number of implantation sites. After weaning of offspring, all parental animals (F_0, F_1) were terminated. In all parental animals, selected organs were weighed. Adrenals were examined microscopically in all animals of every dose group, whereas all other organs were analysed from 10 females and 10 males in the control and highest-dose groups. Serum samples from 10 animals of each sex were selected from F_1 parental animals (at termination) in each dose group and analysed for concentrations of luteinizing hormone

(LH), follicle stimulating hormone (FSH), testosterone for males and progesterone and l7β-estradiol for females. Offspring (F_1 and F_2 pups) were investigated for clinical conditions and survival (at least daily), body weight development (days 0, 7, 14 and 21 during lactation), sexual development (preputial separation analysed daily from PND 35 onward and vaginal opening examined daily from PND 26 onward in F_1 pups; anogenital distance examined on PND 4 in F_2 pups) and sex ratio. Pups were terminated after weaning (on PND 26), the brain, adrenals, spleen, thymus, ovaries and uterus (females only) were weighed for one male and one female per litter and dose group, and the adrenals were examined microscopically.

Table 9. Doses achieved in the multigeneration study in rats

	Doses (mg/kg bw per day)					
	Males			Females		
	150 ppm	500 ppm	1 500 ppm	150 ppm	500 ppm	1 500 ppm
F_0 parents premating	10.4	34.6	100.3	12	39.7	121.6
F_1 parents premating	11.5	38.0	114	12.6	44.1	128.6
F_0 females during gestation	–	–	–	9.2	30.6	95
F_1 females during gestation	–	–	–	8.7	31.4	92
F_0 females during lactation	–	–	–	24.7	85.3	252.4
F_1 females during lactation	–	–	–	24.3	84.4	232

bw: body weight; F_0: parental generation; F_1: first filial generation; ppm: parts per million

Source: Takahashi (2004)

Parental animals: No treatment-related effects on mortality, clinical signs, body weight, feed consumption, mating, fertility, gestation or sperm evaluation were observed. The mean estrous cycle length in the F_0 (4.1 days versus 4.0 days in controls and all other treatment groups) and F_1 generations (4.2 days versus 4.0 in controls and all other treatment groups with statistical significance) was slightly longer at 1500 ppm. The values were within the range, but above the means, of the historical control data of the IET laboratory (nine studies between 2004 and 2009; F_0: mean: 4.04 [range: 4.0–4.1 days]; F_1: mean: 4.15 [range: 4.0–4.3 days]). A statistically significant prolongation of completion of preputial separation in F_1 males at 1500 ppm and of vaginal opening in F_1 females at 500 ppm and higher was noted. All values were around the mean and within the historical control range of the same findings in the IET laboratory (see Table 10). At necropsy, increased incidences of white-coloured and enlarged adrenals were observed in females at 500 ppm and above in F_0 (statistically significant for enlargement of the adrenals at 1500 ppm) and F_1 females (statistically significant for both findings at 1500 ppm). Organ weight analysis revealed a statistically significant increase in absolute and relative adrenal weights in F_0 and F_1 females at 500 ppm and higher and in F_0 and F_1 males at 1500 ppm. Additionally, statistically significant increases in absolute and relative pituitary and ovary weights were observed in F_0 females at 1500 ppm (only relative weights of both organs were statistically significantly increased in F_1 females). Moreover, absolute prostate weights were statistically significantly decreased in F_1 males. Histopathology revealed a statistically significantly increased incidence of hypertrophy of the zona glomerulosa in F_0/F_1 females and F_1 males only (not statistically significant, 9/24 versus 4/24 in controls) at 500 ppm and in both sexes and generations at 1500 ppm. There was a statistically significant increased incidence of hypertrophy of the zona fasciculata in F_0/F_1 females at 1500 ppm. Additionally, a statistically significant increase in interstitial vacuolation of ovaries was observed in females. The following concentrations of hormones were statistically significantly reduced in females: progesterone at 150 ppm and higher, FSH at 500 ppm and higher and 17β-estradiol at 1500 ppm. Testosterone levels were

non-statistically significantly reduced in males in all treatment groups, without a clear dose–response correlation (see Table 11). No treatment-related change in LH levels was observed.

Table 10. Sexual development parameters in F_2 pups (anogenital distance) and F_1 parental rats (preputial separation, vaginal opening)

	Anogenital distance in F_2 males (PND 4)			Preputial separation in F_1 males		Vaginal opening in F_1 females		Historical control data for F_1 (9 studies from IET laboratory; 2004–2009)	
Dietary concentration (ppm)	**BW (g)**	**Absolute AGD (mm)**	**Relative AGD**	**Days of age**	**BW (g)**	**Days of age**	**BW (g)**	**Preputial separation**	**Vaginal opening**
0	11.2	6.12	0.274	40.9	178	29.5	92	**Mean (days):**	
150	10.9	6.49	0.294**	40.5	179	30	95	41.7	30.8
500	11.3	6.4	0.286	41.2	179	30.7*	96	**Range (days):**	
1 500	10.3	6.32	0.291**	41.9*	182	31.0**	95	40.7–42.5	28.2–31.9

AGD: anogenital distance; BW: body weight; F_1: first filial generation; IET: Institute of Environmental Toxicology (Japan); PND: postnatal day; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$

Source: Takahashi (2004)

Table 11. Group mean serum hormone concentrations in F_1 parental rats[a]

Sex	Dietary concentration (ppm)	FSH (ng/mL)	Testosterone (ng/mL)	Progesterone (ng/mL)	17β-Estradiol (pg/mL)
Male	0	9.63 ± 1.67	1.2 ± 0.92	–	–
	150	9.97 ± 1.44	0.81 ± 0.55	–	–
	500	8.39 ± 1.97	0.53 ± 0.47	–	–
	1 500	10.06 ±1.46	0.8 ± 0.89	–	–
Female	0	7.58 ± 0.87	–	15.7 ± 4.1	19.3 ± 4.9
	150	9.32 ± 1.06	–	10.9 ± 4.7*	18.6 ± 6.1
	500	3.70 ± 2.09*	–	9.9 ± 3.5**	17.4 ± 4.8
	1 500	3.69 ± 1.66*	–	8.3 ± 2.4**	12.7 ± 4.9*

–: not measured; FSH: follicle stimulating hormone; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$

[a] Values represent mean ± standard deviation.

Source: Takahashi (2004)

Pups: Male and female F_2 pups revealed a statistically significant decrease in body weight (maximally −8% in relation to controls) on lactation days 7, 14 and 21 at 1500 ppm. A statistically significant elevation in relative anogenital distance was observed at 150 and 1500 ppm in F_2 males only. A statistically significant increase in absolute and relative adrenal weights in F_1/F_2 male and female pups (except for the increase in absolute adrenal weight in F_2 pups, which was not statistically significant) was noted at 500 ppm. Additionally, relative brain weights were statistically significantly increased in male and female F_2 pups. Histopathology showed an increase in incidences of hypertrophy in the zona glomerulosa and zona fasciculata of the adrenals in F_1 and F_2 males at 500 ppm (statistically significant for either zona glomerulosa or zona fasciculata in F_1 and F_2 males, respectively) and in both sexes at 1500 ppm.

The NOAEL for parental toxicity was 150 ppm (equal to 10.4 mg/kg bw per day), based on increased incidences of white-coloured and enlarged adrenals in females, increased adrenal weights in females, elevated incidence of hypertrophy of adrenocortical cells in both sexes, and delayed sexual maturation and sexual hormonal changes in females at 500 ppm (equal to 34.6 mg/kg bw per day). The NOAEL for offspring toxicity was also 150 ppm (equal to 10.4 mg/kg bw per day), based on increased adrenal weights and increased incidences of hypertrophy of adrenocortical cells in males at 500 ppm (equal to 34.6 mg/kg bw per day). The NOAEL for reproductive toxicity was 1500 ppm (equal to 100.3 mg/kg bw per day), the highest dose tested (Takahashi, 2004).

(b) Developmental toxicity

Rats

In a dose range–finding developmental toxicity study, cyflumetofen (batch no. 01C1; purity not provided) was administered by oral gavage from gestation day (GD) 6 to GD 19 to groups of eight female time-mated BrlHan:WIST@Tac(GALAS)Wistar rats (York, 2001b). The dose levels were 0, 100, 500 and 1000 mg/kg bw per day. General clinical observations, body weights and feed consumption were recorded. All rats terminated at day 20 of gestation were given a macroscopic examination, including external observation and an examination of the thoracic, abdominal and pelvic viscera. The following developmental parameters were examined: number of corpora lutea, gravid uterine weight, number and distribution of implantation sites and uterine contents, number of live fetuses, number of early and late intrauterine deaths, weight, gross external alterations and sex of fetuses.

There were no treatment-related effects on mortality, clinical signs, body weights, feed consumption, developmental parameters or macroscopy in maternal animals and no treatment-related effects on litter parameters or gross external alterations in fetuses under the conditions of this range-finding study.

Therefore, the NOAEL for maternal and developmental toxicity was 1000 mg/kg bw per day, the highest dose tested, under the conditions of this range-finding study (York, 2001b).

In a developmental toxicity study, cyflumetofen (batch no. 01D1; purity 97.67%) was administered by oral gavage from GD 6 to GD 19 to groups of 25 female time-mated BrlHan:WIST@Tac(GALAS)Wistar rats (York, 2001a). The dose levels were 0, 50, 250 and 1000 mg/kg bw per day. General clinical observations, body weights and feed consumption were recorded. All rats terminated at day 20 of gestation were given a macroscopic examination, including external observation and an examination of the thoracic, abdominal and pelvic viscera. The placenta, liver and kidneys were weighed, and the adrenals were weighed and examined microscopically. The following developmental parameters were examined: number and distribution of corpora lutea, gravid uterine weight, number and distribution of implantation sites and uterine contents, number of early and late intrauterine deaths, number of live fetuses, and weight, gross external changes, soft tissue and skeletal alterations and sex of fetuses.

There were no treatment-related effects on mortality, clinical signs or feed consumption in dams. Corrected body weight gains on GDs 6–20 were statistically significantly reduced at 1000 mg/kg bw per day. Organ weight analysis revealed a statistically significant increase in absolute and relative weights of the left adrenal at 250 mg/kg bw per day and of the right and left adrenals at 1000 mg/kg bw per day, which were accompanied by a statistically significant increase in slight diffuse hypertrophy of the adrenocortical cells at 1000 mg/kg bw per day and slight or moderate bilateral diffuse cytoplasmic vacuolation of cortical cells (2/25 versus 0/25 in controls) at 250 mg/kg bw per day (non-statistically significant) and at 1000 mg/kg bw per day (24/25). There was no effect on distribution and number of implantations, corpora lutea or resorptions.

There were no treatment-related effects on number of live fetuses or external or visceral alterations. Skeletal changes were confined to an increase in the incidence of wavy ribs, which were

observed in 3.9% (0 mg/kg bw per day), 3.4% (50 mg/kg bw per day), 5.5% (250 mg/kg bw per day) and 10.2% (1000 mg/kg bw per day) of fetuses, with statistical significance at the highest dose, and in 20.8% (0 mg/kg bw per day), 13% (50 mg/kg bw per day), 17.4% (250 mg/kg bw per day) and 28% (1000 mg/kg bw per day) of litters. Moreover, an increased incidence of incompletely ossified sternal centra was observed in 3.3% (0 mg/kg bw per day), 2.7% (50 mg/kg bw per day), 7.9% (250 mg/kg bw per day) and 8.3% (1000 mg/kg bw per day) of fetuses, with statistical significance at the highest dose, and in 16.7% (0 mg/kg bw per day), 8.7% (50 mg/kg bw per day), 39.1% (250 mg/kg bw per day) and 36% (1000 mg/kg bw per day) of litters, with statistical significance at the two highest doses.

The NOAEL for maternal toxicity was 50 mg/kg bw per day, based on increased adrenal weights accompanied by an increased incidence of vacuolation of adrenocortical cells at 250 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 50 mg/kg bw per day, based on an increased incidence of incompletely ossified sternal centra at 250 mg/kg bw per day (York, 2001a).

Rabbits

In a dose range–finding developmental toxicity study, cyflumetofen (batch no. 01D1; purity not provided) was administered by oral gavage to groups of five female 5- to 7-month-old time-mated New Zealand White (Hra:(NZW) SPF) rabbits from GD 6 to GD 28 (York, 2003a). The dose levels were 0, 10, 100, 500 and 1000 mg/kg bw per day. General clinical observations, body weights and feed consumption were recorded. All rabbits terminated on day 29 of gestation were given a macroscopic examination, including external observation and an examination of the thoracic, abdominal and pelvic viscera. The following developmental parameters were examined: number of corpora lutea, gravid uterine and placental weights, number and distribution of implantation sites, number of live fetuses, number of early and late intrauterine deaths, and weight, gross external alterations and sex of fetuses.

There were no treatment-related effects on mortality, feed consumption, developmental parameters or macroscopy in maternal animals and no treatment-related effects on litter parameters or gross external alterations in fetuses under the conditions of this range-finding study. The only adverse clinical sign, which may have been test related, was ungroomed coat in one rabbit at 1000 mg/kg bw per day. Moreover, body weight gains were reduced in the 100, 500 and 1000 mg/kg bw per day groups during the entire dosing period, but not in a treatment-related fashion (78%, 90% and 90% of the control group value, respectively).

Therefore, the NOAEL for maternal and developmental toxicity was 1000 mg/kg bw per day, the highest dose tested, under the conditions of this range-finding study (York, 2003a).

In a developmental toxicity study, cyflumetofen (batch no. 01D1; purity 97.67%) was administered by oral gavage to groups of 25 female time-mated (5–7 months old) New Zealand White (Hra:(NZW) SPF) rabbits from GD 6 to GD 28 (York, 2003b). The dose levels were 0, 50, 250 and 1000 mg/kg bw per day. General clinical observations, body weights and feed consumption were recorded. All rats terminated on day 29 of gestation were given a macroscopic examination, including external observation and an examination of the thoracic, abdominal and pelvic viscera. The placenta, liver, kidneys and adrenals were weighed. Placentae were examined for size, colour and shape. The following developmental parameters were examined: number and distribution of corpora lutea, gravid uterine weight, number and distribution of implantation sites, number of early and late intrauterine deaths, number of live fetuses, and weight, gross external changes, soft tissue and skeletal alterations and sex of fetuses.

There were no treatment-related effects on mortality or clinical signs in dams. A non-statistically significant decrease in body weight gain during GDs 6–29 at 250 and 1000 mg/kg bw per day (76% and 68% of control value, respectively) was observed, which partly correlated with a decrease in feed consumption (93% and 78% of control value, respectively) during GDs 18–21 at the same doses. Organ weights revealed a (non-statistically significant) decrease in absolute and relative liver weights at 250 mg/kg bw per day (by 7.8% and 4%, respectively) and 1000 mg/kg bw per day

(by 8.3% and 6%, respectively). Additionally, the absolute weights of the left and right adrenals were reduced by 11.9% and 6.3%, respectively, at 1000 mg/kg bw per day.

Male, female and combined fetal weights were statistically significantly reduced (by approximately 12% relative to controls) at 1000 mg/kg bw per day. Moreover, placental weights combined by sex were significantly reduced (−12% relative to the control group) at 1000 mg/kg bw per day. A non-statistically significant increase in fetal and litter incidences in flexed downward forepaws at 1000 mg/kg bw per day (three fetuses [1.4%] in two litters [8%] versus 0 in all other treatment groups and controls) was noted. The incidences were within the historical control range of the same laboratory (range in fetuses: 0–4%; mean: 0.12%; range in litters: 0–25%; mean: 0.89%; studies dated from 2002 to 2004). An increased incidence of fetuses (5% versus 1.6% in controls) and statistically significantly increased incidence of litters (40% versus 8.3% in control) with angulated hyoid alae were noted at 1000 mg/kg bw per day; the latter was above the historical control range of litter incidences. Additionally, a statistically significant increase in litter (32% versus 0% in controls) and fetal (7.3% versus 0% in controls) incidences of incompletely ossified sternal centra was seen (above the historical control range) at 1000 mg/kg bw per day. Moreover, the number of ossification sites per fetus per litter was statistically significantly increased in the thoracic and caudal vertebrae and in the ribs (above the historical control range) or was statistically significantly decreased in the case of lumbar vertebrae and of the xiphoid in the sternum (both below the historical control range) at the two highest doses. Overall, a statistically significant increase in the fetal incidence of variations (16.5% versus 7.6%; calculated per litter: 18.6% versus 6.3%) was noted at 1000 mg/kg bw per day.

The NOAEL for maternal toxicity was 50 mg/kg bw per day, based on slight body weight loss at GDs 6–9 and decreased body weight gain during the entire treatment period, which partly correlated with decreased feed consumption at 250 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was also 50 mg/kg bw per day, based on changes in the number of ossification sites in the vertebrae, ribs and sternum at 250 mg/kg bw per day (York, 2003b).

2.6 Special studies

(a) Neurotoxicity

In an acute neurotoxicity study, a single dose of cyflumetofen (batch no. 01H1; purity 97.08%) was administered by gavage to groups of 10 male and 10 female approximately 5-week-old Wistar Crl:WI(Han) rats (Buesen et al., 2010a). The dose levels were 0, 125, 500 and 2000 mg/kg bw. Animals were observed for 14 days. General clinical observations and feed consumption were recorded daily during the dosing period, and body weights were measured prior to treatment and weekly thereafter. Functional observational battery and locomotor activity were recorded prior to treatment and at days 0, 7 and 14. At day 15, animals were killed, and five of each sex per dose were used for neuropathological analysis: brain weights were measured and a gross examination of the nervous system was performed in all animals, and microscopic analysis was performed on the nervous system in the control and highest-dose groups. To show the responsiveness of the test, the applicant conducted positive control studies for acute neurotoxicity in Wistar rats with triethyltin bromide (batch no. 00206DS; purity 97%), methylphenidate hydrochloride (batch no. 1032818; purity 100%) and diazepam (batch no. H06379; purity not provided). All studies revealed clinical and neuropathological effects that were characteristic for each substance (Mellert, Kaufmann & van Ravenzwaay, 2008; Kaspers, 2009).

Clinical examinations, functional observational battery as well as motor activity measurement revealed no test substance–related effects in male or female Wistar rats at any dose level or time point during treatment. Brain weight determination and neuropathological examinations (macroscropy and microscopy) did not reveal any treatment-related neuropathological findings.

The NOAEL for acute systemic and neurotoxicological effects was 2000 mg/kg bw per day, the highest dose tested (Buesen et al., 2010a).

In a subchronic neurotoxicity study, cyflumetofen (batch no. 01H1; purity 97.08%) was administered in the diet to groups of 10 male and 10 female approximately 7-week-old Wistar Crl:WI(Han) rats over a period of 90 days (Buesen et al., 2011a). The dose levels were 0, 500, 1500 and 5000 ppm (equal to 0, 30, 89 and 293 mg/kg bw per day for males and 0, 41, 99 and 353 mg/kg bw per day for females, respectively). Detailed clinical observations were performed prior to treatment and weekly during the dosing period. Body weight and feed consumption were measured prior to treatment and weekly during the dosing period. (Additionally, body weights were recorded on days when the functional observational battery was performed.) Functional observational battery and locomotor activity were recorded prior to study initiation and at days 1, 22, 50 and 85. At day 91, animals were killed, and five animals of each sex per group were used for neuropathological analysis: brain weights were measured and a gross examination of the nervous system was performed in all animals, and microscopic analysis was performed on the nervous system in the control and highest-dose groups. Additionally, adrenal glands were weighed and examined histologically in all treatment groups.

No treatment-related effects on mortality, clinical signs or feed consumption were seen. Overall body weight gain (days 0–91) was non-statistically significantly decreased at 5000 ppm in both sexes. No treatment-related effects on functional observational battery or locomotor activity were observed, and no neuropathological effects were noted. Pathological examination of the adrenals revealed a statistically significant increase in absolute and relative adrenal weights at 5000 ppm in males (141% and 148% of control value, respectively) and at 1500 and 5000 ppm in females (193% and 198% of control value, respectively, at the highest dose). All animals at 5000 ppm showed a light beige discoloration of the adrenals (concomitantly with a moderate enlargement of the adrenals in females). A clear increase in the incidence and severity of diffuse vacuolation in the adrenocortical cells was detected at 1500 ppm and higher compared with the controls and the lowest dose (500 ppm) in both sexes. Generally, diffuse vacuolation was seen in all zonae in the adrenals, but predominantly in the zona fasciculata at 1500 ppm and higher. Increased degrees of severity of vacuolation (microvesicular to macrovesicular patterns) were mainly observed in the zona fasciculata and zona reticularis.

Based on increased adrenal weights in females and increased incidences of histopathological findings in adrenals (vacuolation of adrenocortical cells) in both sexes at 1500 ppm (equal to 89 mg/kg bw per day), the NOAEL for systemic toxicity was 500 ppm (equal to 30 mg/kg bw per day). No treatment-related changes in neurobehaviour or neuropathology were noted. Therefore, the NOAEL for subchronic neurotoxicity was 5000 ppm (equal to 293 mg/kg bw per day), the highest dose tested (Buesen et al., 2011a).

(b) Immunotoxicity

Cyflumetofen (batch no. 01H1; purity 97.08%) was administered in the diet to groups of eight female 6-week-old Crl:WI (Han) rats for 28 days to investigate immunotoxicity. The concentrations in feed were 0, 500, 1500 and 5000 ppm (equal to 0, 33, 107 and 349 mg/kg bw per day, respectively). Cyclophosphamide monohydrate (batch no. 1362353; purity 100%) as immunosuppressant was administered by gavage at 4.5 mg/kg bw per day to a positive control group of eight female Wistar Han rats (Buesen et al., 2011b). All animals were immunized 6 days before blood sampling and necropsy using sheep red blood cells (sRBCs) administered intraperitoneally. Detailed clinical observations were performed before treatment and weekly during treatment, and body weight development was investigated at the start of treatment and twice weekly during treatment. Feed intake was recorded weekly. Blood samples were collected at the scheduled termination, and the primary T cell–dependent antibody response (anti-sRBC immunoglobulin M [IgM] enzyme-linked immunosorbent assay [ELISA]) was measured. Adrenals, spleen and thymus were weighed, macroscopy was performed on all animals and the adrenals were examined microscopically.

There were no treatment-related effects on clinical signs, body weight or feed consumption. Six days after immunization, no changes in the sRBC IgM titres were found in any cyflumetofen-treated groups. Regarding pathology, immune-relevant organs such as thymus and spleen were

without findings. Statistically significant increases in the incidence of absolute and relative weights of the adrenals were noted at 500 ppm (+11% and +12%, respectively) and above (maximally +87% for both parameters). The increases in weight correlated with light discoloration and enlargement of the adrenals and microscopic changes, such as an increased incidence and degree of severity of vacuolation in the adrenocortical cells of the zona fasciculata and zona reticularis at 1500 and 5000 ppm. The oral administration of the positive control substance cyclophosphamide monohydrate led to findings indicative of immunotoxicity, which was reflected by significantly lower sRBC IgM antibody titres as well as reduced spleen and thymus weights.

The NOAEL for the immunotoxicologically relevant effects was 5000 ppm (equal to 349 mg/kg bw per day), the highest dose tested, and the NOAEL for systemic toxicity was 500 ppm (equal to 33 mg/kg bw per day), based on increases in adrenal weight, enlargement and discoloration of adrenals and microscopic changes, such as vacuolation of adrenocortical cells, at 1500 ppm (equal to 107 mg/kg bw per day) (Buesen et al., 2011b).

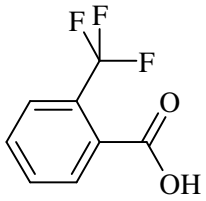
(c) Toxicity of metabolites

B-1 (2-(trifluoromethyl)benzoic acid with CAS no. 433-97-6), a goat and plant metabolite and food processing hydrolysis product, B-3 (2-(trifluoromethyl)benzamide with CAS no. 360-64-5), a soil metabolite, and the impurity AB-13 were tested for acute oral toxicity and potential genotoxic properties. B-1 is also a major metabolite of cyflumetofen in rats (urine: < 10%; faeces: 17%; and bile: < 0.8% of low dose).

Studies with metabolite B-1

Metabolite B-1 (Fig. 3) (batch no. FHE01; purity 99%) was tested in an acute oral toxicity study and in one in vivo and three in vitro genotoxicity tests (see Table 12).

Fig. 3. Structure of B-1 (2-(trifluoromethyl)benzoic acid)



Acute toxicity: The oral LD_{50} in female Wistar (Crl@WI) BR rats was greater than 2000 mg/kg bw. Lethargy, hunched posture, uncoordinated movements and/or piloerection were noted among the animals between days 1 and 3 (Beerens-Heijnen, 2004).

Genotoxicity: With the exception of the mouse lymphoma TK gene mutation assay, which is described in more detail below, the other two in vitro assays and one in vivo DNA repair assay (UDS) were negative. In addition, a toxicity prediction assessment of B-1 (Barentsen, 2008b) revealed no structural alerts (genotoxicity, mutagenicity, chromosome damage and carcinogenicity as major end-points examined) in a DEREK for Windows program (version 10.0.2) analysis.

In the first mouse lymphoma gene mutation assay (Verspeek-Rip, 2004b), B-1 concentrations from 1 to 1900 µg/mL revealed slight cytotoxicity at 1000 µg/mL and above, with reduction of relative total growth by up to 44% with or without S9 mix; a maximally 3-fold increase in mutation frequency (above the historical control range) was observed at the two highest concentrations (1000 and 1900 µg/mL) with and without S9 mix after 3-hour exposure times. In the second experiment, mutation frequency was increased 8-fold relative to controls at the highest concentration without S9 mix after a 24-hour exposure (1901 µg/mL) and above the historical control range, accompanied by marked cytotoxicity (reduction of relative total growth by 91%). No significant increase in mutation

frequency occurred in the second experiment with S9 mix treatment (no cytotoxicity up to the highest dose), and the positive results of the first experiment were therefore not confirmed. As the plates of the positive control without S9 mix addition could not be evaluated, the second experiment was repeated with an additional concentration of 1500 µg/mL, resulting in 2.7-fold and 7.6-fold increases in mutation frequency (again above the historical control range) at 1500 and 1900 µg/mL, respectively, without S9 mix treatment (two highest concentrations) after a 24-hour exposure, which was again accompanied by marked cytotoxicity (82% and 92% reduction in relative total growth). No precipitation occurred up to the highest concentration. In general, the increased mutation frequencies were reflected by an increase in the fraction of small revertant colonies relative to controls (whereas the fractions of large revertant colonies were only marginally increased).

Table 12. Results of genotoxicity studies with B-1

End-point	Test system	Concentration	Batch no.; purity	Result	Reference
Reverse mutation (Ames)	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 *Escherichia coli* WP2 uvrA	3–5 000 µg/plate (±S9)	FHE01; 99%	Negative[a]	Verspeek-Rip (2004c)
Mouse lymphoma TK	L5178Y cell line	1–1 901 µg/mL (±S9)	FHE01; 99%	Positive[b]	Verspeek-Rip (2004b)
Chromosomal aberration	Peripheral human lymphocytes	33–1 901 µg/mL (±S9)	FHE01; 99%	Negative	Buskens (2004b)
In vivo DNA repair assay (UDS)	Male Wistar (Han) rats	1 000 and 2 000 mg/kg bw in 3 males per dose	FHE01; 99%	Negative	Buskens (2009b)

bw: body weight; DNA: deoxyribonucleic acid; S9: 9000 × *g* supernatant fraction from rat liver homogenate; TK: thymidine kinase; UDS: unscheduled DNA synthesis

[a] Precipitation at 3330 and 5000 µg/plate ±S9.
[b] More details are provided in the text.

In conclusion, metabolite B-1 was mutagenic in the mouse lymphoma L5178Y in vitro study in experiments without S9 mix treatment predominantly at prolonged exposure times, at high concentrations and occasionally accompanied by pronounced cytotoxicity.

Studies with metabolite B-3

Metabolite B-3 (Fig. 4) (batch no. MA080115; purity 99.1%) was tested in one in vivo and three in vitro genotoxicity tests (see Table 13). No acute oral toxicity test has been performed with B-3. The acute toxicity of B-3 relative to cyflumetofen was, however, estimated from the UDS assay in rats. Additionally, a 28-day rat study was performed with B-3, and a position paper on the genotoxicity of B-3 and derivation of a metabolite-specific acceptable daily intake (ADI) was provided by the applicant (Meurer, 2014).

Genotoxicity: The results of the in vitro genotoxicity tests are summarized in Table 13. One tester strain of Ames tests, *Salmonella typhimurium* TA100, gave a positive response. B-3 induced 1.7- and 1.9-fold increases in the number of revertants at 3330 and 5000 µg/plate (two highest doses with and without S9 mix), respectively. In a repeat experiment, B-3 induced 1.6- and 2.1-fold increases at the two highest concentrations (3330 and 5000 µg/plate), respectively, without S9 mix pretreatment. As the numbers were at or above the historical control range and concentration

dependent, B-3 was considered to be mutagenic in the TA100 tester strain at high concentrations. No precipitation or bacteriotoxicity was noted. In addition, in a mouse lymphoma TK gene mutation assay, B-3 was positive without S9 mix at high concentrations: B-3 induced an up to 2-fold increase in the mutation frequency at the highest concentration of 1891 µg/plate (without S9 mix and with a 3-hour exposure). In a repeat experiment (with a 24-hour exposure), B-3 induced 2- and 4.4-fold increases in mutation frequency above the historical control range at 1000 and 1891 µg/mL (two highest concentrations without S9 mix), accompanied by moderate cytotoxicity (reduction of relative total growth by 47%) at the highest dose. No positive response was obtained with S9 mix. In conclusion, B-3 was mutagenic in the mouse lymphoma test system in the absence of S9 mix at high concentrations.

Fig. 4. Structure of B-3 (2-(trifluoromethyl)benzamide)

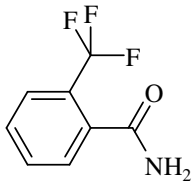


Table 13. Results of genotoxicity studies with B-3

End-point	Test system	Concentration	Batch no.; purity	Result	Reference
Reverse mutation (Ames)	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 *Escherichia coli* WP2 uvrA	3–5 000 µg/plate (±S9)	MA080115; 99.1%	Positive in TA100	Verspeek-Rip (2009b)
Mouse lymphoma TK	L5178Y cell line	1–1 891 µg/mL (±S9)	MA080115; 99.1%	Positive	Verspeek-Rip (2009a)
Chromosomal aberration	Peripheral human lymphocytes	33–1 891 µg/mL (±S9)	MA080115; 99.1%	Negative	Buskens (2009c)
In vivo DNA repair assay (UDS)	Male Wistar (Han) rats	175 and 350 mg/kg bw in 3 males per dose	MA080115; 99.1%	Negative	Buskens (2009a)

bw: body weight; DNA: deoxyribonucleic acid; S9: 9000 × *g* supernatant fraction from rat liver homogenate; TK: thymidine kinase; UDS: unscheduled DNA synthesis

An in vitro chromosomal aberration assay on peripheral human lymphocytes and an in vivo UDS assay did not reveal clastogenic effects or an increased DNA repair rate. Based on the results of the in vivo UDS test in rats, a more acutely toxic effect with B-3 compared with cyflumetofen is expected, as 1/2 males died at 2000 mg/kg bw within 3.5 hours after dosing, and 1/1 male at either 500 or 1000 mg/kg bw had to be terminated for humane reasons in the range-finding study. Moreover, animals at 250 mg/kg bw and higher exhibited clinical signs, such as ataxia and lethargy. A toxicity prediction assessment of B-3 (Barentsen, 2008c) revealed no structural alerts (genotoxicity, mutagenicity, chromosome damage and carcinogenicity as major end-points examined) in a DEREK for Windows program (version 10.0.2) analysis.

Short-term study of toxicity: B-3 (Reg. No. 4288294; batch no. L84-230; purity 99.4%) was administered for 28 days in the diet to groups of five male and five female 6-week-old CRL:WI(Han) rats. The concentrations in feed were 0, 75, 300 and 1200 ppm (equal to 0, 6.1, 24.1 and 85.3 mg/kg bw per day for males and 0, 6.2, 23.9 and 88.2 mg/kg bw per day for females, respectively) (Stark, 2014). General clinical signs were conducted daily. Detailed clinical signs, feed consumption and body weights were recorded weekly. Functional observational battery and motor activity observations were conducted at the end of the study. Clinical pathology (clinical chemistry, urine analysis, haematology) was performed towards the end of the study. At study termination, organ weights, macroscopy and histopathology were performed. Histopathological analysis was performed in the highest-dose and control group animals. The Mallory Heidenhain's stain was performed to assess eosinophilic droplets, and two males of each of the control and highest-dose groups were immunostained with an antibody to α_{2u}-globulin.

Body weight was statistically significantly decreased from study days 7 and 14 onwards in males and females, respectively, reaching maximal reductions of −23.1% and −16.8% on study day 28 in males and females, respectively, at 1200 ppm.

Body weight gain was statistically significantly decreased in males by 16.5% at 300 ppm and in males and females up to −45.7% and −55.4% after 28 days, respectively, and correlated partly with decreases in feed consumption at 1200 ppm. In the functional observational battery, a dose-related reduction in exploration of the area was observed at 300 ppm (one female) and at 1200 ppm (one female and three males versus 0 in the control groups). Additionally, hindlimb grip strength was statistically significantly decreased (−15.8%) in males and forelimb grip strength was decreased by 16.8% in females at 1200 ppm. Clinical chemistry revealed a statistically significant increase in γ-glutamyltransferase activities and potassium levels in both sexes and a statistically significant decrease in chloride (both sexes) and calcium (males only) levels at 1200 ppm. Total protein and albumin levels were statistically significantly increased at 300 ppm (by 5%) and at 1200 ppm (by 11% and 8%, respectively) in both sexes, and globulins were statistically significantly increased in females at 300 and 1200 ppm (~12%) and in males at 1200 ppm (~17%). Cholesterol values were statistically significantly increased by 40% at 300 ppm in females and by 63% and 50% in females and males at 1200 ppm, respectively, whereas triglyceride levels were reduced in males at 1200 ppm. Urine analysis revealed blood in urine in males in the two highest dose groups, and transitional epithelial cells and granulated and epithelial cell casts were found in the urine sediment of animals in all treated groups. Additionally, a decrease in urinary pH (also at 300 ppm) and a statistically significant increase in urine volume were noted in males at 1200 ppm. Organ weight analysis revealed a statistically significant decrease in absolute brain weight in males at 300 ppm and in both sexes at 1200 ppm, a statistically significant decrease in absolute heart weight in both sexes at 1200 ppm and statistically significant increases in relative kidney weight in males at 300 ppm and relative testis and thyroid weights in males at 1200 ppm. Furthermore, a statistically significant increase in absolute liver weight was noted in males at 1200 ppm, and statistically significant increases in relative liver weights were seen in both sexes at 300 ppm and above (maximally by 25% and 64% in males, respectively); in females, statistically significant decreases in absolute spleen weight at 300 ppm and relative spleen weight at 300 ppm and above and a statistically significant decrease in absolute ovary weight at 1200 ppm were observed. Organ pathology showed an increased incidence of liver discoloration in both sexes and focal constriction (males only) at 1200 ppm, partly accompanied by an increased incidence of centrilobular hypertrophy in both sexes at 300 ppm and above. Moreover, an increased incidence of discoloration of kidneys at 300 ppm and above was noted, which was partly associated with increased incidences of tubular degeneration, granular and hyaline casts and higher degrees of eosinophilic droplets in all treated male groups. Immunohistochemical staining for α_{2u}-globulin showed that these effects were due to an α_{2u}-globulin nephropathy, which is considered a male rat–specific effect with no relevance to humans. Additionally, an increased incidence of decreased size of prostate, seminal vesicles and coagulating glands (1/5 males each) and increased incidences of uterine, cervical and vaginal atrophy (3/5 females each) and of decreased cellularity in bone marrow (2/5) were observed.

Based on body weight changes in males, reduced exploration of the area in females, slight changes in clinical chemistry, increases in liver weights and centrilobular hypertrophy in the liver in

both sexes at 300 ppm (equal to 23.9 mg/kg bw per day), the NOAEL was 75 ppm (equal to 6.1 mg/kg bw per day) (Stark, 2014).

Position paper from the applicant: The applicant stressed that the negative in vivo UDS assay in rat liver adequately covered the need for any in vivo follow-up assay for point mutations with the metabolite B-3 (Meurer, 2014). As no elevated DNA synthesis was observed in the UDS assay in rat liver and the liver is supposed to be one of the major target organs of B-3 (as shown in the 28-day rat study), B-3 can be considered not genotoxic in vivo. To underline this conclusion, BASF is performing an in vivo comet assay with metabolite B-3 (final report scheduled for early 2015). An ADI for B-3 of 0.01 mg/kg bw was derived by BASF based on a NOAEL of 6.1 mg/kg bw per day, using a safety factor of 600. An additional safety factor of 6 was considered in the calculation to cover the extrapolation from a subacute to a chronic exposure.

Studies with impurity AB-13

AB-13 (Fig. 5) (batch no. HN0305021; purity 99.2%) was tested in an acute oral toxicity study (Teunissen, 2004) with female Wistar (Crl@WI) BR rats and in three in vitro genotoxicity tests (see Table 14).

Fig. 5. Structure of AB-13

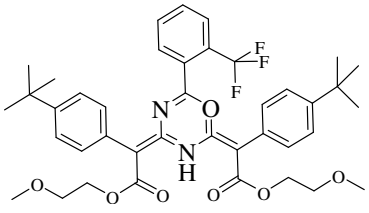


Table 14. Results of genotoxicity studies with AB-13

End-point	Test system	Concentration	Batch no.; purity	Result	Reference
Reverse mutation (Ames)	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 *Escherichia coli* WP2 uvrA	3–5 000 µg/plate (±S9)	HN0305021; 99.2%	Negative	Buskens (2004c)
Mouse lymphoma TK	L5178Y cell line	0.03–100 µg/mL (±S9)[a]	HN0305021; 99.2%	Negative	Verspeek-Rip (2004a)
Chromosomal aberration	Peripheral human lymphocytes	3–333 µg/mL (±S9)	HN0305021; 99.2%	Negative	Buskens (2004a)

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

[a] Precipitation occurred at 100 µg/mL ±S9.

Acute toxicity: The LD_{50} value was greater than 2000 mg/kg bw in female Wistar rats. Hunched posture, uncoordinated movements and shallow respiration were noted among the animals between days 1 and 2.

Genotoxicity: All in vitro genotoxicity assays were negative. In addition, a toxicity prediction assessment of AB-13 (Barentsen, 2008a) revealed no structural alerts for genotoxicity, mutagenicity, chromosome damage or carcinogenicity in a DEREK for Windows program (version 10.0.2) analysis.

(d) Mode of action for Leydig cell adenoma

Five in vitro mechanistic studies were performed to identify a possible mode of action for LCA via perturbation of the estrogen or androgen system.

To determine the potential of cyflumetofen to inhibit aromatase (CYP19) activity (Foster, 2011), the United States Environmental Protection Agency (USEPA)–recommended aromatase assay was performed, as described in the Endocrine Disruptor Screening Program Test Guideline OPPTS 890.1200 (2009). In short, the aromatase human CYP19 + P450 reductase Supersomes™ kit purchased from BD Biosciences was used to investigate the impact of cyflumetofen (batch no. 009020; purity 99.5%) on the conversion of [1β-^{3}H]androstenedione to estradiol by measuring the production of ^3H_2O (representing aromatase activity) by liquid scintillation counting. Differing concentrations of cyflumetofen (10^{-10}–$10^{-4.5}$ mol/L) were compared with a positive control, 4-hydroxyandrostenedione (formestane; 10^{-10}–10^{-5} mol/L) and a negative control (test substance replaced by ethanol), to determine the extent of inhibition of aromatase activity. Incubations were performed in triplicate (except negative controls: quadruplicate) for 15 minutes, and aromatase activities from three independent experiments were calculated.

The results revealed that cyflumetofen was not an inhibitor of CYP19 aromatase activity. The maximum extent of inhibition of CYP19 aromatase activity by cyflumetofen was 8% (92% of full activity control), 9% (91% of full activity control) and 4% (96% of full activity control), in three independent experiments, without any clear concentration-dependent patterns. 4-Hydroxyandrostenedione (the positive control) caused up to a 99% inhibition of the aromatase activity in all experiments in a concentration-dependent fashion (Foster, 2011).

To determine the potential of cyflumetofen to function as an estrogen receptor (ER) agonist, an in vitro human ERα-transcriptional activation system (hERα-HeLa-9903 cell line purchased from JCRB Cell Bank) was used, in which ER activation is reflected by luciferase activity (Steady-Glo® Luciferase Assay System from Promega), as described in the USEPA Endocrine Disruptor Screening Program Test Guideline OPPTS 890.1300 and in Organisation for Economic Co-operation and Development (OECD) Test Guideline 455 (2009). In short, cyflumetofen (batch no. 009020; purity 99.5%) was applied to hERα-HeLa-9903 cells at concentrations of 10^{-11}–10^{-5} mol/L in triplicate in three independent experiments (Akhurst, 2011a). The responsiveness of the test system was assessed in parallel during each test using appropriate concentrations of a strong estrogen (E2, 17β-estradiol), a weak estrogen (17α-estradiol), a very weak agonist (17α-methyltestosterone) and a negative chemical (corticosterone). As a positive and negative control, E2 (10^{-9} mol/L) and dimethyl sulfoxide (DMSO) (0.1% volume per volume [v/v]) were used, respectively (six replicates each). Cytotoxicity was assessed in all cyflumetofen-treated cells and negative control cells. The test chemical was considered to produce a positive response if the RPC_{Max} (the maximum level of response induced by a test chemical concentration relative to the response induced by E2 at 10^{-9} mol/L) was at least 10% and if this exceedance occurred in at least two out of three independent assays.

Cyflumetofen was not cytotoxic at the highest test concentration used. In all three assays, highest RPC_{Max} values were achieved at the maximal cyflumetofen concentration (10^{-5} mol/L). In the first, second and third assays, RPC_{Max} values were 7.8%, 10.4% and 4.3%, respectively. Therefore, cyflumetofen was negative in two out of three assays and only marginally positive (RPC_{Max}: 10.4%) in the remaining assay.

In conclusion, cyflumetofen was considered negative in the hERα-transcriptional activation assay (Akhurst, 2011a).

To determine the potential of cyflumetofen to interact with ER, an in vitro ER-binding assay using rat uterine cytosol was conducted in compliance with the USEPA Endocrine Disruptor Screening Program Test Guideline OPPTS 890.1250 (2009). In short, cytosol protein preparations from uteri (isolated from up to 100-day-old ovariectomized Sprague-Dawley (CD (Crl:CD(SD))) rats) were obtained and pooled, and the final protein concentration suitable for the competitive binding assay was determined (Matthews, 2012). The competitive binding assay was carried out as follows: 1 nmol/L [^{3}H]17β-estradiol, various concentrations of cyflumetofen (10^{-10}–10^{-4} mol/L) and solvents were incubated overnight at 4 °C with 0.3 mg/mL cytosolic uterine protein concentrations (containing ER). After an extensive washing procedure, samples were subjected to liquid scintillation counting (measuring of bound [^{3}H]17β-estradiol). The responsiveness of the test system was assessed in parallel during each test using appropriate concentrations of non-radiolabelled 17β-estradiol, a weak positive control (19-norethindrone) and a negative control (octyltriethoxylsilane). Moreover, a solvent control (ethanol) was incubated as well. Assays were performed in triplicate for each concentration and in three runs. From the fitted protein binding curves, median inhibitory concentrations (IC_{50} values) and relative binding affinities were determined for the test substances. A positive response in this assay is defined as greater than 50% displacement of radiolabelled 17β-estradiol from the receptor at one or more of the test concentrations used.

As cyflumetofen did not compete with 17β-estradiol in protein binding, no [^{3}H]17β-estradiol protein binding curve could be defined in any runs. A maximum of 14% displacement of [^{3}H]17β-estradiol from proteins in the cytosolic uterine preparations was observed at 10^{-4} mol/L (100 µmol/L) on run 1 only. Therefore, cyflumetofen was considered to be "not interactive" for ER binding. The reference standard and the positive and negative control compounds gave results that were consistent with the USEPA criteria for assay performance (Matthews, 2012).

To determine the potential of cyflumetofen to interact with androgen receptors (AR), an in vitro AR binding assay using rat prostate cytosol was conducted as outlined in the USEPA Test Guideline OPPTS 890.1150 (2009). In short, cytosol protein preparations from prostate (isolated from up to 90-day-old castrated Sprague-Dawley rats) were obtained and pooled, and the final protein concentration suitable for the competitive binding assay was determined (Willoughby, 2012). In a preliminary validation assay, it was shown that the AR was present in reasonable concentrations in protein preparations and revealed appropriate affinity for [^{3}H]R1881 (a synthetic androgen, also called methyltrienolone). The competitive binding assay was carried out as follows: 1 nmol/L [^{3}H]R1881, various concentrations of cyflumetofen (10^{-10}–10^{-3} mol/L in the first run and 10^{-11}–10^{-4} mol/L in the remaining runs) and solvents were incubated overnight at 4 °C with 8.8 mg/mL cytosolic prostate protein concentrations. After an extensive washing procedure, samples were subjected to liquid scintillation counting (measuring bound [^{3}H]R1881). All concentrations were tested in replicates of three, and three independent runs were performed. The responsiveness of the test system was assessed in parallel during each run using appropriate concentrations of non-radiolabelled R1881 and a weak positive (dexamethasone) control. Moreover, a solvent control (DMSO) was incubated as well. From the fitted protein binding curves, IC_{50} values and relative binding affinities were determined for the test substances and controls. A positive response in this assay is defined as greater than 50% displacement of radiolabelled R1881 from the receptor at one or more of the test concentrations.

As precipitation was observed at 10^{-3} and 10^{-4} mol/L in the first run, the concentration range of cyflumetofen was shifted to lower concentrations. A maximum of 16.4% and 13.4% displacement of [^{3}H]R1881 was observed from proteins in the cytosolic prostate preparations at the top two concentrations (10^{-3} and 10^{-4} mol/L), respectively, accompanied by precipitation of the test substance in the first run only. In the second and third runs, 15% and 16% displacement of the ligand were noted at the highest dose of 10^{-4} mol/L, respectively, without significant changes at lower concentrations.

As only marginal displacements of the ligand at concentrations at or near precipitation of cyflumetofen were observed, cyflumetofen was considered to be "not interactive" for AR binding.

The reference standards and the solvent control compounds gave results that were consistent with the USEPA criteria for assay performance (Willoughby, 2012).

To determine the potential of cyflumetofen to interact with steroidogenesis, an in vitro H295R steroidogenesis assay was performed, as outlined in the USEPA Test Guideline OPPTS 890.1550 (2009). The objective of the H295R steroidogenesis assay is to detect substances that affect production of 17β-estradiol (E2) and testosterone. H295R cells are human adenocarcinoma cells that express genes encoding all the key enzymes in the steroidogenesis pathway, beginning with the sequence of reactions from cholesterol to the production of E2 and testosterone. The aim of this study was to determine the fold change in testosterone and E2 concentrations in cell culture after treatment with cyflumetofen (Akhurst, 2011b). In short, 24-well plates with H295R cells were incubated with various concentration of cyflumetofen (10^{-11}–10^{-5} mol/L in the first run and 10^{-10}–10^{-5} mol/L in the second and third runs) at 37 °C for 48 hours. All concentrations were tested in triplicate, and three independent runs were performed. After incubation, the supernatant cell culture medium was analysed by ELISA (based on the principle of competitive binding) to measure E2 and testosterone concentrations. The ELISA methods were based on commercially available kits (DRG, catalogue no. EIA-1559 [testosterone] and EIA-2693 Huntingdon Life Sciences LBA002716 [E2]). The responsiveness of the test system was assessed in parallel during each run using 10 µmol/L forskolin, an inducer of steroidogenesis, and 1 µmol/L prochloraz, an inhibitor of steroidogenesis. Moreover, a solvent control (DMSO) was incubated as well. A chemical was judged to be positive if the fold increases or decreases were statistically significantly ($P \leq 0.05$) different from the solvent control.

In the second run, cyflumetofen was cytotoxic at 10 µmol/L, with a viability value of 61% of the solvent control, and therefore this value was excluded from the statistical analysis. In the first run, cyflumetofen caused a statistically significant increase in E2 concentration to 1.50 times the solvent control value at the highest concentration of 10 µmol/L (see Table 15). In the second and third runs, an additional concentration, 5 µmol/L, was included in the dose range in order to further investigate this response. In these remaining runs, cyflumetofen caused a statistically significant increase in E2 concentration to maximally 1.64 times the solvent control value at this highest analysable concentration of 5 µmol/L. In addition, in the first and second runs, cyflumetofen caused a statistically significant decrease in testosterone concentration to maximally 0.63 times the solvent control value at the statistically analysable top concentrations of 1, 5 and 10 µmol/L. In the third run, cyflumetofen caused a statistically significant decrease in testosterone concentration to maximally 0.75 times the solvent control value at 5 and 10 µmol/L.

In conclusion, cyflumetofen demonstrated an influence on the steroidogenesis pathway, acting as an inducer of E2 production at concentrations of 5 µmol/L and above (maximally 1.64-fold) and an inhibitor of testosterone production at concentrations of 1 µmol/L and above (maximally 0.63-fold) (Akhurst, 2011b).

In a position paper from the applicant (van Cott & Meurer, 2013), to elucidate a possible mode of action for the induction of LCA by cyflumetofen, the applicant concluded that a possible treatment-related carcinogenic effect on Leydig cells by cyflumetofen at the highest dose (6000 ppm) in the second carcinogenicity study (Takahashi, 2013) cannot be disregarded; the suggested mode of action included a decrease in testosterone levels (as indicated in the in vitro steroidogenesis assay) and/or enhanced biliary excretion of testosterone due to liver enzyme induction (not mechanistically investigated, however), which could result in a transient compensatory higher LH production along the hypothalamic–pituitary–testicular axis and Leydig cell proliferation and LCA formation in the testis. Fischer rats have a high background incidence of LCA formation. Therefore, as Fischer rats are extremely sensitive to changes in LH levels and subsequent LCA formation compared with humans, as increased incidences of LCA were observed only at high doses in rats and as testicular lesions were not observed in short-term studies in rats or any other species, lifetime exposure risk assessment based on the chronic reference dose will be protective of human health; therefore, cyflumetofen can be considered “not likely to be carcinogenic to humans”, according to the applicant.

Table 15. Mean fold change in estradiol and testosterone concentrations relative to solvent controls after treatment with cyflumetofen (top four/five concentrations shown)

Cyflumetofen concentration	Estradiol mean (±SD) fold change relative to controls			Testosterone mean (±SD) fold change relative to controls		
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
10 µmol/L	1.5 ± 0.07***	1.93 ± 0.09[#]	1.57 ± 0.06***	0.71 ± 0.03***	0.47 ± 0.05[#]	0.76 ± 0.04**
5 µmol/L	–	1.64 ± 0.06***	1.29 ± 0.02***	–	0.63 ± 0.09***	0.75 ± 0.07***
1 µmol/L	0.91 ± 0.05	1.12 ± 0.02	0.98 ± 0.03	0.84 ± 0.07*	0.77 ± 0.02***	0.87 ± 0.05
100 nmol/L	0.89 ± 0.01	0.84 ± 0.02	0.89 ± 0.05	0.95 ± 0.05	0.98 ± 0.07	0.89 ± 0.1
10 nmol/L	0.85 ± 0.05*	0.83 ± 0.11	0.87 ± 0.08	0.98 ± 0.06	0.92 ± 0.05	0.98 ± 0.01

SD: standard deviation; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; [#]: not included in the statistical analysis, as it produced only 61% cell viability

Source: Akhurst (2011b)

(e) Other special studies

Cyflumetofen (batch no. 01D1; purity 97.7%) was administered for up to 28 days in the diet to groups of male and female 6- to 8-week-old Fischer (F344/DuCrlCrj) rats, to investigate the mechanism(s) for the effects on adrenal gland and ovary in a non-GLP and non-guideline study (Takeda, 2006). The concentrations in feed were 0 ppm (10 animals of each sex), 100 ppm (eight animals of each sex) and 5000 ppm (10 animals of each sex), which are equal to 0, 7.44 and 378 mg/kg bw per day for males and 0, 7.59 and 347 mg/kg bw per day for females, respectively. Clinical observations were performed at least daily. Body weights and feed consumption were recorded prior to treatment and once weekly thereafter. Serum hormone levels were measured, including adrenocorticotrophic hormone (ACTH) and corticosterone (main glucocorticosteroid), after 4 weeks using a radioisotope method. Necropsy in males was performed after 4 weeks (females were selected for necropsy on the day of diestrus within an additional 5-day period of treatment). All gross findings and adrenal and ovary weights were recorded in all animals. Two animals of each sex of the 0 and 5000 ppm groups were subjected to ultrastructural examination of the adrenal gland and/or ovary. From the remaining eight animals of each sex per dose, half of the left adrenal gland was used for quantitative analysis of gene expression by reverse transcription and real-time quantitative polymerase chain reaction (PCR) of CYP11A1 (cholesterol monooxygenase, a cholesterol side-chain cleavage enzyme), CYP11B1 (steroid 11β-hydroxylase), neutral cholesteryl ester hydrolase (NCEH) and hormone-sensitive lipase (HSL) genes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard to normalize gene expression levels. The other half of the left adrenals and the left ovaries were subjected to histopathological examination, and the right adrenals were additionally used for quantitative cholesterol measurements.

There were no treatment-related clinical signs or changes in body weight or feed consumption. In addition, no treatment-related differences in serum concentrations of ACTH or corticosterone were observed. At necropsy, all animals revealed enlarged and whitish adrenal glands at 5000 ppm. Absolute and relative adrenal weights were statistically significantly increased in males (+26% and +20% relative to controls, respectively) and females (+65% and 64% relative to controls, respectively). Moreover, relative ovary weight was statistically significantly increased (+10% relative to controls) and absolute ovary weight was non-statistically significantly elevated (+11% relative to controls) at 5000 ppm. However, it was suspected that the effects on the ovary were incidental, because, after excluding two females with spontaneous large ovarian cysts, there were only marginal,

non-statistically significant increases in ovary weights. Histopathologically, diffuse cytoplasmic vacuolation of adrenocortical cells and of the ovary (females only) was noted in all animals at 5000 ppm. Ultrastructurally, the vacuolations turned out to be lipid droplets within the adrenocortical and ovary cells. Gene expression analysis by reverse transcription and real-time quantitative PCR showed a statistically significant increase in absolute and relative (compared with GAPDH) CYP11A1 expression in females (+33% and +36% compared with controls, respectively) and males (+24% and +25% compared with controls, respectively) at 5000 ppm. In addition, a statistically significant decrease in absolute and relative HSL expression in females (−31% compared with controls for both parameters) and males (−21% and −22% compared with controls, respectively) was noted, and a statistically significant decrease (by 30%) in absolute and relative CYP11B1 expression in females was observed at 5000 ppm. Total cholesterol, free cholesterol and cholesteryl ester concentrations in adrenals were increased by 24% (statistically significantly), 20% and 54% in males, respectively, and by 54% (statistically significantly), 39% (statistically significantly) and 126% in females, respectively, at 5000 ppm.

In conclusion, cyflumetofen revealed an increase in total cholesterol (predominantly cholesteryl ester) concentrations in adrenals, which correlated with vacuolation by lipid deposition of the adrenocortical cells and of ovarian interstitial cells at 5000 ppm (equal to 7.44 mg/kg bw per day) in both sexes. Furthermore, cyflumetofen may have an inhibitory effect on HSL expression at 5000 ppm in both sexes; as HSL is involved in cholesterol catabolism, its inhibition might result in cholesterol and other lipid deposition. Catabolism of lipids might be inhibited between 7.44 and 347 mg/kg bw per day, leading to the formation of lipid droplets in adrenals and ovarian cells. As a secondary response, CYP11A1 expression may be enhanced in both sexes at 5000 ppm by the elevated supply of lipids (Takeda, 2006).

To investigate pharmacological safety aspects in dogs, respiratory rate, blood pressure, heart rate and electrocardiogram were examined in conscious dogs. After vehicle treatment of four approximately 14-month-old male Beagle dogs and a withdrawal period of 6 days, the same animals were administered a single dose of cyflumetofen (batch no. 01D1; purity 97.67%) of 0 or 2000 mg/kg bw per day by gelatine capsule (Inui, 2003). A blood pressure–electrocardiogram transmitter was implanted subcutaneously in the flank, and a catheter from a pressure sensor connected to the transmitter was introduced into the femoral artery. Electrocardiographic electrodes were implanted subcutaneously on the right outer side of the chest and left outer side of the abdominal area. Moreover, a respiration pickup was attached at the thorax region, connected to a multitelemeter transmitter (which was placed in a jacket worn by the animal). The respiratory rate, blood pressure (systolic blood pressure, diastolic blood pressure and mean blood pressure), heart rate and electrocardiogram parameters (PQ interval, QRS duration, QT interval and QTc) were measured before administration and at 0.5, 1, 3, 6 and 24 hours after administration telemetrically and analysed with a data acquisition and real-time analysis software. Electrocardiographic parameters were analysed from 20 stable waveforms at the time point of measurements.

Systolic blood pressure, heart rate, electrocardiogram (PQ interval, QRS duration, QT interval, QTc) and respiratory rate in the 2000 mg/kg bw group showed no statistically significant differences when compared with controls at 0.5, 1, 3, 6 and 24 hours after administration. Diastolic blood pressure and mean blood pressure at 1 hour after administration were slightly, but statistically significantly, higher in the treated group; however, values were within the range of variation of post-administration measurements in the control group.

In conclusion, cyflumetofen did not reveal treatment-related adverse effects on the respiratory or cardiovascular system in dogs (Inui, 2003).

3. Observations in humans

Medical examination (including clinical signs, blood pressure, chest X-ray, haematological and clinical chemistry findings and urine analysis) of workers engaged in the production of

cyflumetofen at Kyoyu Agri Co., Ltd and Sugai Chemical Co., Ltd was conducted (16 workers in total) from 2007 to 2008. No remarkable abnormal findings were reported in any workers (Higashida, 2009; Okamoto, 2009a,b).

Comments

Biochemical aspects

The specific metabolism or degradation of the individual enantiomers of the racemic mixture was not investigated.

In a pharmacokinetic study performed in mice, peak concentrations of radiolabel in plasma were reached rapidly (0.5–1 hour after dosing) and were 2.4-fold higher in females than in males. Moreover, in females, a second C_{max} was observed at 2 or 8 hours after dosing, suggesting enterohepatic recirculation. Terminal half-lives were in the range of 22–60 hours. The absorption in mice represented by the AUC was up to 2-fold higher in females than in males and increased sublinearly with increasing doses.

Absorption of cyflumetofen in rats was approximately 69–78% at the low dose (3 mg/kg bw), based on urinary and biliary excretion, with saturation at the high dose (approximately 35–46% absorption at 250 mg/kg bw). There were no remarkable differences in absorption according to sex or the position of the radiolabels used. Saturation of oral absorption was also noted after repeated daily administration at the low dose. Peak concentrations in plasma after a single high-dose application occurred after 1–4 hours. Radioactivity in plasma decreased biexponentially, with a terminal half-life of 12–22 hours. The AUC increased less than proportionally with the dose, confirming saturation of absorption. The AUC was up to 2-fold higher in females than in males at the high dose.

The major route of elimination at the low dose was the urine (58–67%), with lower amounts in faeces (25–33%). At the high dose, the major route of excretion was the faeces (68–80%), with lower amounts in urine (14–26%). Studies in bile duct–cannulated rats showed that at least 30% and 18% were excreted in bile in males and females, respectively, regardless of dose level. A decrease in urinary excretion in cannulated compared with non-cannulated rats suggests that some reabsorption from the intestinal tract after biliary excretion might occur at low doses. Within 72 hours after administration, at least 95% of the absorbed dose was excreted.

In rats, the half-lives for elimination from tissues were in the range of 9–24.5 hours at the low dose and 14–42.5 hours at the high dose, with the longest half-life in adipose tissue, followed by bone marrow. Highest residues were identified in the liver, followed by the kidney, regardless of sex, dose, label position or time point of measurement. Levels of radioactivity in tissues appeared to have reached steady state after 4 days of repeated daily dosing. Residues in erythrocytes and skin declined relatively slowly.

Cyflumetofen was extensively metabolized. The predominant metabolic pathway was cleavage of the *tert*-butylphenyl (A-ring) and trifluorotolyl (B-ring) moieties. Major reactions on the A-ring were cleavage of the methoxyethyl group, hydroxylation at the butyl group, and decarboxylation and glucuronidation at the butyl group. Major reactions on the B-ring were glutathione conjugation at the carboxyl group and further metabolism to mercapturic acid or thiolactic acid. In addition, hydroxylation and oxidation reactions at the butyl group and cleavage of the carboxylic ester moiety were observed on the parent structure (intact A- and B-ring).

Toxicological data

The oral LD_{50} was greater than 2000 mg/kg bw in female rats. The dermal LD_{50} was greater than 5000 mg/kg bw in rats, and the LC_{50} in an inhalation study in rats was greater than 2.65 mg/L. Cyflumetofen was not irritating to the skin of rabbits and was slightly irritating to the eyes of rabbits. Skin sensitization was observed in a maximization assay in guinea-pigs.

In repeated-dose toxicity studies in mice, rats or dogs, the main effects were on the adrenals (vacuolation and hypertrophy of cortical cells) and liver (e.g. hepatocellular hypertrophy). LCA occurred at the highest doses in the rat carcinogenicity study.

In a 4-week mouse feeding study with dietary concentrations of 0, 100, 500, 1000 and 5000 ppm (equal to 0, 13.1, 67.2, 135 and 663 mg/kg bw per day for males and 0, 14.5, 74.9, 150 and 763 mg/kg bw per day for females, respectively), absolute and relative adrenal weights were increased at 5000 ppm. Histopathology showed an increased incidence of diffuse vacuolation and hypertrophy of adrenocortical cells at 5000 ppm. The NOAEL was 1000 ppm (equal to 135 mg/kg bw per day), based on increased adrenal weights and histopathological changes in the adrenals at 5000 ppm (equal to 663 mg/kg bw per day).

In a 13-week mouse feeding study with dietary concentrations of 0, 300, 1000, 3000 and 10 000 ppm (equal to 0, 35.4, 117, 348 and 1200 mg/kg bw per day for males and 0, 45.0, 150, 447 and 1509 mg/kg bw per day for females, respectively), the NOAEL was 1000 ppm (equal to 117 mg/kg bw per day), based on histopathological changes in adrenocortical cells (e.g. increased incidence of diffuse vacuolation in females and of diffuse hypertrophy in males) at 3000 ppm (equal to 348 mg/kg bw per day).

In a 2-week rat feeding study with dietary concentrations of 0, 1000 and 10 000 ppm (equal to 0, 101 and 981 mg/kg bw per day for males and 0, 105 and 1000 mg/kg bw per day for females, respectively), the lowest-observed-adverse-effect level (LOAEL) was 1000 ppm (equal to 101 mg/kg bw per day), the lowest dose tested, based on changes in clinical chemistry, an increase in absolute and/or relative weights of liver and adrenals, hypertrophy of the adrenals in females and histopathological alterations, such as diffuse vacuolation of adrenocortical cells, at all doses. Moreover, all females showed vacuolation of interstitial cells in the ovary; vacuolation of corpora lutea was additionally observed at 10 000 ppm.

In a 4-week rat feeding study with dietary concentrations of 0, 100, 500, 1000 and 5000 ppm (equal to 0, 7.5, 37.6, 75.1 and 384 mg/kg bw per day for males and 0, 8.05, 40.8, 79.8 and 409 mg/kg bw per day for females, respectively), the NOAEL was 500 ppm (equal to 37.6 mg/kg bw per day), based on clinical chemistry changes (decreases in total cholesterol and triglycerides) and increases in organ weights (liver and adrenal) at 1000 ppm (equal to 75.1 mg/kg bw per day). In addition, histopathological effects, such as diffuse hypertrophy of hepatocytes (in all males at 1000 ppm and in both sexes at 5000 ppm), diffuse vacuolation of adrenocortical cells in both sexes accompanied by diffuse hypertrophy in all females and an increased incidence of vacuolation of interstitial cells in the ovaries, were observed at 1000 ppm and above. The results of lipid staining performed on the adrenals of both sexes and ovaries of females indicated that the vacuolation was due to the presence of lipid.

In a 4-week (26 days) rat feeding study with dietary concentrations of 0, 500, 1500, 4000 and 12 000 ppm (equal to 0, 43, 128, 339 and 1028 mg/kg bw per day for males and 0, 46, 132, 351 and 1039 mg/kg bw per day for females, respectively), the NOAEL was 500 ppm (equal to 43 mg/kg bw per day), based on increased adrenal weight in females and elevated incidences of adrenocortical cell vacuolation in both sexes at 1500 ppm (equal to 128 mg/kg bw per day).

In a 13-week rat feeding study with dietary concentrations of 0, 100, 300, 1000 and 3000 ppm (equal to 0, 5.4, 16.5, 54.5 and 167 mg/kg bw per day for males and 0, 6.28, 19.0, 62.8 and 193 mg/kg bw per day for females, respectively), the NOAEL was 300 ppm (equal to 16.5 mg/kg bw per day), based on increased adrenal weights and diffuse hypertrophy of adrenocortical cells in females, mild to moderate diffuse vacuolation of adrenocortical cells in males and vacuolation of ovarian interstitial cells at 1000 ppm (equal to 54.5 mg/kg bw per day).

In a 4-week dog study, cyflumetofen was administered in gelatine capsules at a dose of 0, 100, 300 or 1000 mg/kg bw per day. The NOAEL was 100 mg/kg bw per day, based on increases in adrenal weights in both sexes and increased incidences of fine vacuoles in adrenocortical cells (predominantly in the zona fasciculata and zona reticularis) at 300 mg/kg bw per day. Dark red foci (designated as capillary dilatation) on the right atrioventricular valve of the heart were observed in one female at 1000 mg/kg bw per day.

In a 13-week dog study, cyflumetofen was administered in gelatine capsules at a dose of 0, 30, 300 or 1000 mg/kg bw per day. The NOAEL was 300 mg/kg bw per day, based on a reduction in body weight gain (from day 1 to day 90), elevated absolute and relative adrenal weights in males, increased absolute and relative pituitary weights in females and an increase in absolute and relative testis weights at 1000 mg/kg bw per day. In addition, dark red foci (designated as capillary dilatation) on the right atrioventricular valve of the heart and dark red foci in the mucosa of the urinary bladder in one male and increased incidences of vacuolation of adrenocortical cells in females and males were observed at 1000 mg/kg bw per day.

In a 52-week dog study, cyflumetofen was administered in gelatine capsules at a dose of 0, 30, 300 or 1000 mg/kg bw per day. The NOAEL was 30 mg/kg bw per day, based on increased incidences of vacuolation in the adrenal cortex in both sexes, accompanied by degenerative processes (e.g. interstitial fibrosis and infiltration of brown pigment–laden macrophages) at 300 mg/kg bw per day.

In two 18-month feeding studies in mice, dietary concentrations were 0, 150, 500, 1500 and 5000 ppm (equal to 0, 15.5, 54.3, 156 and 537 mg/kg bw per day for males and 0, 14.3, 48.1, 144 and 483 mg/kg bw per day for females, respectively) in the first study and 0 and 10 000 ppm (equal to 0 and 1143 or 1132 mg/kg bw per day for males and females, respectively) in the second study. The overall NOAEL was 1500 ppm (equal to 144 mg/kg bw per day), based on increased adrenal weight (predominantly in females) and an increase in the incidence of diffuse vacuolation of adrenocortical cells in both sexes at 5000 ppm (equal to 483 mg/kg bw per day). There was no increased incidence of tumours in these studies.

In two 52-week feeding studies in rats, dietary concentrations were 0, 50, 150, 500 and 1500 ppm (equal to 0, 1.9, 5.6, 18.8 and 56.8 mg/kg bw per day for males and 0, 2.3, 6.9, 23.3 and 69.2 mg/kg bw per day for females, respectively) in the first study and 0 and 6000 ppm (equal to 0 and 250 or 319 mg/kg bw per day for males and females, respectively) in the second study. The overall NOAEL for systemic toxicity was 500 ppm (equal to 18.8 mg/kg bw per day), based on a reduction in total cholesterol and triglyceride concentrations in both sexes at several time points, increased liver weight (most pronounced early during the study period) in both sexes and increased adrenal weight in females at 1500 ppm (equal to 56.8 mg/kg bw per day). In addition, histopathology revealed an increased incidence of diffuse vacuolation of adrenocortical cells in males and an increased incidence of diffuse hypertrophy of adrenocortical cells in females at 1500 ppm. Moreover, vacuolation of interstitial gland cells in the ovaries was observed at 1500 ppm and above. In the second study, statistically significant increases in the incidence of hyperplasia of Leydig cells (19/20 versus 6/20 in controls) were observed at 6000 ppm at study termination.

In two 104-week studies in Fischer rats, dietary concentrations were 0, 150, 500 and 1500 ppm (equal to 0, 4.92, 16.5 and 49.5 mg/kg bw per day for males and 0, 6.14, 20.3 and 61.9 mg/kg bw per day for females, respectively) in the first study and 0 and 6000 ppm (equal to 0 and 220 or 287 mg/kg bw per day for males and females, respectively) in the second study. The overall NOAEL for systemic toxicity was 500 ppm (equal to 16.5 mg/kg bw per day), based on an increase in adrenal weights in both sexes, an increase in the incidence of epididymal atrophy and an increased incidence of diffuse hypertrophy and/or vacuolation of adrenocortical cells in both sexes at 1500 ppm (equal to 49.5 mg/kg bw per day). The overall NOAEL for carcinogenicity was 1500 ppm (equal to 49.5 mg/kg bw per day), the highest dose tested in the first study, based on a statistically significantly increased incidence of LCA at 6000 ppm (equal to 220 mg/kg bw per day) in the second study, which was higher than the historical control incidence range. The increase in the incidence of LCA was associated with a statistically significant increase in mass of the testis, with an increase in absolute and relative testis weights at 6000 ppm. There was a slight, non-significant increase in the incidence of C-cell adenomas and adenocarcinomas of the thyroid in male rats. As these tumours are common in rats and there were no accompanying preneoplastic lesions, it was concluded that their occurrence was incidental.

The Meeting concluded that cyflumetofen is carcinogenic in male rats but not in female rats or male or female mice.

Cyflumetofen was tested for genotoxicity in an adequate range of assays, both in vitro and in vivo. A mouse lymphoma gene mutation assay was positive with and without liver enzyme activation at concentrations close to those at which precipitation occurred. Cyflumetofen was not genotoxic in an Ames test or in an in vitro chromosomal aberration assay. There was no evidence of genotoxicity in an in vivo micronucleus assay or in an in vivo UDS assay in rat liver.

The Meeting concluded that cyflumetofen is unlikely to be genotoxic in vivo.

Five in vitro mechanistic studies were performed to identify a possible mode of action for the LCA via perturbation of the estrogen or androgen system. Cyflumetofen was not a significant aromatase inhibitor in a human recombinant cell system, did not significantly interact with AR or ER binding using rat prostate or uterine cytosol protein preparations, respectively, and was not an agonist in a human ER transcriptional activation system. In a steroidogenesis assay, cyflumetofen induced estrogen production at and above 5 µmol/L (maximally 1.64-fold) and inhibited testosterone production at and above 1 µmol/L (maximally 0.63-fold) in human adenocarcinoma cells. Fischer rats are very sensitive to decreased testosterone levels, which lead to a compensatory increase in LH production and subsequent Leydig cell proliferation and LCA progression. It cannot be excluded that this mode of action is relevant to humans. However, as an increased incidence of LCA was observed only at high doses in rats, cyflumetofen can be considered as not likely to be carcinogenic to humans at levels occurring in the diet.

In view of the lack of genotoxicity, the absence of carcinogenicity in mice and the fact that only LCA was observed in a particularly sensitive strain of rat at the highest dose tested, the Meeting concluded that cyflumetofen 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, 150, 500 and 1500 ppm (equal to 0, 10.4, 34.6 and 100.3 mg/kg bw per day for males and 0, 12, 39.7 and 121.6 mg/kg bw per day for females, respectively), the NOAEL for parental toxicity was 150 ppm (equal to 10.4 mg/kg bw per day), based on increased incidences of white/enlarged adrenals and increased adrenal weights in females and an elevated incidence of hypertrophy of adrenocortical cells in both sexes at 500 ppm (equal to 34.6 mg/kg bw per day). In addition, delayed vaginal opening and decreased FSH and progesterone concentrations in serum were noted in females at 500 ppm. In offspring, the NOAEL was also 150 ppm (equal to 10.4 mg/kg bw per day), based on increased adrenal weights in both sexes and increased incidences of hypertrophy of adrenocortical cells in males at 500 ppm (equal to 34.6 mg/kg bw per day). The NOAEL for reproductive toxicity was 1500 ppm (equal to 100.3 mg/kg bw per day), the highest dose tested.

In a range-finding study on the developmental toxicity of cyflumetofen in rats administered 0, 100, 500 or 1000 mg/kg bw per day, no treatment-related developmental or maternal toxicity was observed. In the main study on the developmental toxicity of cyflumetofen in rats at dose levels of 0, 50, 250 and 1000 mg/kg bw per day, the NOAEL for maternal toxicity was 50 mg/kg bw per day, based on increased adrenal weights accompanied by an increased incidence of vacuolation of adrenocortical cells at 250 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 50 mg/kg bw per day, based on an increased incidence of incompletely ossified sternal centra at 250 mg/kg bw per day.

In a range-finding study on the developmental toxicity of cyflumetofen in rabbits at dose levels of 0, 10, 100, 500 and 1000 mg/kg bw per day, no treatment-related developmental or maternal toxicity was observed. In the main study on the developmental toxicity of cyflumetofen in rabbits at dose levels of 0, 50, 250 and 1000 mg/kg bw per day, the NOAEL for maternal toxicity was 50 mg/kg bw per day, based on slight body weight loss at GDs 6–9 and decreased body weight gain relative to controls during the entire treatment period, which partly correlated with a decrease in feed consumption at 250 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was also 50 mg/kg bw per day, based on changes in the number of ossification sites in the vertebrae, ribs and sternum at 250 mg/kg bw per day. An increased incidence of total variations (per fetus and per litter) was noted

at 1000 mg/kg bw per day. Skeletal variations were confined to increased fetal and litter incidences of incompletely ossified sterna centra (above the historical control range of the laboratory) and an increase in fetal and litter incidences of angulated hyoid alae at 1000 mg/kg bw per day.

The Meeting concluded that cyflumetofen is not teratogenic.

In an acute neurotoxicity study in rats administered cyflumetofen at a dose of 0, 125, 500 or 2000 mg/kg bw, the NOAEL for acute toxicity and neurotoxicity was 2000 mg/kg bw, the highest dose tested.

In a 13-week neurotoxicity study in rats administered cyflumetofen in the diet at a concentration of 0, 500, 1500 or 5000 ppm (equal to 0, 30, 89 and 293 mg/kg bw per day for males and 0, 41, 99 and 353 mg/kg bw per day for females, respectively), the NOAEL for systemic toxicity was 500 ppm (equal to 30 mg/kg bw per day), based on an increase in adrenal weights and an increased incidence and higher degree of severity of diffuse vacuolation of the adrenocortical cells (predominantly in the zona fasciculata) in both sexes at 1500 ppm (equal to 89 mg/kg bw per day). The NOAEL for subchronic neurotoxicity was 5000 ppm (equal to 293 mg/kg bw per day), the highest dose tested.

The Meeting concluded that cyflumetofen is not neurotoxic.

In a 4-week mouse immunotoxicity study with dietary concentrations of 0, 500, 1500 and 5000 ppm (equal to 0, 33, 107 and 349 mg/kg bw per day, respectively), the NOAEL for immunotoxicity was 5000 ppm (equal to 349 mg/kg bw per day), the highest dose tested. The NOAEL for systemic toxicity was 500 ppm (equal to 33 mg/kg bw per day), based on an increase in adrenal weights correlating with discoloration and enlargement of the adrenals and microscopic changes, such as vacuolation of adrenocortical cells, at 1500 ppm (equal to 107 mg/kg bw per day).

The Meeting concluded that cyflumetofen is not immunotoxic.

In a mechanistic study to further elucidate the effects on adrenal gland and ovary, male and female rats were fed diets containing 0, 100 or 5000 ppm (equal to 0, 7.44 and 378 mg/kg bw per day for males and 0, 7.59 and 347 mg/kg bw per day for females, respectively) for up to 28 days. Cyflumetofen caused enlarged and discoloured adrenals with increased weights. An increase in total cholesterol concentration, predominantly cholesteryl esters, in adrenals correlated with vacuolation by lipid deposition within the adrenocortical and ovarian interstitial cells at 5000 ppm in both sexes. Furthermore, a slight inhibitory effect on HSL expression, which is involved in cholesterol catabolism and therefore might result in lipid deposition, was noted at 5000 ppm in both sexes. In addition, CYP11A1 (cholesterol side-chain cleavage enzyme) expression was slightly enhanced in both sexes at 5000 ppm, probably due to the elevated supply of lipids. No effects were seen on ACTH or corticosterone levels in serum. In conclusion, catabolism of lipids might be inhibited at cyflumetofen doses above 7.44 mg/kg bw per day, leading to the formation of lipid droplets in adrenals and ovarian cells.

In a safety pharmacology study, respiratory rate, blood pressure and heart rate (including electrocardiogram) were measured in male dogs after a single-dose application of 0 or 2000 mg/kg bw. Cyflumetofen did not cause any treatment-related adverse effects on the respiratory or cardiovascular system in dogs under the conditions of the study.

Toxicological data on metabolites and/or degradates

Acute toxicity and genotoxicity studies were performed for B-1, a goat and plant metabolite and food processing hydrolysis product. B-1 is also a major metabolite in the rat (occurring at up to 28% of the applied dose).

B-1 was of low acute oral toxicity (LD_{50} > 2000 mg/kg bw per day).

The potential genotoxicity of B-1 was tested in an adequate range of in vitro and in vivo assays. A mouse lymphoma gene mutation assay was positive at 1000 µg/mL without liver enzyme

activation and in one of two experiments at 1000 µg/mL with enzyme activation. B-1 was not mutagenic in an Ames test and was not genotoxic in an in vitro chromosomal aberration assay. There was no evidence of genotoxicity in an in vivo UDS assay in rat liver.

The Meeting concluded that metabolite B-1 is unlikely to be genotoxic in vivo.

B-3, a soil metabolite, and AB-13, an impurity, have not been identified as food residues and are therefore not relevant for dietary risk assessment.

For AB-1, a goat metabolite and food processing hydrolysis product, no toxicological data were provided. AB-1 is an intermediate in the metabolism of rats and occurs at less than 1% of the applied dose in bile. It is further transformed to AB-3 and AB-2. In sum, AB-1, AB-2 and AB-3 and their glucuronidated derivatives account for greater than 20% of the applied dose in rats. As AB-1 is structurally similar to the parent and is transformed to metabolites, which represent a large portion of the metabolism in rats, its toxicity can therefore be considered to be covered by that of the parent.

Human data

In reports on manufacturing plant personnel, no adverse health effects were noted.

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

Toxicological evaluation

The Meeting established an ADI of 0–0.1 mg/kg bw per day on the basis of a NOAEL of 10.4 mg/kg bw per day in the two-generation rat feeding study, based on parental and offspring toxicity at 34.6 mg/kg bw per day. A safety factor of 100 was applied. The margin between the upper bound of the ADI and the LOAEL of 220 mg/kg bw per day for LCA in rats is 2200.

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

Levels relevant to risk assessment of cyflumetofen

Species	Study	Effect	NOAEL	LOAEL
Mouse	Eighteen-month studies of toxicity and carcinogenicity[a,b]	Toxicity	1 500 ppm, equal to 144 mg/kg bw per day	5 000 ppm, equal to 483 mg/kg bw per day
		Carcinogenicity	5 000 ppm, equal to 483 mg/kg bw per day[c]	–
Rat	Ninety-day study of toxicity[a]	Toxicity	300 ppm, equal to 16.5 mg/kg bw per day	1 000 ppm, equal to 54.5 mg/kg bw per day
	Two-year studies of toxicity and carcinogenicity[a,b]	Toxicity	500 ppm, equal to 16.5 mg/kg bw per day	1 500 ppm, equal to 49.5 mg/kg bw per day
		Carcinogenicity	1 500 ppm, equal to 49.5 mg/kg bw per day	6 000 ppm, equal to 220 mg/kg bw per day
	Two-generation study of reproductive	Reproductive toxicity	1 500 ppm, equal to 100.3 mg/kg bw per	–

Species	Study	Effect	NOAEL	LOAEL
	toxicity[a]		day[c]	
		Parental toxicity	150 ppm, equal to 10.4 mg/kg bw per day	500 ppm, equal to 34.6 mg/kg bw per day
		Offspring toxicity	150 ppm, equal to 10.4 mg/kg bw per day	500 ppm, equal to 34.6 mg/kg bw per day
	Developmental toxicity study[d]	Maternal toxicity	50 mg/kg bw per day	250 mg/kg bw per day
		Embryo/fetal toxicity	50 mg/kg bw per day	250 mg/kg bw per day
Rabbit	Developmental toxicity study[d]	Maternal toxicity	50 mg/kg bw per day	250 mg/kg bw per day
		Embryo/fetal toxicity	50 mg/kg bw per day	250 mg/kg bw per day
Dog	One-year study of toxicity[e]	Toxicity	30 mg/kg bw per day	300 mg/kg bw per day

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

Estimate of acceptable daily intake (ADI)

0–0.1 mg/kg bw

Estimate of acute reference dose (ARfD)

Unnecessary

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

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

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

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid, 68% at low dose (3 mg/kg bw), 35% at high dose (250 mg/kg bw)
Dermal absorption	20% at low concentration (0.2 g/L), 27% at high concentration (200 g/L)
Distribution	Widely distributed, highest levels in liver, followed by kidney and bone marrow
Potential for accumulation	No evidence of accumulation
Rate and extent of excretion	≥ 95% within 72 hours, mainly in urine, partly in bile

Metabolism in animals	Extensive, primarily cleavage between tolyl and phenyl moieties, hydroxylation and conjugation
Toxicologically significant compounds in animals and plants	Cyflumetofen, metabolite B-1 (goat, rat, plant and food processing hydrolysis product) and AB-1 (goat, rat and food processing hydrolysis product)
Acute toxicity	
Rat, LD_{50}, oral	> 2 000 mg/kg bw per day
Rat, LD_{50}, dermal	> 5 000 mg/kg bw per day
Rat, LC_{50}, inhalation	> 2.65 mg/L
Rabbit, dermal irritation	Not irritating
Rabbit, ocular irritation	Slightly irritating
Guinea-pigs, dermal sensitization	Sensitizing (maximization test)
Short-term studies of toxicity	
Target/critical effect	Adrenals: weight and histopathological changes (rat, mouse, dog)
Lowest relevant oral NOAEL	16.5 mg/kg bw per day (rat)
Lowest relevant dermal NOAEL	1 000 mg/kg bw per day, highest dose tested
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Adrenals: weight and histopathological changes; testis: LCA at highest dose (rats)
Lowest relevant NOAEL	16.5 mg/kg bw per day (rat)
Carcinogenicity	Unlikely to pose a carcinogenic risk from the diet
Genotoxicity	
	Unlikely to be genotoxic
Reproductive toxicity	
Target/critical effect	No reproductive toxicity
Lowest relevant parental NOAEL	10.4 mg/kg bw per day
Lowest relevant offspring NOAEL	10.4 mg/kg bw per day
Lowest relevant reproductive NOAEL	100.3 mg/kg bw per day, highest dose tested
Developmental toxicity	
Target/critical effect	Skeletal variations
Lowest relevant maternal NOAEL	50 mg/kg bw per day (rat and rabbit)
Lowest relevant embryo/fetal NOAEL	50 mg/kg bw per day (rat and rabbit)
Neurotoxicity	
Acute neurotoxicity NOAEL	2 000 mg/kg bw per day, highest dose tested
Subchronic neurotoxicity NOAEL	293 mg/kg bw per day, highest dose tested
Developmental neurotoxicity NOAEL	No data
Other toxicological studies	
Mechanistic studies	Inhibition of cholesterol catabolism possibly due to a decrease in hormone-sensitive lipase expression in the adrenals of rats
	No significant effect on respiratory/cardiovascular systems in

	dogs
	No significant effect in vitro on the aromatase and estrogen/adrenal receptor system
	Induction of 17β-estradiol synthesis and inhibition of testosterone synthesis in an in vitro steroidogenesis assay
	Possible mode of action of LCA: Testosterone level reduction and subsequent compensatory processes
Immunotoxicity NOAEL	349 mg/kg bw per day, highest dose tested
Studies on metabolites	B-1:
	LD_{50}: > 2 000 mg/kg bw
	Unlikely to be genotoxic in vivo
	Studies on B-3 and AB-13 were submitted, but these compounds are not relevant to a dietary risk assessment.
Medical reports	
	Three medical reports: No abnormal findings

Summary

	Value	Study	Safety factor
ADI	0–0.1 mg/kg bw	Two-generation reproductive toxicity study (rat)	100
ARfD	Unnecessary	–	–

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DICHLOBENIL

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Explanation

Dichlobenil is the International Organization for Standardization (ISO)–approved common name for 2,6-dichlorobenzonitrile (International Union of Pure and Applied Chemistry), with Chemical Abstracts Service number 1194-65-6. It belongs to the group of benzonitrile compounds, which are used as herbicides on cranberry bogs, dichondra, ornamentals, blackberry, raspberry and blueberry fields, apple, pear, filbert and cherry orchards, vineyards, hybrid poplar–cottonwood plantations and rights-of-way to control weeds; and in sewers to remove roots. It inhibits the germination of actively dividing meristems and acts primarily on growing points and root tips.

Dichlobenil 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.

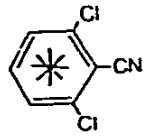
Some of the critical studies do not comply with good laboratory practice (GLP), as the data were generated before the implementation of GLP regulations. Overall, however, the Meeting considered that the database was adequate for the risk assessment.

Evaluation for acceptable daily intake

1. Biochemical aspects

The absorption, distribution, metabolism and excretion of dichlobenil were studied in rats following a single oral low dose, a single oral high dose, a single oral daily dose repeated for 11 days followed by a radioactive dose and intravenous administration.

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



* denotes the position of the uniformly labelled phenyl ring

1.1 Absorption, distribution and excretion

In an absorption, distribution, excretion and metabolism study, Sprague-Dawley rats (five of each sex per dose) were administered dichlobenil as a single intravenous dose of 5.6 mg/kg body weight (bw) or a single oral gavage dose of 6.7 mg/kg bw. A separate group of four male bile duct–cannulated rats received dichlobenil as a single oral gavage dose of 6.7 mg/kg bw. In this study, [phenyl-U-^{14}C]dichlobenil (purity 99.7%) was used. For oral dosing, ^{14}C-labelled dichlobenil was suspended in 1% tragacanth gum; for intravenous dosing, it was suspended in polyethylene glycol 400/benzylalcohol/isopropylidene glycol (15%/2%/60% weight per volume [w/v]) in water. Animals were exposed as described above and held in excretion cages. Urine was collected at 6, 24, 48, 72, 96 and 168 hours after administration. Faeces were collected at 24, 48, 72, 96 and 168 hours after dosing. Bile was collected from bile duct–cannulated animals at 2, 5 and 24 hours after administration, and urine was collected at 5 and 24 hours. At 168 hours, all intact animals were euthanized, and blood and liver were collected. Radioactivity in cage washes, urine, faeces, plasma and liver was measured by combustion followed by liquid scintillation counting.

The total recovery of radioactivity was about 85% and 96% of the administered dose by the oral and intravenous routes of exposure, respectively (Table 1). Seven days after intravenous administration, male rats had excreted about 70% of the dose in the urine and 25% of the dose in the faeces. In females, about 65% and 31% of the dose were excreted in the urine and faeces, respectively. Similar results were obtained 7 days after oral administration. Male and female rats excreted about 65% and 20% of the dose in urine and faeces, respectively. The rate of urinary excretion was rapid; excretion was about 95% complete in 24 hours. Residual radiolabel in the liver, 168 hours after oral or intravenous administration, did not differ significantly between routes or sexes. The bile duct cannula was broken in one rat, so the results of three rats were reported. Approximately 79% of the administered dose was excreted in the bile in 24 hours. Based on a comparison of urinary excretion following oral and intravenous administration, approximately 89% and 97% of the oral dose were absorbed in male and female rats in 7 days, respectively (Koorn, Kaldenberg & De Lance, 1987).

The elimination and time course tissue distribution of [phenyl-U-^{14}C]dichlobenil (purity 99.6%) were studied following a single gavage dose of 2.5 mg/kg bw to male and female Sprague-Dawley rats. The compound was suspended in 1% tragacanth gum and administered to 14 rats of each sex. Two rats of each sex were terminated at 1, 3, 6, 24, 72, 168 and 336 hours after dosing, and various tissues were collected for the analysis of radioactivity. Daily excretion was also determined in rats killed after 7 and 14 days.

Table 1. Cumulative excretion of radioactivity in rats 24 and 168 hours after oral or intravenous exposure to [phenyl-U-^{14}C]dichlobenil

	Excretion (% of administered radiolabel)				
	0–24 h			0–168 h	
Fraction	**Single oral intact (M/F)**	**Single oral cannulated (M)**	**Intravenous intact (M/F)**	**Single oral intact (M/F)**	**Intravenous intact (M/F)**
Urine	62[a]/63[a]	20	67/62	65[a]/65[a]	70[a]/65[a]
Faeces	18/19	NC	25/30	19/21	25/31
Bile	NC	79	NC	NC	NC
Total excreted[b]	80/82	99	92/92	84/86	96/96

F: female; M: male; NC: not collected

[a] Including final cage wash.

[b] Slight differences may occur between the sums of the individual values and the totals listed because of rounding.

Source: Koorn, Kaldenberg & De Lance (1987)

Table 2. Cumulative excretion of radioactivity in rats 168 hours after oral exposure to [phenyl-U-^{14}C]dichlobenil

	Excretion (% of administered radiolabel ± SD)	
Fraction	**Males (n = 4)**	**Females (n = 4)**
Urine[a]	75.1 ± 4.8	55.8 ± 7.1
Faeces	23.8 ± 10.5	20.6 ± 3.1
Total excreted	98.7 ± 7.4	76.4 ± 7.3

SD: standard deviation

[a] Excluding cage wash.

Source: Kaldenberg & de Lange (1988)

The elimination of radioactivity in urine and faeces 7 days after dosing is shown in Table 2. Seven days after dosing, male rats eliminated 75.1% and 23.8% of the administered dose in the urine and faeces, respectively. Excretion was slightly lower in female rats. In females, only 55.8% and 20.6% of the administered dose were excreted in the urine and faeces, respectively.

In all tissues and organs, the highest concentrations of radioactivity were found between 1 and 3 hours after administration (Table 3). No large differences in distribution were observed between males and females for most organs and tissues at most time points, except for plasma and brown fat concentrations at 168 and 336 hours: in males, plasma concentrations were approximately 3 times higher than in females, whereas the reverse was encountered for brown fat.

In conclusion, the results show that dichlobenil is rapidly absorbed and distributed. After the highest concentrations have been reached, the concentrations decline rapidly, showing no potential for accumulation (Kaldenberg & de Lange, 1988).

The elimination of radioactivity following oral administration of [phenyl-U-^{14}C]dichlobenil (purity > 99%) as single or multiple doses (11 days) at 3.75, 30 or 240 mg/kg bw was studied. The radioactive test material was suspended in 1% tragacanth and administered to five Sprague-Dawley rats of each sex per dose. The radiolabelled dichlobenil was given as a single oral dose on day 1 in one study and on days 1 and 11 in a multiple-dose study, with rats receiving unlabelled test material on days 2–10.

Table 3. Levels of radioactivity in selected organs and tissues after oral administration of a single gavage dose of ^{14}C-labelled dichlobenil in male and female rats at various time intervals post-dosing

	Levels of radioactivity (kBq/g)						
	1 h	**3 h**	**6 h**	**24 h**	**72 h**	**168 h**	**336 h**
Males							
Plasma	0.848	0.315	0.197	0.023	0.014	0.016	0.014
Blood	0.571	0.211	0.124	0.016	0.008	0.006	0.005
Liver	2.866	1.332	0.615	0.158	0.074	0.032	0.008
Kidney	3.332	10.005	0.588	0.056	0.019	0.011	0.006
Brown fat	0.782	0.393	0.103	0.017	0.004	0.019	< 0.004
Kidney fat	3.099	4.412	0.200	0.023	< 0.008	0.051	0.013
Females							
Plasma	0.763	0.323	0.283	n.s.	0.006	0.005	< 0.004
Blood	0.500	0.208	0.186	0.020	0.010	0.008	0.005
Liver	1.792	0.098	1.011	0.194	0.078	0.018	0.012
Kidney	3.413	1.302	1.533	0.087	0.027	0.014	0.010
Brown fat	0.956	0.264	0.312	0.016	0.007	0.054	0.015
Kidney fat	3.277	0.609	0.910	0.208	0.012	0.074	0.019

n.s.: not sampled

Source: Kaldenberg & de Lange (1988)

Animals were exposed as described above and held in metabolism cages. Urine was collected at 6 hours after each radioactive dose and daily thereafter up to the time of termination, 192 hours after the last dose was administered. Faeces were collected daily up to the time of termination. At termination, whole blood, plasma, residual carcass, liver and kidneys were removed for analysis. Radioactivity in all biological samples and cage washes was measured using (combustion) liquid scintillation counting.

Cumulative excretion is shown in Tables 4 and 5. Total recoveries at the two lowest doses were between 89% and 92%. At the high dose, total recoveries accounted for 77–83% of the administered dose. It appears that there is some saturation occurring at the high dose, but it could be due to low total radioactivity recoveries in the high-dose group.

Tissue levels 9 days after the last of 11 oral doses of dichlobenil in selected tissues were dose dependent (Table 6). The highest residue levels were noted in the liver in animals receiving all doses. There were no apparent sex-related differences in tissue distribution.

In conclusion, no consistent differences appear to exist between the different dose groups or between sexes. Some differences are observed between the amounts of radiolabel recovered from urine in the various groups, but they seem to reflect the lower recovery in those groups (Cameron, Mutch & Scott, 1988).

Table 4. Cumulative excretion of radioactivity in rats 24 hours after the first and last doses of dichlobenil in a repeated oral exposure study

	Excretion (% of administered radioactivity)					
	First dose: 0–24 h			Last dose: 240–264 h		
	3.75 mg/kg bw per day	30 mg/kg bw per day	240 mg/kg bw per day	3.75 mg/kg bw per day	30 mg/kg bw per day	240 mg/kg bw per day
Fraction	**M/F**	**M/F**	**M/F**	**M/F**	**M/F**	**M/F**
Urine	64/66	60/63	40/28	55/58	61/56	47/41
Cage wash	2.7/2.6	4.9/2.0	1.3/1.7	3.6/2.2	1.8/1.6	2.5/3.0
Faeces	16/15	11/9	3.5/4.1	22/17	15/20	18/16
Total excreted[a]	83/84	76/74	45/34	81/77	78/78	68/60

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

[a] Slight differences may occur between the sums of the individual values and the totals listed because of rounding.

Source: Cameron, Mutch & Scott (1988)

Table 5. Distribution and cumulative excretion of radioactivity in rats 0–240 and 240–432 hours after repeated oral exposure to dichlobenil

	Distribution and excretion (% of administered radiolabel, single dose)					
	First dose: 0–240 h			Last dose: 240–432 h		
	3.75 mg/kg bw per day	30 mg/kg bw per day	240 mg/kg bw per day	3.75 mg/kg bw per day	30 mg/kg bw per day	240 mg/kg bw per day
Fraction	**M/F**	**M/F**	**M/F**	**M/F**	**M/F**	**M/F**
Urine	67/69	65/69	64/55	59/62	64/57	52/47
Cage wash	3.3/3.2	5.7/2.6	3.1/3.5	4.2/4.6	2.8/2.4	3.3/4.0
Faeces	20/20	18/18	15/18	25/20	18/22	23/21
Total excreted[a]	90/92	89/90	83/77	87/87	85/81	78/72
Residual carcass	NC	NC	NC	0.33/0.33	0.32/0.22	0.22/0.22
Tissues/organs	NC	NC	NC	< 0.01/ < 0.01	< 0.01/ < 0.01	< 0.01/ < 0.01
Recovery[a]	90/92	89/90	83/77	87/87	86/83	78/72

bw: body weight; F: female; M: male; NC: not collected

[a] Slight differences may occur between the sums of the individual values and the totals listed because of rounding.

Source: Cameron, Mutch & Scott (1988)

Table 6. Total radioactivity in liver, kidney, whole blood and plasma of rats (n = 5 of each sex) 9 days after the last of 11 doses of dichlobenil

	Total radioactivity (mg eq/kg (L))		
	3.75 mg/kg bw per day	30 mg/kg bw per day	240 mg/kg bw per day
Organ/tissue	**M/F**	**M/F**	**M/F**
Liver	0.06/0.07	0.31/0.39	2.65/2.62
Kidney	0.02/0.03	0.12/0.22	1.98/2.19
Whole blood	0.01/0.02	0.06/0.07	0.30/0.43
Plasma	< 0.01/< 0.01	0.01/0.01	0.20/0.28

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

Source: Cameron, Mutch & Scott (1988)

1.2 Biotransformation

The metabolism of [^{14}C]dichlobenil was studied in Sprague-Dawley rats following single oral and repeated oral doses (11 days) of 3.75, 30 and 240 mg/kg bw. In this study (Cameron, Mutch & Scott, 1988), metabolites from urinary and faecal samples were identified. For metabolite fingerprints, the 0- to 48-hour and 240- to 288-hour urinary samples were pooled separately for males and females at the three dose levels. For faeces, the 0- to 24-hour or 0- to 48-hour and 240- to 264-hour or 240- to 288-hour samples were also pooled. For isolation and identification of metabolites, the 0- to 48-hour and 240- to 288-hour urinary and faecal samples of the highest-dose group were pooled. Radioactivity in urinary and faecal samples was measured using (combustion) liquid scintillation counting. Samples of urine were hydrolysed with β-glucuronidase and arylsulfatase. Metabolites were identified by high-performance liquid chromatographic analysis, nuclear magnetic resonance, mass spectrometry, gas chromatography/mass spectrometry and infrared spectroscopy.

This study revealed that the radioactive compound was absorbed to a significant extent following the first of 11 daily oral doses of dichlobenil. An average of more than 60% of the administered radioactivity was recovered in the urine of rats from the three dose groups. Excretion via faeces accounted for less than 25% of the administered radioactivity (at all dose levels) following the first dose of dichlobenil.

Dose rising and multiple dosing tend to saturate glutathione conjugation. The amount of parent compound recovered from faeces increases with dose. At the middle dose, there is a particularly great difference in recovery of parent compound from faeces between the first and last doses, approximately 22% and 61% of the recovered radiolabel (average of both sexes), respectively.

The metabolite patterns in urine and faeces after the last dose of the repeated-dose study are presented in Table 7. Ten metabolites were identified in the urine, and five metabolites in the faeces. No parent compound was detected in the urine. At the high dose, a significant quantity of parent compound was identified (93% male and female combined) in faeces (Cameron, Mutch & Scott, 1988).

From the chemical structures of the metabolites, two metabolic pathways are involved in the degradation of dichlobenil in rats following oral gavage administration (Fig. 2). One pathway includes hydroxylation at the 3 or 4 position, followed by glucuronidation or sulfation, and the second pathway includes substitution of one chlorine atom by glutathione. The contribution of glutathione-conjugated metabolites decreases with increasing doses, indicating saturation of the metabolic pathway (Vincent et al., 1989).

2. Toxicological studies

2.1 Acute toxicity

The results of acute toxicity studies with dichlobenil (including skin and eye irritation and dermal sensitization studies) are summarized in Table 8.

(a) Oral administration

Rats

Five male and five female fasted Wistar albino rats were dosed orally, by gavage, with dichlobenil (purity 99.8%) ground and mixed with corn oil (10 g/40 mL) at a single dose of 2000 mg/kg bw and observed 1, 2 and 4 hours post-dosing and once daily thereafter for mortality and clinical signs. Body weights were recorded on days 0, 7 and 14. A gross necropsy was performed on all animals.

Nine of 10 animals survived the 2000 mg/kg bw dose level. One male was terminated on day 2 due to its moribund condition. Clinical signs in the surviving animals included lethargy, chromorhinorrhoea, ataxia, sagging eyelids, soiling of the anogenital area, diarrhoea, red staining of the nose/mouth, wetness of the anogenital area and localized alopecia. Piloerection, flaccidity and

Table 7. Metabolite patterns in rats 192 hours after the last dose following repeated oral exposure to dichlobenil

	% of total radioactivity recovered from respective excretion product					
	Urine			Faeces		
	3.75 mg/kg bw per day	30 mg/kg bw per day	240 mg/kg bw per day	3.75 mg/kg bw per day	30 mg/kg bw per day	240 mg/kg bw per day
Metabolite	**M/F**	**M/F**	**M/F**	**M/F**	**M/F**	**M/F**
A (conjugate)	3/3	4/5	5/6	–	–	–
B (conjugate)	11/6	5/4	3/4	–	–	–
C (conjugate)	2/4	3/3	8/3	–	–	–
D (conjugate)	20/12	29/25	30/14	19/8	5/7	–
E (conjugate)	6/2	4/2	2/1	–	–	–
F_1 (conjugate)	2/2	4/3	3/1	–	–	–
F_2 (conjugate)	18/13	14/10	9/14	14/5	5/9	–
G (conjugate)	16/24	11/13	5/7	–	–	–
H	13/24	16/27	22/37	28/25	8/11	5[a]
I	2/3	3/4	4/6	–	–	–
K	–	–	–	–	–	< 3[a]
L	–	–	–	–	–	< 3[a]
Dichlobenil (parent compound)	–	–	–	6/4	60/62	93[a]
Total	93/93	93/96	91/93	67/42	78/89	98[a]

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

[a] Male and female fractions combined.

Source: Vincent et al. (1989)

coma were the other physical signs noted in the animal that was terminated early. Body weight changes in the surviving animals were normal. Necropsy of the survivors was normal in 2/9 animals. Kidney abnormalities were noted in 7/9 survivors. Necropsy of the early terminated animal revealed abnormalities of the intestines, as well as red staining of the nose/mouth area.

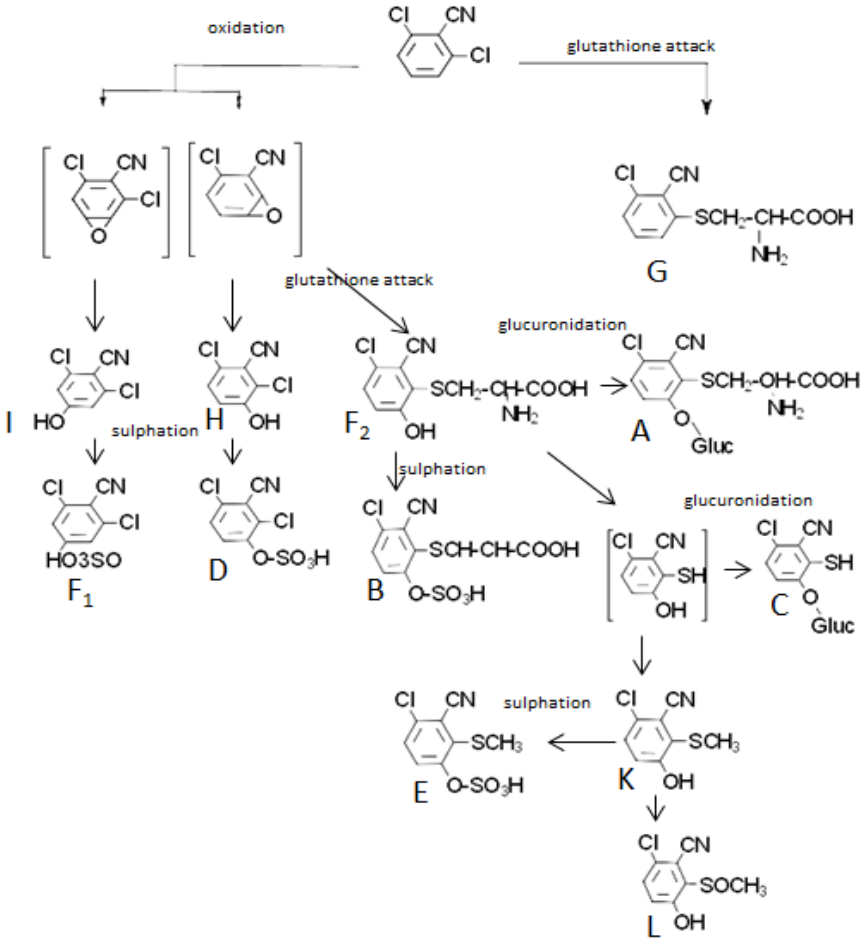
Based on these results, the acute oral median lethal dose (LD_{50}) of dichlobenil was greater than 2000 mg/kg bw (Graver, 1999).

(b) Dermal application

Rabbits

Five female New Zealand White rabbits were dosed dermally with dichlobenil (purity 100%) at a dose of 2000 mg/kg bw. The compound was applied to the prepared site at a dose of 2000 mg/kg bw under a four-layered surgical gauze patch measuring 10 cm × 15 cm. The torso was wrapped with plastic, which was secured with non-irritating tape to keep the test article in contact with the skin for 24 hours, at which time the wrappings were removed and the application sites were cleaned and evaluated according to the Draize method (24 hours, 7 and 14 days). Animals were observed for mortality and clinical signs 1, 2 and 4 hours post-dosing 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.

Fig. 2. Proposed metabolic pathway in rats



No deaths or clinical signs of an adverse reaction to treatment occurred. Dermal reactions were absent to slight (erythema in one rabbit) at 24 hours and absent on days 7 and 14. Body weight changes were normal. Necropsy results were normal.

Based on these results, the acute dermal LD_{50} of dichlobenil was greater than 2000 mg/kg bw in female rabbits (Cerven, 2000).

In a separate study, five male and five female New Zealand White rabbits were dosed dermally with dichlobenil (purity 98.8%) at 2000 mg/kg bw based on the results of a preliminary study in two rabbits. The application site was moistened with water, and the dichlobenil was applied to an area of 12 cm × 14 cm. The application site was covered by an occlusive dressing for 24 hours. After a 24-hour exposure period, the application site was cleaned and evaluated according to the Draize method.

Two female rabbits were found dead on day 5. Antemortem signs comprised underactivity, pallor and cold to touch, pigmented staining of the snout and closed eyes. No systemic or local sign of reaction was observed (except one female, which showed pallor on day 4) in the surviving animals. Body weights of all treated rabbits achieved expected gains. Necropsy of the decedents revealed blood staining around the mouth and nares, dark tracheal contents and dark areas on the left lung lobe in one animal, and dark fluid in the thorax and abdomen of the other animal. Necropsy of the surviving animals revealed no significant internal macroscopic lesions, although one animal had multiple dark areas in the musculature below the application site.

Table 8. Acute toxicity of dichlobenil

Species	Strain	Sex	Route	Purity; vehicle	Result	Reference
Rat	Wistar	M + F	Oral	99.8%; corn oil	LD_{50} > 2 000 mg/kg bw	Graver (1999)
Rabbit	New Zealand White	F	Dermal	100%	LD_{50} > 2 000 mg/kg bw	Cerven (2000)
Rabbit	New Zealand White	M + F	Dermal	98.8%	LD_{50} > 2 000 mg/kg bw	Johnson (1994)
Rat	CD (Sprague-Dawley derived)	M + F	Inhalation (whole body)	–	LC_{50} (4 h) > 3.2 mg/L	Cracknell (1994)
Rat	Wistar	F	Inhalation	–	Not a respiratory irritant at 0.372 mg/L	Janssen (1986)
Rat	Wistar	M + F	Inhalation	99.8%	LC_{50} (4 h) > 2.5 mg/L	Janssen & de Rooy (1985)
Rabbit	New Zealand White	M	Skin irritation	98.8%; 1% tragacanth	Not irritating	Koopman (1985)
Rabbit	New Zealand White	M	Eye irritation	98.8%	Not irritating	Koopman (1983)
Guinea-pig	Dunkin Hartley	F	Skin sensitization (Magnusson-Kligman)	99.4%; paraffin oil	Not sensitizing	Cuthbert & Carr (1987)

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

Based on these results, the acute dermal LD_{50} of dichlobenil was greater than 2000 mg/kg bw in rabbits (Johnson, 1994).

(c) Exposure by inhalation

Rats

Five male and five female CD (Sprague-Dawley) rats were exposed in a chamber to an atmosphere containing a maximum technically achievable concentration of 3.21 mg/L of dichlobenil (purity not reported). The mass median aerodynamic diameter (MMAD) of dichlobenil technical was greater than 5.5 µm, with a geometric standard deviation (GSD) of 13.8 ± 2.1. The animals were subjected to a single 4-hour, continuous, snout-only exposure. 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.

No deaths occurred during the exposure or observation period. During the exposure, slow and shallow respiration was seen for all animals; wet and soiled fur was recorded for one male and two females. During the 2 hours immediately following the exposure, underactivity, piloerection, hunched posture, staggering gait and wet fur were seen for all animals. Subsequently, all animals were normal in appearance and behaviour. Body weight losses were evident for four males on the day following exposure, but their weight gains were normal thereafter. Body weight gains for the remaining male and for all females were unaffected by treatment. There were no macroscopic findings at necropsy, and lung, liver and kidney weights were unaffected.

Based on these results, the acute median lethal concentration (4-hour; LC_{50}) was estimated to be greater than 3.21 mg/L air (Cracknell, 1994).

In a separate study, the respiratory irritation of dichlobenil (purity not reported) was evaluated in three male Wistar rats. The respiratory movements of the rats were recorded before, during and after a nose-only exposure to an aerosol of dichlobenil. From these recordings, the respiratory frequencies were calculated and expressed as a percentage of the value before exposure. It was concluded that an airborne concentration of 0.372 mg/L (time not given) has no irritating action on the respiratory tract of Wistar rats (Janssen, 1986).

In a third study, a group of Wistar rats (five of each sex) were exposed nose-only (aerosol) to an atmosphere containing dichlobenil (purity 98.8%) for 4 hours. Group I and group II (two of each sex) were exposed to dichlobenil in acetone, and group III (one of each sex) was exposed to acetone only. In the highest-dose group, dichlobenil levels between 2.5 and 3.25 mg/L were measured. The test substance was administered to the animals as an aerosol. The MMAD and GSD were determined twice. The MMAD values for group I were 2.57 and 2.61 µm, and the GSD values were 3.13 and 3.14. In group II, the MMAD values were 2.11 and 2.00 µm, and the GSD values were 2.89 and 2.90. The post-exposure observation period was 14 days.

Decreased respiratory frequency and temporarily decreased reactivity and locomotor activity were observed in the test groups, indicating an irritating action of the test atmosphere on the respiratory system and suggesting a slight action on the nervous system when compared with control rats. No effects on body weight were seen following exposure. No mortalities occurred during the 14-day observation period. There were no macroscopic findings at necropsy.

Based on these results, the acute median lethal concentration (4-hour; LC_{50}) was estimated to be greater than 2.5 mg/L in rats (Janssen & de Rooy, 1985).

(d) Dermal irritation

In a study of primary dermal irritation, three male New Zealand White rabbits were dermally exposed to 0.5 g of dichlobenil (purity 98.8%) slightly moistened with 1% tragacanth suspension and placed onto the shorn skin on the back of each rabbit under a 6 cm^2 patch of aluminium foil 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 wiped, and the dermal irritation was scored at 30–60 minutes and at 24, 48 and 72 hours after patch removal using the Draize method. The animals were observed for 14 days post-treatment.

There was no erythema or oedema at any time point.

Based on the results of this study, dichlobenil was not irritating to the skin of rabbits (Koopman, 1985).

(e) Ocular irritation

Rabbits

In a primary eye irritation study, a sample of 100 mg of dichlobenil (purity 98.8%) was instilled into the left eye of three male New Zealand White rabbits. The eyes were not washed. The ocular reactions were evaluated at 24, 48 and 72 hours and on day 7.

No effects were seen on cornea, iris or conjunctiva during the 7 days of observation.

Based on the results of this study, dichlobenil is not irritating to the eyes of rabbits (Koopman, 1983).

(f) Dermal sensitization

Guinea-pigs

The skin sensitization potential of dichlobenil (purity 99.4%) was investigated in 40 female Dunkin Hartley guinea-pigs using the maximization test. The test group and controls consisted of 20 guinea-pigs each. For the intradermal phase of induction, 2% (w/v) dichlobenil in paraffin oil was

selected. The control animals received the same treatment, but with paraffin oil replacing the test article. Twenty-four hours after injection, the dose sites were scored for irritation. Six days after the intradermal injection, all the test and control sites were wetted with 10% aqueous sodium lauryl sulfate to provoke a mild inflammatory response. On day 7, the test group received 10% (w/v) test substance in paraffin oil, and the control group received paraffin oil via topical application during the second phase of induction. Twenty-four hours after patch removal, the treated sites of both test and control groups were assessed for irritancy. Two weeks after the topical induction, animals (both test and control) were challenged with the test article at 25% (w/v) and with the vehicle (paraffin oil) by epidermal application. The application sites were assessed at 24 and 48 hours after removal of the test item and scored for severity of reaction.

The test animals exhibited slight to moderate reactions after the induction phase. The control animals had a slight reaction. None of the animals showed a positive reaction to the treatment after the challenge phase.

Under the conditions of the guinea-pig maximization study, there was no indication of delayed contact hypersensitivity induced by dichlobenil (Cuthbert & Carr, 1987).

2.2 Short-term studies of toxicity

(a) Oral administration

Mice

In a 90-day oral toxicity study, dichlobenil (purity 99.4%) was administered to Charles River CD-1 mice (10 of each sex per dose group) in the diet at a concentration of 25, 125, 625 or 3125 parts per million (ppm) (equal to 3.8, 19, 91 and 447 mg/kg bw per day for males and 4.8, 24, 114 and 512 mg/kg bw per day for females, respectively). The control group consisted of 20 mice of each sex. Stability, homogeneity and dietary concentrations were measured periodically. Treated animals were inspected twice daily on weekdays and once daily during weekends. Body weight, feed consumption and water consumption were recorded weekly. Ophthalmoscopic examination was conducted at the beginning and end of the study for controls and high-dose animals. Blood was collected at the end of the study from all animals. Some neurological evaluations (e.g. motor activity) were recorded as part of the clinical examinations.

Dietary concentrations were 96–107% of the nominal concentrations. Diets were stable and homogeneously distributed.

No treatment-related effects were observed on mortality, ophthalmoscopic examination, haematology or gross pathology.

During the first 2 weeks of treatment, the following clinical signs (slight) were present at 3125 ppm: reduced locomotor activity (females only), hunched posture, piloerection and ptosis. The onset of clinical signs was not reported. At 3125 ppm, body weight gains were significantly decreased ($P < 0.05$) by 80% in the males compared with controls during week 1 and by 56% during week 4. At termination, the body weight gains in males had returned to normal. In the females at 3125 ppm, body weight gains were decreased by 58% ($P < 0.05$) during week 1 and by 52% in weeks 9–13. There were no treatment-related differences in overall (weeks 1–13) body weight gains. The decreases in the body weight gains at week 1 were not considered adverse, as the changes in body weight gains were minor and occurred together with significant reductions in feed consumption. Feed consumption at 3125 ppm was significantly ($P < 0.01$) decreased by 10–19% in the males and by 14–26% in the females during the first 4 weeks of treatment. Additionally in the females at this dose, cumulative feed consumption was decreased by 14% ($P < 0.01$) compared with controls for weeks 5–8 and weeks 9–13. The decrease in overall feed consumption for weeks 1–4 was coincident with early study decreases in body weight gain. In the 3125 ppm females, water consumption was increased ($P < 0.01$) by 58% during week 1 and by 23% during week 2, resulting in an increase of 21% ($P < 0.01$) over controls for weeks 1–4.

At 625 ppm, feed consumption was decreased ($P < 0.05$) by 8% in the males during week 2 and by 10% in the females during weeks 2 and 4 compared with controls. Cumulative feed consumption in these animals was decreased ($P < 0.05$) by 8% for weeks 1–4 and by 7% for weeks 5–8. Glucose was decreased ($P < 0.05$) in the 625 ppm (16%) and 3125 ppm (10%) males. Treatment-related effects on the liver were observed. At 3125 ppm, increases ($P < 0.01$) were observed in cholesterol in the males (50%) and females (43%) and in phospholipids in the males (46%) and females (40%). Significant ($P < 0.01$) increases in the absolute and relative (to body weight) liver weights were observed in males (17–20%) and females (18–23%) at 3125 ppm. The severity of centrilobular swelling of parenchymal cells was increased at 625 and 3125 ppm in both sexes. Glycogen storage (either diffusely throughout the liver or in isolated single parenchymal cells) was increased in incidence and/or severity in both sexes at 3125 ppm.

In the kidneys, the severity of pyelitis was increased in the 625 and 3125 ppm males; however, the incidence of this finding did not increase in a dose-dependent manner. Additionally in the 3125 ppm males, the incidence of marginal to slight interstitial nephritis was increased over controls.

The only findings at 625 ppm were minor, transient decreases in feed consumption in both sexes, decreased glucose in males and minor increases in severity of centrilobular hepatocyte swelling in both sexes and pyelitis in the kidneys in males. However, the incidences of these microscopic findings were not dose dependent. At this dose, the incidences of centrilobular hypertrophy were not accompanied by increased liver weights or changes in clinical chemistry. Therefore, these changes are likely an adaptive response to the test material and are not considered adverse at this dose level.

The nose (a potential target organ) was not examined histopathologically in this study.

The no-observed-adverse-effect level (NOAEL) was 625 ppm (equal to 91 mg/kg bw per day), based on transient clinical signs of toxicity, decreased body weight gains, decreased feed consumption and liver toxicity (increased liver weights, clinical chemistry, severity of centrilobular hypertrophy and glycogen storage) in both sexes at 3125 ppm (equal to 447 mg/kg bw per day) (Kemp, de Haan & Dawes, 1987).

Hamsters

In a 90-day oral toxicity study, dichlobenil (purity 98.5%) was administered to 10 F1D Alexander Syrian hamsters (supplier Bio Breeders Inc., USA) of each sex per dose in the diet at a concentration of 41, 209, 1289 or 7500/4648 ppm (equivalent to 3, 16, 79 and 395/263 mg/kg bw per day, respectively). Twenty animals of each sex were tested as a control group. Ten hamsters of each sex from the control and high-dose groups (7500/4648 ppm) were kept on study for an additional 4-week recovery period, during which the animals received diet without dichlobenil. As a result of excessive weight loss (13–25%) in both sexes after 2 weeks at 7500 ppm, all high-dose animals received 4648 ppm dichlobenil from week 3 until week 13. Animals were inspected twice daily for signs of toxicity and mortality on weekdays and once on weekends. Animals were weighed weekly. Feed consumption was recorded daily. Ophthalmological examination was performed at study initiation and during week 11. Blood was collected before the treatment and at termination on week 13 or week 17 for haematology and clinical chemistry measurements. All animals were subjected to gross pathological examination. Selected organs were weighed, and tissues were examined histopathologically.

There were no treatment-related effects on survival, and no adverse clinical signs or ophthalmological changes were observed. Body weight gains in high-dose males did not rebound to control levels until week 10; however, mean body weights in 4648 ppm males remained decreased (21%) at the end of treatment. Mean feed consumption was decreased ($\geq 11\%$) in 4648 ppm males after dose lowering. Mean serum cholesterol and phospholipids were increased by 55% each ($P < 0.01$) in females at 4648 ppm. Concentrations of these clinical chemistry parameters, body weight gain and feed consumption returned to control levels during the recovery period. Other changes in haematological or clinical chemistry parameters were slight and not considered toxicologically

significant. Mean absolute liver weights were increased by at least 13% ($P < 0.01$) in males at and above 1289 ppm and by 16% ($P < 0.01$), 34% ($P < 0.01$) and 85% ($P < 0.01$) in females at 209, 1289 and 4648 ppm, respectively. Mean absolute prostate weight was reduced by at least 19% ($P < 0.05$) at and above 209 ppm, whereas mean absolute seminal vesicle and testicular weights were reduced by 16% or greater at 209, 1289 and 4648 ppm. Statistically significant changes in other mean absolute and/or relative organ weights (e.g. kidney, spleen, brain and uterus) were observed at 4648 ppm; however, in the absence of histopathological correlates, these changes were not considered toxicologically significant.

At necropsy, livers of 5/10 ($P < 0.01$) males were enlarged at 4648 ppm, whereas 5/10 or 10/10 ($P < 0.01$) females had enlarged livers at 1289 or 4648 ppm, respectively. Rough surface of the liver was also noted for 3/10 and 8/10 ($P < 0.01$) females at 1289 or 4648 ppm, respectively. Black grit was found in the gallbladders of 2/10 ($P < 0.01$) females at 4648 ppm at the end of the treatment. Control incidences for each of these hepatobiliary observations were 0/20. At 4648 ppm, small testis was observed in 3/9 males ($P < 0.01$), whereas small epididymis was seen in 2/9 males ($P < 0.01$); the control incidence for each of these observations was 0/20. Slight haemorrhagic area in the epididymis and seminal vesicle was also observed in 1/9 males (versus 0/20 controls) at 4648 ppm. Bile granules in the gallbladder were observed in 2/10 males and 3/10 females in the high-dose recovery group (versus 0/10 and 1/10, respectively, in controls).

Histopathological observation revealed a marked decrease in the number of spermatocytes at the end of treatment in 5/9 males at 4648 ppm (versus 1/20 in controls). Marginal to moderate prostatic mineralization was observed in 3/10, 4/10 or 3/9 males at 209, 1289 and 4648 ppm, respectively, after 13 weeks (versus 1/20 in controls). Marginal to marked tubular degeneration in the testes was observed in 7/9 ($P < 0.01$) males at 4648 ppm (versus 2/20 in controls). Calculi in the gallbladder were observed at the end of treatment in 7/10 males ($P < 0.01$) and 10/10 ($P < 0.01$) females at 4648 ppm (versus 0/20 in each control group). Swollen hepatocytes were noted in 2/9 males at 4648 ppm and in 2/10 females at 209 ppm (marginal), 4/10 females at 1289 ppm (marginal to slight; $P < 0.01$) and 4/10 females at 4648 ppm (marginal to moderate; $P < 0.01$). Control incidences for swollen hepatocytes were 1/20 for males and 0/20 for females.

The nose (a potential target organ) was not examined histopathologically in this study.

In conclusion, the NOAEL was 41 ppm (equivalent to 3 mg/kg bw per day), based on decreased weight and mineralization of the prostate and decreased mean absolute seminal vesicle and testicular weights in males at 209 ppm (equivalent to 16 mg/kg bw per day) (Kemp, de Haan & Jager, 1987).

Rats

In a 90-day oral toxicity study, dichlobenil (purity 90.5%) was administered in the diet to albino Rochester Wistar rats (12 of each sex per dose) at a concentration of 0, 100, 1000 or 3000 ppm (equivalent to 0, 10, 100 and 300 mg/kg bw per day, respectively) for 3 months. An additional six males were treated with 10 000 ppm (equivalent to 1000 mg/kg bw per day) for 3 months. Histopathological examination was performed on major organs, and five animals per group were examined.

Five (of six) males treated with 10 000 ppm dichlobenil died by week 7 of the study (causes undetermined). Mortality, due to either respiratory infection or “other causes”, was evenly distributed across males in all other groups (2/12, 2/12, 1/12 and 2/12 at 0, 100, 1000 and 3000 ppm, respectively); 2/12 females died on study at 1000 ppm only. Mean body weights were decreased in males (≥ 14%) by the end of the study at and above 3000 ppm. There were no treatment-related effects on urinary protein or glucose up to 3000 ppm. In addition, no adverse effects on haematological parameters were observed. There was an increase in absolute kidney weights at 3000 ppm. Mean absolute liver weights were increased in males (39%) and females (52%) at 3000 ppm. The effect was dose dependent and is considered adverse, as granular swelling and fine foamy vacuolation of the hepatocytes were observed in all rats of each sex at 3000 ppm and in all males at 10 000 ppm. Necrosis was observed in only one 3000 ppm group male. An inflammatory, necrotic

focus was also observed in the liver of one male at 1000 ppm, whereas vacuolar swelling was observed in a second animal at the same dose.

The nose (a potential target organ) was not examined histopathologically in this study. Histopathological results were not reported for all animals, clinical chemistry analysis was not performed, there was a lack of initial haematological measurements, feed consumption was not measured and there was a lack of analysis of test substance concentration, stability and homogeneity. These deficiencies would not be expected to alter the conclusions of this study.

The NOAEL was 100 ppm (equivalent to 10 mg/kg bw per day), based on hepatocytic inflammation and necrosis at 1000 ppm (equivalent to 100 mg/kg bw per day) in males (Hodge, 1961).

Dogs

In a 90-day oral toxicity study, dichlobenil (purity 95.5%) was administered in the diet to two Beagle dogs of each sex per dose group at a concentration of 0, 50, 150 or 450 ppm (equivalent to 0, 1.3, 3.8 and 11 mg/kg bw per day) for at least 90 days; the 450 ppm dose group consisted of three males and one female.

No adverse effects of treatment were observed on mortality, clinical signs, body weights, body weight gains, feed consumption, haematology, urine analysis or gross pathology. Liver effects were observed at 450 ppm. Alkaline phosphatase, alanine aminotransferase (ALT), kidney weights and liver weights were increased at 450 ppm. The study authors reported a finely foamy appearance of the liver cell cytoplasm that was attributed to vacuoles resulting from glycogen storage and concluded that the observed glycogen storage in treated dogs was unrelated to the test substance, because only mild glycogenic infiltration of the hepatic parenchymal cells was observed in dogs in the 450 ppm dose.

The nose (a potential target organ) was not examined histopathologically in this study.

The NOAEL was 150 ppm (equivalent to 3.8 mg/kg bw per day), based on increased alkaline phosphatase, ALT, and liver and kidney weights at 450 ppm (equivalent to 11 mg/kg bw per day) (Til, Feron & de Groot, 1967).

In a 52-week toxicity study, dichlobenil (purity 98.8%) was administered by capsule to five Beagle dogs of each sex per dose at a dose of 0, 1, 6 or 36 mg/kg bw per day. Animals were observed twice daily for morbidity and mortality. Animals were observed for adverse reactions daily. Detailed clinical examinations were conducted on all animals before the initiation of treatment and during weeks 26 and 52. Individual body weights were recorded at least once weekly. Individual feed consumption and water consumption were measured daily up to week 16 of the treatment period, then daily during weeks 20, 24, 28, 32, 36, 40, 44, 48 and 52. Eyes were examined by ophthalmoscopy and fundus photography prior to the initiation of treatment (week −1) and during week 52. Blood was collected from the jugular vein during weeks −1, 26/27 and 52/53. Urine was collected from fasted animals during weeks −1, 26/27 and 52/53. All animals were killed and necropsied after 52 weeks of treatment; selected organs were weighed, and tissue samples were fixed and preserved at necropsy. Selected tissues from all dogs killed post-study were examined histopathologically.

All animals survived to the scheduled termination date. No treatment-related clinical signs of toxicity were observed in any dogs, and no compound-related effects were noted on feed or water consumption, ophthalmoscopic examination, urine analysis or gross pathology. Lower mean body weights (12–16%) and body weight gains (36–44%; $P < 0.05$) were observed throughout the study in males at 36 mg/kg bw per day (relative to controls), whereas no biologically significant changes in body weight or body weight gain were observed in females. Haemoglobin, haematocrit and erythrocyte counts during treatment were slightly decreased in males at and above 6 mg/kg bw per day at weeks 26 and 52. However, as the magnitudes of change (6–12%), although statistically significant, were slight and as haemosiderin was observed in the spleen without dose dependence at

and above 6 mg/kg bw per day, the observed parameters were not considered toxicologically significant. Statistically or biologically significant changes in these same haematological parameters were not observed in females. Increased serum cholesterol, triglyceride, phospholipid and alkaline phosphatase levels were observed in males and females at and above 6 mg/kg bw per day (Table 9). Gamma-glutamyltransferase was also increased in males only. Increased ($\geq$ 18%) absolute and relative liver weights were observed in mid- and high-dose males and females (Table 10). This effect corresponded to periportal hepatocytic hypertrophy in males at and above 6 mg/kg bw per day and at 36 mg/kg bw per day in females. Decreased (78%; $P < 0.05$) uterine weights were also observed in females at 36 mg/kg bw per day. This effect was accompanied by delayed maturation of the uterus at this dose. Other changes in absolute or relative organ weights were not considered treatment related, as the effects were not dose related or were not accompanied by histopathological alterations. No adverse effects were observed in the nasal cavity of treated or control animals at termination.

In conclusion, the NOAEL was 1 mg/kg bw per day, based on increased liver weights and increased serum cholesterol, triglycerides, phospholipids and alkaline phosphatase in males and females and increased serum gamma-glutamyltransferase and periportal hypertrophy of the hepatocytes in males at 6 mg/kg bw per day (Pickersgill, 1995).

In a 2-year toxicity study, dichlobenil (purity 95.5%) was administered in the diet to Beagle dogs (four of each sex per dose) at a concentration of 0, 20, 50 or 350 ppm (equivalent to 0, 0.5, 1.3 and 8.8 mg/kg bw per day, respectively). Body weights were recorded weekly for 16 weeks and then every other week. Feed consumption was measured weekly. Blood was collected before treatment and at 13, 26, 52, 78 and 102 weeks for haematological and clinical chemistry measurements. Urine was collected before treatment and at 12, 26, 78 and 102 weeks. All animals were terminated, and a gross pathological examination was conducted. Selected organs were weighed and examined microscopically.

No animals died during the study, and there were no treatment-related clinical signs of toxicity. No adverse effects were observed on body weight, feed consumption or haematological parameters or at necropsy.

At 350 ppm, mean concentrations of serum alkaline phosphatase were increased 2–3 times over controls in males and females (Table 11). Mean ALT levels in females were 2 times higher than in controls at 350 ppm during the second half of the study, whereas mean ALT concentrations in males were decreased at this same dose and time period. An increase in the number of erythrocytes (moderate to high) in the urine was observed in 3/4 high-dose females at 350 ppm at week 102 only. Mean absolute and relative liver weights were increased by 35% and 28% in males and by 67% and 46% in females at 350 ppm (Table 11). Increases in mean absolute kidney weight (10% in males; 24% in females) and thyroid weight (57% in males; 111% in females) were not considered toxicologically significant at the high dose in the absence of histopathological correlates and also because mean body weight was increased by 45% and 86% in males and females, respectively, at this dose. In addition, no difference in mean relative kidney weight was observed in any treatment group, relative to controls. Mean absolute and/or relative glucose-6-phosphatase and glucose-6-phosphate dehydrogenase activities were increased in the liver at 350 ppm, thereby reflecting the increased metabolic activity of hepatocytes at this dose (Table 11). Moderately severe leukocytic infiltration around the central veins of the liver was observed in 2/4 males and 3/4 females at 350 ppm. “One or a few tiny foci of necrosis” were also observed in 3/4 males at 350 ppm (versus 1/4 controls).

The nose (a potential target organ) was not examined histopathologically in this study.

The NOAEL was 50 ppm (equivalent to 1.3 mg/kg bw per day), based on increased liver weight (both sexes), serum alkaline phosphatase (both sexes) and serum ALT (females only); liver histopathology (leukocytic infiltration around the central veins [both sexes] and necrosis [males only]); and an increase in the number of erythrocytes in the urine (females only) at 350 ppm (equivalent to 8.8 mg/kg bw per day) (Til et al., 1969).

Table 9. Selected clinical chemistry changes in male and female dogs (52-week study)

Parameter	Week	0 mg/kg bw per day	1 mg/kg bw per day	6 mg/kg bw per day	36 mg/kg bw per day
Males					
Cholesterol (g/L)	−1	1.71 ± 0.60	1.72 ± 0.12	1.58 ± 0.19	1.56 ± 0.27
	26	1.37 ± 0.40	1.65 ± 0.15 (20)[a]	2.05 ± 0.37** (50)	3.00 ± 0.76** (119)
	52	1.23 ± 0.36	1.54 ± 0.17	1.90 ± 0.29** (54)	2.56 ± 0.45** (108)
Triglycerides (g/L)	−1	0.29 ± 0.11	0.40 ± 0.10	0.38 ± 0.06	0.34 ± 0.07
	26	0.33 ± 0.11	0.38 ± 0.11 (15)	0.48 ± 0.13* (45)	1.85 ± 1.56** (461)
	52	0.32 ± 0.07	0.43 ± 0.07* (34)	0.46 ± 0.10* (44)	1.55 ± 0.67** (384)
Phospholipids (g/L)	−1	3.22 ± 0.99	3.25 ± 0.20	2.96 ± 0.28	3.05 ± 0.43
	26	3.01 ± 0.59	3.58 ± 0.43 (19)	3.90 ± 0.31** (30)	5.33 ± 1.17** (77)
	52	2.78 ± 0.76	3.45 ± 0.33* (24)	3.86 ± 0.31** (39)	4.94 ± 0.76** (78)
Alkaline phosphatase (IU/L)	−1	278 ± 43	283 ± 71	269 ± 41	268 ± 67
	26	133 ± 42	156 ± 67	191 ± 34* (44)	575 ± 320** (332)
	52	110 ± 54	129 ± 53	191 ± 50* (74)	525 ± 224** (377)
GGT (IU/L)	−1	3 ± 1	4 ± 1	4 ± 1	4 ± 1
	26	3 ± 2	4 ± 1	4 ± 2	5 ± 1* (67)
	52	0 ± 0	2 ± 2	2 ± 1*	3 ± 2*
Females					
Cholesterol (g/L)	−1	1.36 ± 0.22	1.42 ± 0.36	1.27 ± 0.21	1.40 ± 0.19
	26	1.55 ± 0.36	1.85 ± 0.57	2.27 ± 0.31** (46)	3.06 ± 0.41** (97)
	52	1.72 ± 0.53	1.74 ± 0.70	2.14 ± 0.31 (24)	2.99 ± 0.67** (74)
Triglycerides (g/L)	−1	0.33 ± 0.06	0.32 ± 0.08	0.34 ± 0.11	0.34 ± 0.06
	26	0.45 ± 0.11	0.43 ± 0.12	0.64 ± 0.23 (42)	1.13 ± 0.16** (151)
	52	0.51 ± 0.10	0.52 ± 0.10	0.66 ± 0.12* (29)	1.34 ± 0.35** (163)
Phospholipids (g/L)	−1	2.77 ± 0.33	2.85 ± 0.54	2.65 ± 0.41	2.84 ± 0.44
	26	3.43 ± 0.51	3.80 ± 0.97	4.27 ± 0.41* (24)	5.47 ± 0.44** (59)
	52	3.69 ± 1.07	3.53 ± 0.99	4.36 ± 0.38 (18)	5.39 ± 0.83** (46)
Alkaline phosphatase (IU/L)	−1	253 ± 49	281 ± 41	298 ± 54	293 ± 66
	26	135 ± 65	177 ± 49	174 ± 40 (29)	404 ± 118** (199)
	52	142 ± 78	174 ± 72	178 ± 60 (25)	462 ± 65** (225)

bw: body weight; GGT: gamma-glutamyltransferase; IU: International Units; *: $P < 0.05$; **: $P < 0.01$

[a] Numbers in parentheses are per cent change, relative to control (reviewer calculated these values only where there are significant results, to examine dose–response relationships).

Source: Data taken from pp. 121–138 of Pickersgill (1995)

Table 10. Selected absolute and relative organ weights in dogs (52-week study)

Organ	Absolute (g) and relative (% of body weight) organ weights			
	0 mg/kg bw per day	1 mg/kg bw per day	6 mg/kg bw per day	36 mg/kg bw per day
Males				
Terminal body weight	10 380 ± 1 275	10 080 ± 1 199	9 700 ± 480 (7)[a]	8 820 ± 853* (15)
Liver	281.1 ± 30.4	287.6 ± 27.6	332.1 ± 22.4* (18)	476.7 ± 95.9** (70)
% body weight	2.73 ± 0.34	2.87 ± 0.28	3.42 ± 0.13** (25)	5.37 ± 0.66** (97)
Kidneys	45.60 ± 3.60	44.80 ± 4.16	45.47 ± 4.87	55.16 ± 5.22** (21)
% body weight	0.444 ± 0.053	0.446 ± 0.028	0.471 ± 0.064	0.627 ± 0.053** (41)
Thyroids	0.691 ± 0.207	0.695 ± 0.309	0.764 ± 0.179	1.080 ± 0.206* (56)
% body weight	0.006 7 ± 0.002 0	0.007 0 ± 0.003 5	0.007 9 ± 0.001 8	0.012 3 ± 0.002 2** (84)
Testes	13.28 ± 1.75	14.99 ± 3.55	14.77 ± 1.07	17.70 ± 4.85 (33)
% body weight	0.129 ± 0.019	0.149 ± 0.035	0.152 ± 0.010* (18)	0.201 ± 0.052** (56)
Prostate	10.168 ± 4.125	8.755 ± 1.863	7.918 ± 2.468 (22)	6.375 ± 1.070* (37)
% body weight	0.095 3 ± 0.034 8	0.087 1 ± 0.018 4	0.081 7 ± 0.025 0 (14)	0.073 1 ± 0.015 5 (23)
Females				
Terminal body weight	8 660 ± 730	8 860 ± 666	9 620 ± 733	8 040 ± 1 088
Liver	252.9 ± 21.1	300.8 ± 38.6 (19)	378.3 ± 56.5** (50)	391.1 ± 60.7** (55)
% body weight	2.92 ± 0.14	3.41 ± 0.51* (17)	3.92 ± 0.43** (34)	4.86 ± 0.18** (66)
Thyroids	0.560 ± 0.065	0.749 ± 0.104** (34)	0.744 ± 0.113** (33)	0.840 ± 0.084** (50)
% body weight	0.006 5 ± 0.001 1	0.008 5 ± 0.001 5	0.007 8 ± 0.001 6	0.010 6 ± 0.001 5** (63)
Thymus	4.477 ± 0.906	2.702 ± 1.003	4.648 ± 0.661	3.086 ± 0.222*
% body weight	0.051 5 ± 0.008 3	0.030 3 ± 0.011 3*	0.048 4 ± 0.006 5*	0.038 7 ± 0.003 3*
Ovaries	1.340 ± 0.289	1.361 ± 0.665	1.828 ± 0.746	0.676 ± 0.145** (50)
% body weight	0.015 7 ± 0.004 2	0.015 5 ± 0.007 9	0.018 7 ± 0.006 7	0.008 5 ± 0.001 9 (46)
Uterus	15.226 ± 6.506	11.522 ± 8.928	17.280 ± 10.842	3.423 ± 0.734* (78)
% body weight	0.179 9 ± 0.085 5	0.132 1 ± 0.105 6	0.175 7 ± 0.106 7	0.042 7 ± 0.009 2* (76)

bw: body weight; *: $P < 0.05$; **: $P < 0.01$

[a] Numbers in parentheses are per cent change, relative to control (reviewer calculated these values only where there are significant results, to examine dose–response relationships).

Source: Data taken from pp. 316–321 of Pickersgill (1995)

(b) Dermal application

Rabbits

In a 21-day dermal toxicity study, dichlobenil technical (purity 98.8%) was administered topically (i.e. spread evenly over the intact skin of the clipped dorsal region of animals in treatment groups, then moistened with water) to New Zealand White rabbits (five of each sex per dose) at a dose

Table 11. Clinical chemistry changes in male and female dogs receiving dichlobenil for 2 years

	Males				Females			
Week	**0 ppm**	**20 ppm**	**50 ppm**	**350 ppm**	**0 ppm**	**20 ppm**	**50 ppm**	**350 ppm**
Serum alkaline phosphatase (Bessey-Lowry units)								
0	1.0	1.0	1.0	1.1	0.9	0.9	0.9	0.8
12	1.6	1.9	2.0	3.2	2.1	1.6	1.9	4.1
26	1.6	1.7	2.5	2.5	2.2	1.4	2.0	3.9
52	1.0	0.9	1.4	2.6	1.7	1.2	1.5	3.3
78	1.3	1.7	1.6	3.5	1.6	1.1	1.7	4.1
102	1.2	1.5	1.6	3.3	1.5	1.1	1.7	3.5
Serum ALT (rheumatoid factor units)								
0	12.4	11.4	12.4	12.3	12.5	11.0	15.0	12.8
12	9.8	12.4	12.8	11.9	12.8	11.8	11.9	13.1
26	21.8	19.3	26.0	10.5	16.9	14.4	13.1	21.9
52	16.0	25.9	15.0	11.8	13.1	11.9	12.6	18.4
78	22.0	22.3	17.4	9.5	12.6	11.8	7.4	31.4
102	23.9	16.3	25.0	12.5	16.3	15.0	10.5	41.6
Terminal liver weights[a]								
Absolute	452	416	466	610	368	364	408	615
Relative	3.35	3.12	3.07	4.29	2.96	2.90	3.17	4.32
Terminal kidney weights[a]								
Absolute	73	73	81	80	51	56	62	63
Relative	0.54	0.55	0.53	0.56	0.41	0.45	0.48	0.44
Liver enzyme activity								
G6Pase[b]	30.0	34.5	33.6	42.9	27.6	27.6	29.4	37.1
G6PD[c]	5.1	6.8	7.2	15.1	4.7	4.3	5.6	11.8

ALT: alanine aminotransferase; bw: body weight; G6Pase: glucose-6-phosphatase; G6PD: glucose-6-phosphate dehydrogenase; NADPH: nicotinamide adenine dinucleotide phosphate (reduced); ppm: parts per million

[a] Absolute organ weight: g; relative organ weight: g/100 g bw.
[b] Units: µmol glucose-6-phosphate hydrolysed per minute per 100 g bw at pH 6.6 and 30 °C.
[c] Units: µmol NADPH produced per minute per 100 g bw at pH 7.4 and 30 °C.

Source: Til et al. (1969)

of 0, 100, 300 or 1000 mg/kg bw per day, 6 hours/day, for 3 weeks. At the end of the 6-hour exposure period, the skin application was washed with warm water to remove any residues.

Administration of 100, 300 or 1000 mg/kg bw per day produced no adverse clinical signs or skin irritation. No animals died during the study. During the last week of the study, three rabbits (one low-dose male and female and one mid-dose female) were euthanized because they showed hind limb paralysis. All three rabbits showed haemorrhage of the skeletal muscle in the lumbar region around the lower spinal column, and two rabbits showed fractured lower lumbar vertebrae. These findings were considered to be due to trauma, but the cause of the trauma was not established in the report and may have been due to handling. There were no treatment-related effects on body weight, feed consumption, haematology, blood chemistry, organ weights, or gross or microscopic pathology. An increase in mean serum cholesterol (49%, $P < 0.01$) was observed in high-dose females. In the absence of liver histopathology, this effect was not considered toxicologically significant. The nose (a potential target organ) was not examined histopathologically in this study. In addition, an ophthalmological examination was not performed.

The NOAEL for dermal irritation and systemic toxicity in rabbits was 1000 mg/kg bw per day, the highest dose tested; a lowest-observed-adverse-effect level (LOAEL) could not be determined (Allan, 1995).

(c) Exposure by inhalation

In a 7-day inhalation toxicity study, Sprague-Dawley [Crl:CD(SD)IGS BR] rats (five of each sex per concentration) were exposed by nose-only inhalation to dichlobenil (purity 98.1%) as a dust aerosol at an achieved concentration of 0, 21, 77 or 200 mg/m^3 for 6 hours/day for 7 days. The study served as a dose-range finding study for the 28-day inhalation study.

There were no treatment-related effects on survival. Decreased general activity was observed in 1/5 males at each of 77 and 200 mg/m^3. Difficulty breathing was observed in 1/5 and 2/5 males at 77 and 200 mg/m^3, respectively. Mean body weight was decreased in females at 200 mg/m^3, compared with controls. Mean feed consumption was decreased in both sexes at 77 and 200 mg/m^3, compared with controls. No adverse effects were observed at necropsy. Mean absolute and relative liver weights were increased in both sexes at 77 and 200 mg/m^3, respectively, compared with controls. Trace to mild nasal degeneration was observed in at least 1/5 animals of each sex at and above 21 mg/m^3 (versus 0/5 animals of each sex in controls). This response was expected, as dichlobenil is an olfactory toxicant.

The LOAEL was 21 mg/m^3, the lowest dose tested. A NOAEL was not established in this study (Newton, 2002).

In a 28-day inhalation toxicity study, Sprague-Dawley rats (10 of each sex per concentration) were exposed by nose-only inhalation to dichlobenil (purity 98.1%) as a dust aerosol at a concentration of 0, 2.3, 5.1 or 12 mg/m^3 (equivalent to 0, 0.0023, 0.0051 and 0.012 mg/L, respectively) for 6 hours/day, 5 days/week, for 4 weeks. These concentrations were selected based on the results of the 7-day inhalation toxicity study described above.

There were no treatment-related effects on survival, clinical observations, functional observational battery, motor activity, body weights, body weight gains, feed consumption, ophthalmoscopy, haematology, clinical chemistry, organ weights, gross pathology or histopathology in either sex at any concentration.

In conclusion, the NOAEL was 12 mg/m^3 (equivalent to 0.012 mg/L), the highest dose tested. A LOAEL was not observed in this study (Newton, 2002).

2.3 Long-term studies of toxicity and carcinogenicity

Hamsters

In a carcinogenicity study, dichlobenil (purity 99.4%) was administered in the diet for 91 weeks (males) or 78 weeks (females) to F1D Alexander Syrian hamsters (50 of each sex per dose in the treatment groups and 100 of each sex in the control group) at a concentration of 0, 675, 1500 or

3375 ppm (equal to 0, 51, 117 and 277 mg/kg bw per day for males and 0, 55, 121 and 277 mg/kg bw per day for females, respectively).

No treatment-related mortality or clinical signs were observed. Overall body weight gain was decreased for all treated groups compared with controls. In the 3375 ppm dose group, a large drop in body weight (16% decrease) was seen as early as 2 weeks after the initiation of the study and was sustained throughout the study. No treatment-related effects were noted in eyes during ophthalmoscopic examination. A statistically significant increase in body weight–adjusted liver weights was observed in females in all treatment groups and in mid- and high-dose males at termination. Macroscopic examination revealed enlargement of the liver in both sexes receiving 3375 ppm diet, together with a slightly increased incidence of liver masses in males receiving 3375 ppm. A slight increase in the incidence of pale areas on the liver together with a reduction in adipose tissue were also noted in males in all treated groups. In the high-dose males, an increased incidence of benign liver tumours (6/50 versus 0/100 controls) and one malignant liver tumour were observed. One benign tumour was observed in males at 1500 ppm (Table 12). Eosinophilic hepatocytes were observed in males receiving 1500 ppm diet (not statistically significant) or 3375 ppm diet; minimal centrilobular hepatocyte enlargement was seen in all treated male groups and in females receiving 3375 ppm diet. Hepatitis was observed in both sexes receiving 3375 ppm diet and in females receiving 1500 ppm diet, as were pigmented giant cells in males and females receiving 3375 ppm diet, pigmented sinusoidal cells in males and females receiving 3375 ppm diet and brown pigments in hepatocytes in males receiving 1500 or 3375 ppm diet. The nose (a potential target organ) was not examined histopathologically in this study.

Table 12. Selected liver lesions in carcinogenicity study in hamsters

	Incidence of finding							
	Males				**Females**			
Observation	**0 ppm**	**675 ppm**	**1 500 ppm**	**3 375 ppm**	**0 ppm**	**675 ppm**	**1 500 ppm**	**3 375 ppm**
Microscopic non-neoplastic lesions in liver								
Centrilobular hepatocyte enlargement	7/100	15/50*	10/50*	14/50*	7/100	5/49	5/50	11/50*
Finely vacuolated hepatocytes	1/100	3/50	6/50*	8/50*	0/100	0/49	0/50	0/50
Hepatitis	1/100	1/50	2/50	5/50*	0/100	0/49	3/50*	4/50*
Pigmented giant cells	10/100	4/50	5/50	20/50*	17/100	5/49	11/50	17/50*
Pigmented sinusoidal cells	5/100	1/50	3/50	9/50*	7/100	4/49	2/50	9/50*
Brown pigment in hepatocytes	9/100	7/50	16/50*	19/50*	0/100	0/49	0/50	0/50
Eosinophilic hepatocytes	0/100	0/50	1/50	5/50*	0/100	0/49	0/50	0/50
Liver tumours								
Hepatocellular adenoma	0/100	0/50	1/50	6/50*	0/100	0/49	0/50	0/50
Hepatocellular carcinoma	0/100	0/50	0/50	1/50	0/100	0/49	0/50	0/50

ppm: parts per million; *: $P < 0.05$ (Fisher's exact test)

Source: Chambers et al. (1992b)

In conclusion, a NOAEL could not be identified in this study, based on decreased body weight gain in males and females and increased relative liver weight in females at 675 ppm (equal to 51 mg/kg bw per day), the lowest dose tested. The NOAEL for carcinogenicity in male hamsters was 1500 ppm (equal to 117 mg/kg bw per day), based on an increased incidence of hepatocellular adenoma and carcinoma at 3375 ppm (equal to 277 mg/kg bw per day). No treatment-related tumours were observed in female hamsters (Chambers et al., 1992b).

In a separate carcinogenicity study, dichlobenil (purity 99.4%) was administered in the diet for 88 weeks (males) or 80 weeks (females) to F1D Alexander Syrian hamsters (50 of each sex per dose) at a concentration of 5, 26, 132 or 675 ppm (equal to 0.34, 1.69, 9.39 and 45.6 mg/kg bw per day for males and 0.35, 1.78, 9.20 and 48.9 mg/kg bw per day for females, respectively). Control groups consisted of 100 animals of each sex. The criterion for study termination in males was 50% mortality in controls.

Diets were prepared weekly. The test compound was stable and homogeneously distributed in the diet, and measured concentrations were within an acceptable range.

Mortality in control females at termination was 42%. There were no dose-dependent differences in survival of males or females at termination. Mean body weight was decreased (10–11%) in males at 675 ppm from weeks 80 to 88 only of the study. Mean overall body weight gain was decreased at 675 ppm by 96% ($P < 0.01$) in males from weeks 0 to 88 and by 14% in females from weeks 0 to 80. No differences in feed consumption or feed efficiency were observed across the doses. No adverse effects were observed on differential leukocyte counts or ophthalmological end-points or at necropsy.

Mean relative liver weights were increased in females by 15% ($P < 0.01$) at 675 ppm; however, the effect showed no relationship with dosing. Non-neoplastic histopathological observations are summarized in Table 13. Increased incidences ($P < 0.05$) of reduced secretion of the prostate and seminal vesicles were observed in males at and above 132 ppm, at which the dose–response curve plateaued for both end-points. The dose–response curve for the increased incidence ($P < 0.05$) of hyperplasia of the non-glandular region of the stomach in males also plateaued at and above 26 ppm. The toxicological relevance of this end-point is questionable, however, as humans lack a homologue for the rodent non-glandular stomach. Increased incidences ($P < 0.05$) of hyperplasia of the non-glandular region of the stomach were also observed in females; however, the effect was not dose dependent. Increased incidences of hyperplasia ($P < 0.05$) were also observed at several other sites at 675 ppm, including pancreatic islet cells and skin (acanthosis) in males and adrenal cortex, small intestine and bone marrow (sternum) in females. Hypertrophy of the skin (hyperkeratosis) and hepatocytes were also observed at the high dose in females. The nose (a potential target organ) was not examined histopathologically in this study.

The NOAEL was 26 ppm (equal to 1.69 mg/kg bw per day), based on reduced secretion of the prostate and seminal vesicles in males at 132 ppm (equal to 9.39 mg/kg bw per day) (Hooks et al., 1991a,b).

An additional study was conducted to collect background data on incidences of spontaneously occurring tumours in hamsters of the F1D Alexander Syrian strain, supplied by Bio Breeders Inc., USA. This strain was used in the carcinogenicity studies with dichlobenil by Chambers et al. (1992b) and Hooks et al. (1991a,b). The conditions and procedures used in this study were designed to duplicate those of the two studies. This study included one untreated control group consisting of 50 male and 50 female hamsters monitored from about 6 weeks of age until approximately 50% survival was reached in each group, at which time all surviving animals were euthanized. This occurred after 77 weeks for females and 90 weeks for males. Additional data were also generated on survival, body weight change and feed consumption, with limited haematological and organ weight parameters assessed.

Table 13. Non-neoplastic histopathological observations in hamsters

	Incidence of finding				
	0 ppm	**5 ppm**	**26 ppm**	**132 ppm**	**675 ppm**
Males					
Reduced secretion of the prostate	5/100	1/21	2/27	5/21*	9/48*
Reduced secretion of the seminal vesicles	9/100	5/34	5/32	7/27*	10/48*
Islet cell hyperplasia of the pancreas	1/99	0/21	1/26	0/20	4/50*
Epithelial hyperplasia of the stomach (non-glandular region)	18/100	6/50	18/50*	22/50*	20/50*
Acanthosis of the skin	1/100	0/21	0/27	0/25	7/50*
Females					
Centrilobular hepatocytes	1/100	1/50	1/50	1/50	6/50*
Cortical hyperplasia of the adrenals	49/99	25/50	23/49	29/49	36/49*
Epithelial hyperplasia of the stomach (non-glandular region)	29/100	30/50*	21/50	19/50	27/50*
Epithelial hyperplasia of the stomach (limiting ridge)	20/100	18/50*	18/50*	13/50	21/50*
Submucosal inflammatory cells in the stomach (non-glandular region)	12/100	12/50	5/50	11/50	17/50*
Peritonitis	1/100	2/50	2/50	1/50	5/50*
Epithelial hyperplasia of the duodenum	13/100	2/30	3/37	2/37	13/49*
Epithelial hyperplasia of the ileum	9/100	0/27	1/31	2/36	11/49*
Epithelial hyperplasia of the caecum	3/100	3/33	1/32	1/37	9/50*
Prominent mucous cells in the caecum	9/100	4/33	2/32	3/37	11/50*
Limiting dilation of the rectum	3/98	0/28	0/32	0/36	8/50*
Hyperkeratosis of the skin	1/100	0/29	0/35	1/36	6/50*
Hyperplasia of the bone marrow in the sternum	33/100	11/28	11/32	11/36	25/50*

ppm: parts per million; *: $P < 0.05$ (Fisher's exact test)

Source: Data adapted from Table 9, pp. 83–148, of Hooks et al. (1991a)

Based on the results of this study, the study authors (Chambers et al., 1992a) concluded that values for all parameters investigated in this study were essentially similar to those of the respective control groups of the previously mentioned carcinogenicity studies in hamsters (Hooks et al., 1991a,b; Chambers et al., 1992b).

Rats

In a combined chronic toxicity and carcinogenicity study, dichlobenil (purity 96.7%) was administered in the diet to Fischer 344 rats (50 of each sex per dose) for 110 weeks at a concentration of 0, 50, 400 or 3200 ppm (equal to 0, 3.2, 29 and 241 mg/kg bw per day for males and 0, 3.2, 26 and 248 mg/kg bw per day for females, respectively). Interim terminations of 10 additional animals of each sex per dose took place at each of 52 and 78 weeks. Diets were prepared weekly, and dietary concentration, stability and homogeneity were evaluated periodically. Animals were inspected daily for clinical signs and mortality. Body weights were measured weekly for 26 weeks and then biweekly. Feed consumption and water consumption were measured weekly. Ophthalmoscopic examination of

eyes was not performed. Blood was collected from 10 rats of each sex per dose at 52, 78 and 110 weeks. Urine was collected at 52, 73 and 110 weeks from 10 animals of each sex per dose. Animals that died and at termination were subjected to gross pathological examination, and selected tissues were collected for histopathological examination. Selected organs were removed and weighed.

Diets were homogeneous and stable for 7 days, and the concentrations were within an acceptable range.

Mortality was 100% in males by week 98 at 3200 ppm. Survival of males at the middle and low doses was similar to that of controls and of females at all doses tested. No treatment-related clinical signs of toxicity were observed. At 3200 ppm, body weight was decreased by 33% ($P < 0.001$) by week 88 in males and by 34% ($P < 0.001$) by week 104 in females. Body weight decrements were proportional to time on study in both sexes. Similarly, mean body weight gain in males at 3200 ppm was decreased by 19% from weeks 1 to 13 and by 160% from weeks 52 to 66. In females, mean body weight gain at 3200 ppm was decreased by 24% from weeks 1 to 13 and by 40% from weeks 52 to 66. No differences were observed in feed consumption when corrected for body weight, whereas feed efficiency was decreased by 35% in males and by 29% in females at 3200 ppm. Water consumption (corrected for body weight) in high-dose animals was increased by 30% and 55% in males and by 22% and 39% in females at weeks 5 and 15, respectively. Urinary volume was also over 2-fold higher in high-dose males at week 78 ($P < 0.01$). No toxicologically significant effects on haematological parameters were observed. Mean blood urea nitrogen (BUN) was 3-fold higher ($P < 0.01$) at week 78 in males treated with 3200 ppm; in males treated with 400 ppm, mean BUN was increased by 75% ($P < 0.05$) at termination (Table 14). Mean serum uric acid levels were also increased by 35% ($P < 0.01$) at 3200 ppm in males. Mean cholesterol in males treated with 3200 ppm was increased by 91% ($P < 0.05$) at week 52 and by 176% ($P < 0.01$) at week 78; in males treated with 400 ppm, mean cholesterol was increased by 136% ($P < 0.01$) at termination. In females treated with 3200 ppm, mean BUN was over 3-fold higher ($P < 0.05$) at week 110. Mean cholesterol in females treated with 3200 ppm was increased by 168% ($P < 0.01$), 157% ($P < 0.01$) and 83% ($P < 0.01$) at weeks 52, 78 and 110, respectively. No treatment-related changes in mean serum uric acid levels were observed in females.

Mean absolute and relative liver weights were increased in males by 48% ($P < 0.01$) and 118% ($P < 0.01$), respectively, after 78 weeks at 3200 ppm (changes at 78 weeks were similar to those at 52 weeks and at termination); however, the increased relative liver weight was likely due to a combination of increased absolute liver weight and decreased body weight (Table 15). In females of the 400 ppm group, mean absolute liver weights were increased by 16% ($P < 0.01$) at 52 and 78 weeks, and relative liver weights were increased by 23% ($P < 0.01$) at 78 and 110 weeks. At 3200 ppm, mean absolute liver weights of females were increased by 50% and above at 52, 78 and 110 weeks, and relative liver weights were increased by 113% and above at 52, 78 and 110 weeks. Absolute kidney weights were increased by 16% ($P < 0.05$) at 78 weeks in both males and females at 3200 ppm, as was the absolute weight of the adrenals in males (22%, $P < 0.01$) at 78 weeks at the same dose. At terminal necropsy, dose-dependent increases in the incidences of enlarged liver and kidneys were observed in males and females. The incidences of enlarged liver in males were 3/50, 9/50, 15/50 and 45/50 at 0, 50, 400 and 3200 ppm, respectively, whereas enlarged liver was observed in 4/50, 3/50, 11/50 and 44/50 females, respectively. The incidence of enlarged kidney was 2/50, 5/50, 8/50 and 40/50 in males and 0/50, 0/50, 2/50 and 13/50 in females at 0, 50, 400 and 3200 ppm, respectively.

Non-neoplastic histopathological observations are summarized in Table 16. Incidences of fatty metamorphosis (the presence of microscopically visible droplets of fat in the cytoplasm of cells) of the liver were increased above controls at and above 50 ppm at week 52 in males and females; however, dose dependence was lacking for the effect in both sexes at week 78. At study termination, increased incidences of fatty metamorphosis (with increased severity) of the liver were clearly observed in males (45/50) and females (39/40) at 3200 ppm. Liver necrosis was increased in males at 3200 ppm at week 52 only and in females at weeks 52 and 78, but without a dose–response relationship. Cytological alterations (polyploidy with hepatocytic swelling) were increased in females at and above 400 ppm at study termination, whereas increased incidences of “hepatic nodules”

(hyperplasia) were observed in males and females at 3200 ppm at study termination. Clear increases in calcification of the kidney were observed at 3200 ppm in both males (24/50) and females (46/47) at study termination, whereas nephrosis (kidney damage) was observed in 100% of males at and above 400 ppm (increased severity at high dose) and in 98% of females at 3200 ppm at study termination. Nephrosis was also increased in both sexes at both interim termination times at and above 400 ppm. Last, parathyroid hyperplasia was increased at study termination only at and above 50 ppm in males and at 3200 ppm in females. Parathyroid hyperplasia was likely a response to the reported kidney damage (nephrosis), to maintain normal serum calcium concentrations. The nose (a potential target organ) was not examined histopathologically in this study.

There was an increased incidence of hepatocellular tumours at the high dose in both sexes (Table 17). In female rats, there were statistically significant increases in adenomas and combined adenomas/carcinomas by pairwise comparison. There were statistically significant positive dose-related trends for adenomas and carcinomas, alone and combined in both sexes. In male rats, there were no statistically significant increases in hepatocellular tumours at any dose by pairwise comparison.

The NOAEL for systemic toxicity was 50 ppm (equal to 3.2 mg/kg bw per day), based on changes in clinical chemistry (increased BUN, cholesterol), gross pathology (enlarged liver, enlarged kidney) and histopathology (nephrosis, parathyroid hyperplasia) in males; and increased liver weight, enlarged liver and cytological alterations (polyploidy with hepatocytic swelling) in the liver in females at 400 ppm (equivalent to 26 mg/kg bw per day). The NOAEL for carcinogenicity was 400 ppm (equivalent to 26 mg/kg bw per day), based on an increased incidence of hepatocellular tumours at 3200 ppm in both sexes, reaching statistical significance only in females (Inoue & Enomoto, 1984).

Table 14. Selected clinical chemistry values in a chronic toxicity and carcinogenicity study in rats

	Males				Females			
Week	**0 ppm**	**50 ppm**	**400 ppm**	**3 200 ppm**	**0 ppm**	**50 ppm**	**400 ppm**	**3 200 ppm**
Phosphorus (mg/dL)								
52	4.7	4.5	4.5	5.3*	3.9	3.5	4.2	4.3
78	5.2	5.0	5.3	7.9*	4.2	4.5	4.7	5.2*
110	5.5	5.0	5.8	–	5.1	6.0	4.8	8.6
BUN (mg/dL)								
52	19	17	18	25	20	20	18	18
78	19	20	22	59**	20	19	18	26*
110	20	30	35*	–	20	21	18	64*
Creatine (mg/dL)								
52	0.60	0.58	0.54*	0.52**	0.60	0.58	0.53	0.58
78	0.68	0.69	0.71	0.84	0.62	0.60	0.57**	0.63
110	0.60	0.58	0.85*	–	0.61	0.61	0.52**	0.83
Cholesterol (mg/dL)								
52	101	108	143*	193*	120	118	147	321**
78	129	141	177	357**	141	147	185	362**
110	151	187	357**	–	241	205	224	442**

BUN: blood urea nitrogen; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$

Source: Inoue & Enomoto (1984)

Table 15. Selected organ weight changes in a chronic toxicity and carcinogenicity study in rats

	Absolute (g) and relative (g/100 g bw) organ weights							
	Males				Females			
Week	**0 ppm**	**50 ppm**	**400 ppm**	**3 200 ppm**	**0 ppm**	**50 ppm**	**400 ppm**	**3 200 ppm**
Liver								
Absolute								
52	9.90	10.47	11.54**	13.23**	5.27	5.32	6.16**	8.72**
78	10.40	11.03	11.92	15.41**	6.28	6.42	7.30**	11.01**
110	9.87	10.78	12.19**	–	7.76	7.79	8.65*	11.64**
Relative								
52	2.53	2.60	3.05**	4.34**	2.28	2.91	2.72**	4.86**
78	2.40	2.59	2.99*	5.25**	2.35	2.49	2.90**	5.17**
110	2.63	2.79	3.66**	–	2.85	2.70	3.29**	6.62**
Kidney								
Absolute								
52	2.38	2.66*	2.66*	2.99**	1.60	1.62	1.76	2.00**
78	2.80	2.88	2.90	3.24*	1.92	1.92	2.01	2.23**
110	2.84	2.95	3.25**	–	2.23	2.28	2.34	2.43
Relative								
52	0.61	0.66	0.70**	0.96**	0.69	0.70	0.78	1.11**
78	0.65	0.69	0.73	1.10**	0.72	0.75	0.79	1.14**
110	0.76	0.77	0.98**	–	0.83	0.80	0.89	1.41**

bw: body weight; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$

Source: Inoue & Enomoto (1984)

2.4 Genotoxicity

The results of studies of genotoxicity with dichlobenil are summarized in Table 18. All the studies, either in vitro or in vivo, were negative. Dichlobenil is not considered to possess any mutagenic or genotoxic potential.

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

Rats

In a two-generation reproductive toxicity study, dichlobenil technical (purity 99.4%) was administered in the diet to Crl:CD(SD)BR rats (30 of each sex per dose in the P generation; 25 of each sex per dose in the F_1 generation) at a concentration of 0, 60, 350 or 2000 ppm (equivalent to 0, 4, 23 and 130 mg/kg bw per day, respectively) for two consecutive generations. Stability and homogeneity of the prepared diet were measured periodically. Animals were observed once per day for overt signs of toxicity. Body weights and feed consumption were measured weekly and on gestation days (GDs) 0, 6, 12, 15 and 20. Pups were weighed on days 1, 4, 7, 14 and 21 of lactation. Adults were necropsied and examined macroscopically, and histopathological examination was conducted on selected tissues.

Table 16. Selected non-neoplastic lesions in rats

	Incidence of lesions							
	Males				Females			
Week	0 ppm	50 ppm	400 ppm	3 200 ppm	0 ppm	50 ppm	400 ppm	3 200 ppm
Liver								
Fatty metamorphosis								
52	1/10[a]	5/10	8/10	9/10+	0/10	2/10	6/10	10/10+
78	9/10	6/10	7/10	9/10+	5/10	10/10	6/10	10/10+
All others[b]	15/47	17/47	13/47	45/50+	42/47	34/47	36/47	39/40+
Necrosis								
52	0/10	0/10	0/10	5/10	0/10	4/10	1/10	7/10
78	0/10	0/10	0/10	1/10	0/10	0/10	1/10	2/10
All others	2/47	4/47	0/47	1/50	1/47	1/47	1/47	0/47
Cytological alterations								
52	1/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
78	0/10	0/10	1/10	0/10	0/10	0/10	0/10	0/10
All others	0/47	3/47	0/47	1/50	3/47	3/47	14/47	25/47
Nodules								
52	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
78	0/10	0/10	1/10	0/10	1/10	0/10	0/10	0/10
All others	1/47	0/47	1/47	6/50	1/47	2/47	0/47	5/47
Kidney								
Calcification								
52	3/10	0/10	3/10	1/10	10/10	10/10	10/10	10/10
78	0/10	3/10	1/10	5/10	10/10	9/10	10/10	9/10
All others	3/47	2/47	5/47	24/50	22/47	21/47	25/47	46/47
Nephrosis								
52	0/10	0/10	5/10	10/10+	0/10	2/10	7/10	10/10+
78	6/10	8/10	10/10	10/10+	1/10	0/10	2/10	10/10+
All others	36/47	38/47	47/47	50/50+	22/47	21/47	25/47	46/47+
Parathyroid								
Hyperplasia								
52	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
78	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
All others	3/44	9/45	16/46	37/48	0/47	4/47	1/47	17/46

+: increased severity; ppm: parts per million

[a] Tissues affected/tissues examined.

[b] All others = all animals examined except those terminated at 52 and 78 weeks.

Source: Inoue & Enomoto (1984)

Table 17. Liver tumours in rats at 104 weeks

Liver tumour	Males				Females				Historical controls (104 weeks)
	0 ppm	50 ppm	400 ppm	3 200 ppm	0 ppm	50 ppm	400 ppm	3 200 ppm	
Adenoma	0/47	0/47	0/47	4/50	0/47	0/47	2/47	6/47*	5/474 (0.0–0.2%)
Carcinoma	0/47	0/47	0/47	1/50	0/47	0/47	0/47	1/47	3/474 (0.0–2.2%)
Total	1/67[a]	0/67	0/67	5/70	0/67	0/67	2/67	8/67**[b]	

ppm: parts per million; *: $P = 0.014$; **: $P < 0.01$ (Fisher's exact test)

[a] One adenoma (1/10) was observed in controls terminated at 52 weeks.

[b] One carcinoma (1/10) was observed in the 3200 ppm dose group terminated at 78 weeks.

Source: Inoue & Enomoto (1984)

Table 18. Results of studies of genotoxicity with dichlobenil

Type of study	Organism/cell line	Dose range tested	Purity (%)	Result	Reference
In vitro					
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA1538	40–5 000 µg/plate (in DMSO)	Not reported	Negative ±S9 mix	Sekèt (1983)
Chromosomal aberration	Human lymphocytes	0–1 µg/mL (in DMSO)	Not reported	Negative ±S9 mix	Allen et al. (1984)
Gene mutation (CHO/HGPRT)	Chinese hamster ovary cells	0.15–670 µg/mL (in DMSO)	99.3	Negative ±S9 mix	Clarke (2007)
Chromosomal aberration (CHO-WBL)	Chinese hamster ovary cells	10–100 µg/mL (in DMSO)	≥ 99	Negative ±S9 mix	Murli (1990)
DNA repair (UDS)	HeLa S3 cells	0.05–102.4 µg/mL (in DMSO)	Not reported	Negative ±S9 mix	Allen & Proudlock (1984)
Gene mutation	BALB/3T3 cells	62.5–8 000 µg/mL (in DMSO)	Not reported	Negative ±S9 mix	Matthews (1984)
Gene mutation in mammalian cells	Mouse lymphoma L5178Y cells	5–280 µg/mL in DMSO	Not reported	Negative ±S9 mix	Richold et al. (1983)
In vivo					
Mouse micronucleus	Male and female Swiss mice	0, 300, 600 and 1 000 mg/kg bw (gavage in 1% tragacanth)	Not reported	Negative	Jens (1983)

bw: body weight; CHO: Chinese hamster ovary; DMSO: dimethyl sulfoxide; DNA: deoxyribonucleic acid; HGPRT: hypoxanthine–guanine phosphoribosyltransferase; S9: 9000 × *g* supernatant fraction from rat liver homogenate; UDS: unscheduled DNA synthesis

Dietary concentrations were within the acceptable range (85–103% of the nominal), and the test material was homogeneously distributed in the diets.

One (of 30) high-dose P male was found dead during week 14 of the study. The cause of death was liver necrosis and haemorrhage. There were no treatment-related clinical signs of toxicity in the study. During the 10-week premating period for P animals, mean cumulative body weight gains were decreased by 25–26% ($P < 0.05$) at 2000 ppm in both males and females. Similarly, mean overall body weight gains for F_1 males were decreased by 25% during the 10-week premating period and by 18% for F_1 females at 2000 ppm. During gestation, mean body weight gains were decreased by 13–14% in both P and F_1 females at 2000 ppm. Decrements in body weight gain were not observed in adult females of either generation during lactation. Mean feed consumption at 2000 ppm was decreased by 11–33% ($P < 0.001$) in P males and by 17–28% ($P < 0.001$) in P females during premating. Mean feed consumption during premating was decreased by 16–21% ($P < 0.001$) in F_1 males and by 16–20% ($P < 0.001$) in F_1 females at 2000 ppm. Mean feed consumption for P and F_1 females during gestation and lactation was not reported. No treatment-related effects on fertility index, fecundity index, gestation index or mean gestation length were observed in either P or F_1 females. The mean number of implantations per dam was unreported for P females and decreased at 2000 ppm in F_1 females (12.3 versus 14.6 in controls).

No treatment-related effects on live birth index, viability index, lactation index or sex ratio were observed in either F_1 or F_2 offspring (Table 19). In addition, the mean number of pups born live per litter was unaffected in F_1 offspring but decreased ($P < 0.001$) at 2000 ppm in F_2 offspring (11.0 versus 13.7 in controls). This was likely due to the decreased number of implantations per F_1 dam at 2000 ppm. At and above 350 ppm, the mean pup body weight of F_1 offspring was decreased by 16–23% ($P < 0.05$) from postnatal day (PND) 4 (precull) to PND 21, compared with controls. The effect was dose dependent. In F_2 offspring, mean pup body weight was also dose-dependently decreased by 19–22% ($P < 0.05$) from PNDs 14 to 21. An increased incidence of pelvic cavitation of the kidney at 2000 ppm (3% versus 0%) was observed during necropsy in weanling F_2 offspring; however, the incidence was similar to the sporadic incidence observed in P and F_1 animals and was not considered toxicologically significant.

The NOAEL for parental systemic toxicity was 350 ppm (equivalent to 23 mg/kg bw per day), based on decreased body weight gains during premating (males and females) and gestation (females) in both generations, decreased feed consumption during premating in both generations (males and females) and a decreased number of implantations per dam in F_1 females at 2000 ppm (equivalent to 130 mg/kg bw per day). The NOAEL for reproductive toxicity was 350 ppm (equivalent to 23 mg/kg bw per day), based on a decreased number of implantations per dam in F_1 females at 2000 ppm (equivalent to 130 mg/kg bw per day). The NOAEL for offspring toxicity was 60 ppm (equivalent to 4 mg/kg bw per day), based on decreased body weight during weaning in both generations at 350 ppm (equivalent to 23 mg/kg bw per day) (Leeming, 1989; Barker, 1992).

(b) Developmental toxicity

Rats

In a prenatal developmental toxicity study, dichlobenil (purity not reported) was administered by gavage in 1% gum tragacanth to 25 pregnant Wistar Cpb:WU rats per dose from GD 6 to GD 15 inclusive at a dose level of 0, 20, 60 or 180 mg/kg bw per day. Animals were terminated on GD 21, and uteri were examined for live fetuses and intrauterine deaths. Fetuses were weighed and examined for external, visceral and skeletal alterations.

No treatment-related mortality or clinical signs of toxicity were observed in pregnant does in the study. Twenty-nine per cent ($P < 0.01$) and 36% ($P < 0.01$) decreases in mean body weight gain were observed during the dosing period in does treated at 60 and 180 mg/kg bw per day, respectively. This effect was accompanied by a 15% ($P < 0.01$) and 21% ($P < 0.01$) decrease in feed consumption at 60 and 180 mg/kg bw per day, respectively, as well as a respective 19% ($P < 0.05$) and 24% ($P < 0.01$) decrease in feed efficiency. During the post-dosing period (GDs 16–21), body weight gain, feed consumption and feed efficiency values in mid- and high-dose animals rebounded to levels that were similar to or greater than those of controls.

Table 19. Summary of effects of dietary administration of dichlobenil on F_1 reproductive parameters, offspring survival and pup body weights in rats

Parameter	0 ppm	60 ppm	350 ppm	2000 ppm
Number of matings (P parents)	25	25	25	25
Number of pregnancies	25	20	24	25
Fertility index: female (%)	100	80	96	100
Gestation index	100	100	100	100
Mean gestation length (days)	21.5	21.6	21.7	21.7
Mean number of implantations/dam	14.6	14.4	14.2	12.3
Total number of live pups				
Day 0	342	245	306	274
Day 4	237	180	232	238
Day 21	152	116	153	178
Mean number of live pups/litter				
Day 0	12.0	13.7	13.8	11.2
Day 4 precull	9.3	10.2	11.0	8.9
Day 21	6.6	6.9	6.9	6.0
Live birth index	82.7	92.7	31.2	92.9
Viability index	100	100	100	99.4
Lactation index	71.0	67.7	62.8	67.0
Mean pup body weight (g)				
Day 1	6.1	5.7	5.5**	5.6*
Day 4 precull	8.6	7.9	7.2***	6.9***
Day 14	29.8	29.1	27.4**	21.9***
Day 21	49.8	49.5	46.7*	38.2***
Sex ratio (% males)	48	50	51	48

*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$

Source: Leeming (1989); Barker (1992)

No treatment-related changes were observed in caesarean section parameters (death, altered growth) for either embryos or fetuses. It is unclear from the study report whether statistical analysis was performed for litter incidences of external, skeletal or visceral observations. With regard to external anomalies, a shallow, dose-dependent increase in the incidence of small subcutaneous haemorrhage or petechia was observed (0/23, 1/22, 2/22 and 3/21 litters at 0, 20, 60 and 180 mg/kg bw per day); however, the increase in the number of fetuses affected (0–3) was only slightly increased across doses and was not statistically significant. An increased litter (13.6–14.3%) and fetal (3.1–3.5%) incidence of unilateral supernumerary rib (14th) was observed at and above 60 mg/kg bw per day. The fetal incidences were not statistically significantly different from those of concurrent controls. The litter incidence (19%) of bilateral supernumerary rib (14th) was also increased at 180 mg/kg bw per day, as was the fetal incidence (4.7%; $P < 0.05$). However, both the litter and fetal incidences of supernumerary (14th) rib were below the historical control incidences (32.26% and 10.86%, respectively). An increase ($P < 0.01$) in the "degree" of absence of ossification of the sternebrae (expressed as transformed ossification values per litter) was also observed at 180 mg/kg bw per day (7.03 versus 0.59 in controls). The increase was outside the historical control range (0–0.31).

However, given that the concurrent control value (0.59) was above the upper limit of the historical control range and a dose–response relationship for the effect was lacking, the calculated value is not considered toxicologically significant. With respect to visceral anomalies, a very slight increase in the malformations unilateral microphthalmia and intestinal alteration of the situs viscerum (combined with focal fibrosis of the peritoneum and mesentery) was observed at the high dose in one animal in 1/21 litters only. The malformation soft consistency of the lens/unilateral folded retina was observed in a different animal in 1/21 litters only. The incidence of each effect was above that of the historical controls (1/2691 fetuses; 1/401 litters); however, fetal incidences were not statistically significant, and a dose–response relationship was lacking at the doses tested.

The NOAEL for maternal toxicity was 20 mg/kg bw per day, based on decreased body weight gain, feed consumption and feed efficiency during the dosing period at 60 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 180 mg/kg bw per day, the highest dose tested (Koëter, 1984).

Rabbits

In a prenatal developmental toxicity study, dichlobenil technical (purity 98.5%) was administered by gavage in 1% gum tragacanth to 16–17 pregnant New Zealand White rabbits per dose from GD 7 to GD 19 inclusive at a dose level of 0, 15, 45 or 135 mg/kg bw per day. A total of three, four, four and eight animals died or were killed following abortions at 0, 15, 45 and 135 mg/kg bw per day, respectively. Owing to an inadequate number of litters for developmental evaluations, the study was repeated (Barker, 1989a).

In a repeat prenatal developmental toxicity study, dichlobenil technical (purity 98.5%) was administered by gavage in 1% gum tragacanth to 18 pregnant New Zealand White rabbits per dose from GD 7 to GD 19 inclusive at a dose level of 0, 15, 45 or 135 mg/kg bw per day.

No treatment-related mortality, clinical signs of toxicity (including abortions) or gross pathology was observed in the study. A decrease (129%; $P < 0.05$) in body weight gain was observed during the dosing period only (GDs 7–19) in does treated at 135 mg/kg bw per day. This effect was accompanied by a 30% ($P < 0.01$) decrease in feed consumption at the high dose during the dosing period only. Body weight gain and feed consumption in high-dose animals rebounded during the post-dosing period (GDs 19–29).

Increases in total resorptions per dam (1.3) and post-implantation loss (17.9%) were observed at 135 mg/kg bw per day. Although the effects were not dose dependent, the incidences were outside the historical control range and considered treatment related. Although generally occurring at very low incidences (1–3 fetuses), several external, visceral and skeletal defects or anomalies were reported at 135 mg/kg bw per day (Table 20). These effects were not observed in either concurrent or historical controls or were observed at incidences outside historical control ranges and were therefore considered toxicologically significant. External anomalies included bilateral open eye (3/115 fetuses; 3/14 litters), cleft palate and adactyly. High-dose visceral anomalies included abnormal cystic gallbladder and distended ureter with bilateral severe hydronephrosis. Skeletal defects at 135 mg/kg bw per day consisted of malformed and malpositioned right scapula, right radius absent with malpositioned ulna and humerus, fused cervical vertebral arches, asymmetrically ossified and fused cervical vertebra centra, abnormally shaped cranium with enlarged and misshapen fontanelle, enlarged fontanelle (19/115 fetuses; 13/14 litters), misshapen frontals (2/115 fetuses; 2/14 litters), skull and frontals foreshortened and nasal malpositioned, and major fusion of sternebrae (3/115 fetuses; 3/14 litters).

The NOAEL for maternal toxicity was 45 mg/kg bw per day, based on decreased body weight gain and feed consumption during the dosing period at 135 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 45 mg/kg bw per day, based on an increased number of total resorptions per dam, increased post-implantation loss and increased incidences of external, visceral and skeletal malformations at 135 mg/kg bw per day (Barker, 1989b).

Table 20. Selected skeletal, visceral and external anomalies in rabbits

	0 mg/kg bw per day	15 mg/kg bw per day	45 mg/kg bw per day	135 mg/kg bw per day
No. of fetuses (litters) examined	142 (16)	154 (16)	146 (16)	135 (14)
Skeletal findings (no. of fetuses showing major effects/no. examined)	1/142	0/154	0/146	24/115*
Fontanelle enlarged	0	0	0	19 (13)
Frontals misshapen	0	0	0	2 (2)
Skull and frontals foreshortened	0	0	0	1
Major fusion of sternebrae	0	0	0	3 (3)
No. of fetuses showing minor skeletal defects	33	45	36	15
External and visceral observations (no. of fetuses showing major effects/no. examined)	0/142	1/154	1/164	11/115*
Open eye(s) bilateral	0	0	0	3 (3)
Open eye(s) unilateral	0	0	0	1
Arthrogryposis; adactyly	0	0	0	1
Cleft palate	0	0	0	1
External hydrocephaly	0	0	0	1
Gallbladder cystic	0	0	0	1
Distended ureter with bilateral hydronephrosis	0	0	0	1
Aortic distended	0	1	1	1
Aortic arch	0	0	0	1
No. of fetuses showing minor external/visceral defects	5	14	2	12

bw: body weight; *: $P < 0.01$

Source: Barker (1989b)

2.6 Special studies

(a) Immunotoxicity

Rats

In an immunotoxicity study, dichlobenil technical (purity 99.0%) was administered daily to groups of 10 male Sprague-Dawley rats by oral gavage at a dose level of 0, 20, 60 or 180 mg/kg bw per day for 28 days. A concurrent positive control group of 10 male Sprague-Dawley rats received the positive control cyclophosphamide monohydrate at a concentration of 15 mg/kg bw per day by intraperitoneal injection on days 23–28. All rats received a single intravenous injection of 1×10^8

sheep red blood cells (sRBCs) on day 23. Body weight, feed intake and water intake were monitored weekly or biweekly during the study. At the end of the study, blood was collected to determine anti-sRBC immunoglobulin M (IgM) concentration, and absolute and relative (to body weight) liver, spleen and thymus weights were measured.

There was no mortality in the study, nor were there any adverse clinical signs. Feed consumption was decreased by 17% at 180 mg/kg bw per day during week 1. Terminal body weights were decreased by 8% in the 180 mg/kg bw per day group. No treatment-related effects on absolute or relative spleen or thymus weights were found in dichlobenil-treated rats. Absolute and relative liver weights were increased in a dose-related manner. Relative liver weights were increased by 14–62% in the treated groups.

Treatment with dichlobenil doses up to 180 mg/kg bw per day for 28 days did not suppress humoral response, as indicated by the anti-sRBC IgM response. The cyclophosphamide monohydrate positive control responded appropriately, by decreasing the absolute and relative (to body weight) spleen and thymus weights and decreasing the anti-sRBC IgM concentrations.

The LOAEL for systemic toxicity in male Sprague-Dawley rats treated with dichlobenil was 20 mg/kg bw per day, the lowest dose tested, based on increased liver weights at all doses. The NOAEL for immunotoxicity was 180 mg/kg bw per day, the highest dose tested (Johnson, 2014).

(b) Olfactory response evaluations

Oral exposure to dichlobenil

No effects were observed upon histopathological evaluation of nasal tissue in a 1-year dog oral (capsule) toxicity study up to a dose level of 36 mg/kg bw per day (Pickersgill, 1995).

Dermal exposure to dichlobenil

A single dermal application of dichlobenil in acetone caused olfactory damage in mice at doses of 50 mg/kg bw and higher; however, five sequential applications of 25 mg/kg bw had no effect on the olfactory mucosa (Deamer, O'Callaghan & Genter, 1994).

Inhalation exposure to dichlobenil

There appear to be no published studies evaluating the effects of inhalation exposure to dichlobenil.

A 1-week range-finding study in rats was conducted at concentrations of 21, 77 and 200 mg/m^3. Degeneration of the olfactory epithelium in the dorsal meatus of the nose was seen in males and females from all groups (Newton, 2002).

Crompton Corporation completed a 28-day inhalation study in rats at concentrations of 2.3, 5.1 and 12 mg/m^3. Histopathology on the nasal tissue showed that there was no effect of dichlobenil at doses up to 12 mg/m^3 (Newton, 2002).

The results of the above two studies show that 12 mg/m^3 is a no-observed-effect concentration for dichlobenil-induced nasal toxicity for an exposure period up to 28 days. However, an exposure to 21 mg/m^3 for 7 days appears to exceed the detoxification capabilities of the nasal mucosa and induces tissue degeneration. The range of toxicity and margin of safety seen in these studies are similar to those seen with dermal exposure to dichlobenil (see above).

Intraperitoneal exposure to dichlobenil

In a published study, single intraperitoneal injections of dichlobenil were administered to C57Bl mice and Sprague-Dawley rats at 12, 25 or 50 mg/kg bw. In mice, necrosis of Bowman's glands was evident 8 hours after the lowest dose of 12 mg/kg bw. The olfactory region was covered by an attenuated surface epithelium or by a respiratory-like epithelium 3–7 days after dosing. Seven to 20 days after dosing, there was fibrosis of the olfactory region. Partial regeneration of the olfactory

epithelium and scattered intact Bowman's glands were observed after 20 days. The study authors proposed that dichlobenil induces a primary lesion in Bowman's glands resulting from the pronounced binding of a metabolite in these glands (Brandt, Eriksson & Brittebo, 1991).

A single intraperitoneal dichlobenil dose of 50 mg/kg bw was found to cause extensive damage to the olfactory epithelium and Bowman's glands of rats. Histological evaluations showed that Bowman's glands in rats were reduced or eliminated 4 days after the intraperitoneal administration, but began to reappear at day 11 and were fairly normal 25 days after treatment (Hastings, Andringa & Miller, 1993).

Studies have been done to evaluate the effect of olfactory mucosal destruction on actual sensory function. No effects were seen in an odour discrimination test in rats after a single intraperitoneal dichlobenil dose of 50 mg/kg bw. Two weeks later, the rats were given 100 mg/kg bw, and again there were no effects on sensory function. After a single dichlobenil injection of 200 mg/kg bw, it was found that performance on the odour trials dropped to 50%; however, performance on the odour task was nearly normal 5 days after exposure (Hastings, Andringa & Miller, 1993).

In another study, dichlobenil (100 mg/kg bw) given intraperitoneally to rats previously trained in an olfactory task of finding feed buried in bedding caused a transient increase in the mean latency time to find the feed pellet. Treated animals were normal by day 28 (Genter et al., 1996). This type of olfactory sensory recovery has also been seen after methyl bromide exposure at 200 ppm (Hurtt et al., 1988). These studies demonstrate that the nasal mucosa has the ability to regenerate after chemical-induced destruction and that sensory function returns with this tissue regeneration.

Covalent binding to the olfactory mucosa after intraperitoneal administration of [^{14}C]dichlobenil to mice at 12 mg/kg bw was 6 times greater than the binding seen after administration of 6 mg/kg bw (Brittebo, Eriksson & Brandt, 1992), establishing a threshold for the covalent binding that corresponds with the no-observed-effect level of 6 mg/kg bw determined in previous studies (Brandt et al., 1990; Brandt, Eriksson & Brittebo, 1991; Brittebo et al., 1991). However, depletion of glutathione with phorone prior to intraperitoneal treatment with dichlobenil at 6 mg/kg bw resulted in increased covalent binding and extensive toxicity in the olfactory mucosa (Brittebo, Eriksson & Brandt, 1992). These data demonstrate the protective role of glutathione in the olfactory mucosa and that the levels of glutathione in the mucosal tissue are adequate to support detoxification of tissue levels of dichlobenil associated with an intraperitoneal dose of up to 6 mg/kg bw.

Intraperitoneal exposure to chlorthiamid and BAM

The toxic effects of the herbicide 2,6-dichlorothiobenzamide (chlorthiamid), a dichlobenil analogue, and its major environmental metabolite 2,6-dichlorobenzamide (BAM), which is also an environmental metabolite of dichlobenil, were examined in the nasal passages of C57Bl mice following single intraperitoneal injections. The doses administered to mice were 25, 50 and 100 mg/kg bw for BAM and 6, 12, 25 and 50 mg/kg bw for chlorthiamid. BAM (100 mg/kg bw) and chlorthiamid (12 mg/kg bw and above) induced an extensive destruction of the olfactory region, similar to that observed with the analogue dichlobenil at 12 mg/kg bw and above. Necrosis of Bowman's glands was evident first, whereas degeneration and necrosis of the olfactory neuroepithelium developed less rapidly (Brittebo et al., 1991). The toxicity caused by dichlobenil and chlorthiamid to the olfactory neuroepithelium may be secondary to the destruction of Bowman's glands (Brandt et al., 1990).

In vitro studies with dichlobenil

In vitro studies with subcellular homogenates of the olfactory mucosa from C57Bl mice and Sprague-Dawley rats showed a highly efficient cytochrome P450–dependent metabolic activation of dichlobenil in the olfactory mucosa. Based on similar toxicity and the ability of chlorthiamid to inhibit the covalent binding of dichlobenil in vitro, the authors proposed that dichlobenil and chlorthiamid are metabolized to a common cytotoxic product or to closely related cytotoxic products (Brandt, Eriksson & Brittebo, 1991).

Mechanistic studies on the olfactory response produced by dichlobenil

It has also been reported that dichlobenil is metabolized in mouse olfactory mucosa homogenates to a greater degree than in liver homogenates (Eriksson & Brittebo, 1991). This is considered to be a cytochrome P450–dependent mechanism (Brandt et al., 1990; Brandt, Eriksson & Brittebo, 1991; Eriksson & Brittebo, 1991, 1993; Walters, Buchheit & Maruniak, 1993a,b) that results in the formation of an electrophilic intermediate in the olfactory mucosa, which covalently binds to tissue proteins (Brandt et al., 1990; Eriksson & Brittebo, 1993, 1995; Ding, Sheng & Bhama, 1994). Administration of 3-aminobenzene, a probable inhibitor of cytochrome P450, decreased the toxicity of dichlobenil in mice, and the addition of glutathione or the P450 inhibitor metyrapone prevented the binding of [^{14}C]dichlobenil to olfactory microsomal proteins (Eriksson, Busk & Brittebo, 1996). [^{14}C]Dichlobenil binds irreversibly to Bowman's glands to an even greater extent than in the olfactory epithelium (Brandt et al., 1990).

Conclusion

The results of these studies demonstrate that dichlobenil and/or BAM, the major soil metabolite of dichlobenil (see section 2.7(a) below), produce harmful effects on nasal mucosa (olfactory response) via the intraperitoneal route. Dichlobenil is also shown to produce an olfactory response via dermal, inhalation and oral routes of exposure. If the level is exceeded, it has been shown that animals can recover from the harmful effects of dichlobenil on the nasal mucosa, and also there is a threshold for dichlobenil and BAM below which the effects are not observed.

2.7 Studies on metabolites

(a) 2,6-Dichlorobenzamide (BAM)

The substance 2,6-dichlorobenzamide (BAM; CAS No. 2008-58-4) is the major soil metabolite of dichlobenil and the major residue observed in plants and livestock matrices, such as milk, kidney, liver, fat and muscle. It was not detected in a rat metabolism study.

Biochemical aspects

No absorption, distribution or metabolism studies for BAM were submitted. However, in a published study (Bakke et al., 1988), oral absorption, distribution and metabolism of BAM were studied following a single gavage dose of radioactive BAM at 5 mg/kg bw administered to male Sprague-Dawley rats, and excretion was measured. Absorption and excretion of BAM were also studied in male bile duct–cannulated Sprague-Dawley rats. The distribution of radioactive BAM at 48 hours in rats was approximately 62%, 14.6%, 1.8% and 14.9% of the administered dose in urine, faeces, gastrointestinal tract and carcass, respectively. The distribution of radioactive BAM in male bile duct–cannulated rats at 48 hours was approximately 27.6%, 33.5%, 2.2%, 2.4% and 24.6% of the administered dose in urine, bile, faeces, gastrointestinal tract and carcass, respectively. In a metabolism study in rats, the major component in the urine and bile was the unchanged parent compound BAM, and BAM was the only compound detected in tissues. The other metabolites were 2-monohydroxy BAM, 2-chloro-5-hydroxy-6-(methylthio)benzamide and 2-chloro-5-hydroxy-6-[*S*-(*N*-acetyl)cysteinyl]benzamide.

Whole-body radioautography and microautography showed the accumulation of non-extractable residues from BAM in nasal mucosa in mice after a single intravenous administration of BAM at 7 mg/kg bw (Bakke et al., 1988).

BAM is a metabolite of fluopicolide in rats and plants. BAM was evaluated as part of the 2009 JMPR assessment of fluopicolide (Annex 1, reference *118*). Some of these studies were not made available to the present Meeting. As stated in the 2009 monograph, absorption, distribution and excretion were similar following a single oral dose of 10 or 150 mg/kg bw or following repeated dosing of 10 mg/kg bw per day for 14 days in Sprague-Dawley rats. The radiolabel was eliminated mostly in the urine (approximately 82% of the administered dose), with low levels eliminated in the faeces (approximately 13% of the administered dose). The highest concentrations in tissues were seen in the kidney and liver after the 10 mg/kg bw dose and in the skin and fur, kidneys and liver after the

150 mg/kg bw dose. Tissue concentrations increased by approximately 5-fold for a 15-fold increase in dose. The rat metabolism study suggested that biotransformation of BAM consisted of 1) hydrolysis of the amide group, 2) hydroxylation and subsequent conjugation with either glucuronic acid or sulfate and 3) the loss of a chlorine atom, followed by glutathione conjugation and further metabolism of the glutathione group to the mercapturic acid or *S*-methyl metabolites (Annex 1, reference *118*).

Acute toxicity

In an acute oral toxicity study, groups of five male and five female fasted Swiss mice were given a single oral dose of BAM (batch no. FUN81D15A; purity not reported) in a 1% tragacanth suspension at a dose of 0, 156, 312, 625, 1250, 2500 or 5000 mg/kg bw and observed for up to 13 days. Animals were observed periodically for clinical signs. Body weights were recorded 2 days prior to treatment and at 2, 7 and 13 days post-treatment. All animals were examined macroscopically at the end of the study.

All mice in the 5000 mg/kg bw group, 4/5 males and 4/5 females in the 2500 mg/kg bw group and 2/5 males and 4/5 females in the 1250 mg/kg bw group died between 30 minutes and 24 hours following dosing. Clinical signs at lethal or sublethal doses (625–5000 mg/kg bw) included locomotor impairment and dyspnoea. These signs appeared within 5 minutes of dosing and lasted between 1 and 4 days post-dosing. The mice at 156 and 312 mg/kg bw were subdued, with slight locomotor impairment, which lasted for 24 hours post-dosing. No individual animal observations or onset of clinical signs was reported. Males at 2500 and 1250 mg/kg bw and females at 1250, 625 and 312 mg/kg bw lost weight in the first few days after dosing and recovered thereafter. No macroscopic findings were noted at necropsy.

The acute oral LD_{50} of BAM in mice was 1538 mg/kg bw (95% confidence interval 992–2385 mg/kg bw) and 1144 mg/kg bw (95% confidence interval 722–1813 mg/kg bw) for males and females, respectively (Koopman, 1981).

Short-term studies of toxicity

In a 13-week oral toxicity study, BAM technical (batch no. 133/2/4/104; purity not reported) was administered to 10 Wistar rats of each sex per dose in the diet at a concentration of 0, 50, 180, 600 or 2300 ppm (equal to 0, 4, 14, 49 and 172 mg/kg bw per day, respectively). Animals were observed daily for general condition. Body weight and feed consumption were measured weekly. The effect of the compound on skeletal muscle tone was measured at four intervals during the experiment. Haematology, blood chemistry and urine analysis were performed at intervals during the experiment. No ophthalmological examinations were performed. At the end of the experiment, the clearance of bromosulfthalein by the liver and blood clotting time were measured. Postmortem analysis included recording the weight of the principal organs and their histopathology together with that of other tissues and an estimation of liver glycogen content.

There were no treatment-related effects on survival. There was significant hair loss in the female rats in the 600 and 2300 ppm dose groups. Mean terminal body weights were decreased in both males (18%) and females (11%) at 2300 ppm, relative to controls. Mean body weight gain in males treated at 2300 ppm was decreased by 30% ($P < 0.01$) during weeks 2–11, whereas feed consumption in the same group was decreased by 16% ($P < 0.01$). In females, mean body weight gain was decreased by 18% ($P < 0.01$) after 11 weeks at 600 ppm and by 30% ($P < 0.01$) at 2300 ppm. Feed consumption in females was decreased by 17% ($P < 0.01$) at 2300 ppm only. Feed efficiency in the 2300 ppm dose group was reduced in females, but not in males. Increased relaxation scores (muscle hypotonus; $P < 0.05$) were observed at 600 ppm on day 4 only in males and on days 91 and 92 only in females (Table 21). At 2300 ppm, increases in relaxation scores ($P < 0.05$) were observed at each measurement (days 4, 21, 91 and 92) in both sexes. No treatment-related changes were observed in haematological or urine analysis parameters. A 19% ($P < 0.02$) decrease in mean blood coagulation time in males at 2300 ppm was not considered clinically significant and was also not dose dependent. Mean serum urea concentration in males was increased by 55% ($P < 0.001$) after 6 weeks at 2300 ppm. After 12 weeks in males, mean serum urea concentration was increased by 53% ($P <$

0.04) at 600 ppm and by 69% ($P < 0.04$) at 2300 ppm. In the absence of histopathological correlates in the kidney or liver, increased urea concentrations were not considered toxicologically significant. No biologically significant changes in clinical chemistry were observed in females. There was also no difference in bromosulfthalein serum retention (measure of liver function) between control and high-dose animals. Mean absolute thymus, heart and brain weights were decreased in males by 28% ($P < 0.01$), 21% ($P < 0.01$) and 7% ($P < 0.01$), respectively, whereas mean absolute spleen, thymus and heart weights were decreased in females by 13% ($P < 0.05$), 23% ($P < 0.01$) and 9% ($P < 0.01$), respectively. However, there was no difference in mean relative weights for these organs in either sex, and the differences from control values are likely related to the observed decreases in body weight. For these reasons and because histopathological correlates were lacking in these organs, the changes in organ weights were not considered toxicologically significant. The nose (a potential target organ) was not examined histopathologically in this study.

Table 21. Muscle relaxation scores in rats

	Total score							
	Day 4		Day 21		Day 91		Day 92	
Group	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
Control	4	10	5	10	6	7	7	6
50 ppm	7	7	6	7	11	2	11	6
180 ppm	6	19	13	8	11	9	11	10
600 ppm	17*	17	16	13	10	21*	16	23*
2 300 ppm	27*	30*	34*	33*	34*	25*	36*	27*

ppm: parts per million; *: $P < 0.05$

Source: Boschman et al. (1967)

In conclusion, the NOAEL was 180 ppm (equal to 14 mg/kg bw per day), based on reduced skeletal muscle tone (males and females) and decreased body weight gain (females) at 600 ppm (equal to 49 mg/kg bw per day) (Boschman et al., 1967).

In a 2-year toxicity study, BAM (batch no. 133-2-4-104; purity 97%) was administered in the diet to Beagle dogs (four of each sex per dose) at a concentration of 0, 60, 100, 180 or 500 ppm (equivalent to 0, 1.5, 2.5, 4.5 and 12.5 mg/kg bw per day, respectively). Stability and homogeneity analyses of the active ingredient in the feed were not performed. Dogs were observed daily for general health and behaviour. Body weights were recorded weekly. Selected haematological and clinical chemistry parameters were measured prior to study initiation and every 3 months during the study. Urine analysis and ophthalmological examinations were not performed. Necropsies were performed on animals, selected organs were removed and weighed and histopathological examination was carried out.

No animals died during the study, and it was reported that there were no treatment-related clinical signs of toxicity. Mean body weight in males treated at 500 ppm was decreased (non-statistically significantly) at weeks 54 (14%) and 104 (12%), whereas in females, mean body weight was decreased ($P < 0.01$) at weeks 15 (12%), 54 (21%) and 104 (23%) at 500 ppm, relative to controls. Mean cumulative body weight gains (weeks 0–104) in males were 19% and 38% lower than control values at 180 and 500 ppm, respectively. However, neither was statistically significantly different from controls. Similarly, in females, mean cumulative body weight gains at 100, 180 and 500 ppm were 27%, 32% and 69% lower than control values, respectively, without statistical significance. Changes in body weight or body weight gain at or below 180 ppm were not considered toxicologically significant based on the wide variation in these measured parameters. No adverse

effects were observed on haematological or clinical chemistry parameters, at necropsy or on organ weights. Microscopic pathology was not observed as a function of dose.

In conclusion, the NOAEL was 180 ppm (equivalent to 4.5 mg/kg bw per day), based on decreased body weight and body weight gain at 500 ppm (equivalent to 12.5 mg/kg bw per day) (Wilson & Thorpe, 1971).

Long-term studies of toxicity and carcinogenicity

In a carcinogenicity study, BAM (batch no. 195; purity 9.5%) was administered in the diet for 106 weeks to Crl:CD®BR rats (35 of each sex per dose) at a concentration of 0, 60, 100, 180 or 500 ppm (equal to 0, 2.2, 3.6, 6.5 and 19 mg/kg bw per day for males and 0, 2.8, 4.7, 8.5 and 24 mg/kg bw per day for females, respectively). Stability and homogeneity of prepared diets were determined periodically. The frequency of clinical observations was not stated. It was stated that all signs of ill-health or reaction to treatment were recorded. Animals were weighed at initiation and weekly throughout the study. The quantity of feed consumed by each cage of rats (n = 5) was recorded daily. Eyes of all rats in the control and high-dose groups were examined using a Keeler indirect ophthalmoscope at 0, 13, 26, 52 and 104 weeks. Blood was collected by orbital sinus puncture from 10 animals of each sex in the control and high-dose groups at 0, 13, 26, 39, 52 and 103 weeks, and selected haematological and clinical chemistry parameters were evaluated. Urine was collected from five fasted animals of each sex in the control and high-dose groups at 13, 26, 52 and 103 weeks. All animals that died or were terminated on schedule were subjected to gross pathological examination, and the selected tissues were collected from all rats for histological examination. Selected organs were weighed.

Homogeneity of the diet at 60 and 500 ppm was within 3% and 4% of the nominal concentrations, respectively. Measured dietary concentrations were 99.7% and 94.7% at 60 and 500 ppm. The stability of the diet formulations was confirmed during storage at ambient temperature for 18 days for the 500 ppm nominal concentration; however, a loss in concentration of approximately 12% was observed for the 60 ppm nominal concentration (Johnson, 1996).

No treatment-related clinical signs of toxicity, mortality or effects on feed consumption, feed efficiency, ophthalmoscopic examinations, clinical chemistry parameters, urine analysis or macroscopic findings were observed in this study. Body weight, relative to controls, at 500 ppm was decreased by 13% ($P < 0.05$) in males at termination and by 10–21% in females from week 13 until the end of the study. At 180 ppm, body weight in females was decreased by 13% at termination only. At 500 ppm, mean body weight gain was decreased by 10–16% in males and by 16–26% in females from week 26. Body weight gain in females treated at 180 ppm was also decreased by 10–17% during the second half of the study. In the 500 ppm dose group, there were statistically significant decreases in mean haematocrit levels, mean haemoglobin concentration, mean erythrocyte counts and mean corpuscular volume in male and female rats and decreased haemoglobin concentration in female rats. Although statistically significant, the magnitude of change in the haematological parameters (3–9% less than controls) was not biologically significant. A 25% increase in mean relative liver weight was observed at 500 ppm in females, as was a 48% increase in mean relative adrenal weight. The increase in liver weight was considered toxicologically significant due to the presence of liver histopathology at 500 ppm. Non-neoplastic histopathological observations in the liver are summarized in Table 22. Dose dependence was lacking for several parameters measured in the liver (Table 22; Wheldon et al., 1971).

The microscopic examination of tissues in the study by Wheldon et al. (1971) was incomplete, but the tissues had been fixed and stored. In 1996, a new study was undertaken to re-evaluate the tissues. On reanalysis of the non-neoplastic liver findings (Connick, Crome & Gopinath, 1996), increased incidences of eosinophilic (focal and areas) and basophilic (focal and areas) hepatocytes were detected in treated rats from the 100, 180 and 500 ppm BAM dose groups. The effects were generally more pronounced in female rats. No treatment-related histopathological effects were observed in any other examined organ.

Table 22. Non-neoplastic and neoplastic lesions in rats

	Incidence of finding									
	Males					Females				
	0 ppm	**60 ppm**	**100 ppm**	**180 ppm**	**500 ppm**	**0 ppm**	**60 ppm**	**100 ppm**	**180 ppm**	**500 ppm**
No. of animals examined	*27*	*29*	*32*	*26*	*35*	*26*	*28*	*28*	*32*	*35*
Fat deposition										
None	0	7	2	3	5	4	3	1	4	1
Occasional/minimal	13	7	10	7	12	8	11	12	11	7
Moderate	7	6	11	6	9	6	2	7	5	14
Marked	3	5	6	3	3	4	9	4	4	9
Fat degeneration										
Marked	0	2	0	0	0	4[a]	3	1	3[a]	3[a]
Necrosis	0	0	0	1	0	1	1	0	0	0
Non-neoplastic lesions										
Total no. of livers examined	*26*	*28*	*32*	*25*	*34*	*25*	*28*	*28*	*32*	*35*
Eosinophilic hepatocytes: focal	5	12	17**	11	21**	5	4	7	16*	23**
Eosinophilic hepatocytes: areas	1	3	0	2	4	2	2	1	5	18**
Basophilic hepatocytes: focal	7	11	5	6	9	9	10	6	14	23**
Basophilic hepatocytes: areas	1	0	1	0	1	3	3	0	2	5
Hepatocyte vacuolation: centrilobular	5	7	10	5	16*	5	7	5	8	11
Neoplastic lesions										
Total no. of livers examined	*26*	*28*	*32*	*25*	*34*	*24*	*28*	*27*	*32*	*35*
Hepatocellular adenoma	1	0	1	0	1	0	1	0	0	5
Hepatocellular carcinoma	2	1	2	1	0	0	0	0	0	0
Pairwise comparison *P*-value							0.25	0.50	0.50	0.049
P-value for trend test									0.65	0.003

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Fisher's exact test)

[a] Similar findings with Oil Red O and haematoxylin/eosin staining, recorded for one animal in each group, were tallied only once.

Source: Connick, Crome & Gonipath (1996)

With respect to neoplastic observations, upon reanalysis of the tumour data (Connick, Crome & Gopinath, 1996), a marginally significant increase ($P = 0.049$ for the pairwise comparison) in the incidence of hepatocellular adenomas was observed in females at 500 ppm (Table 22). Statistical analysis using the time to tumour method demonstrated evidence ($P = 0.003$ for the trend test) of an

increase in hepatocellular tumours in the 500 ppm females. No effect was seen at the 180 ppm dose level. No hepatocellular carcinomas were observed in female rats, and neither adenomas nor carcinomas were observed in males.

Previously, JMPR (Annex 1, reference *118*) established a NOAEL of 60 ppm (equal to 2.0 mg/kg bw per day) from the above study for the systemic toxicity of BAM based on body weight reductions, increased incidences of eosinophilic and basophilic foci and fat deposition and cellular degeneration in the livers at 100 ppm (equal to 3.5 mg/kg bw per day).[1] However, the current Meeting re-evaluated the data and concluded that the LOAEL was 500 ppm (equal to 19 mg/kg bw per day) in males, based on decreased body weight and body weight gain and an increased incidence of hepatocellular alteration (eosinophilic foci); and 180 ppm (equal to 8.5 mg/kg bw per day) in females, based on decreased body weight and body weight gain and an increased incidence of hepatocellular alteration (eosinophilic foci). Body weight changes at 100 ppm were minor and were not considered adverse by the Meeting, and the hepatocellular degeneration (eosinophilic foci) in males has no clear dose–response relationship. Therefore, the Meeting concluded that the NOAEL for systemic toxicity in this 2-year toxicity and carcinogenicity study in rats was 100 ppm (equal to 4.7 mg/kg bw per day).

The NOAEL for carcinogenicity was 180 ppm (equal to 8.5 mg/kg bw per day), based on a marginally significant increase in the incidence of hepatocellular adenomas seen in females at 500 ppm (equal to 25 mg/kg bw per day) (Weldon et al., 1971; Connick, Crome & Gopinath, 1996; Johnson, 1996).

Genotoxicity

Results of studies of genotoxicity with BAM are summarized in Table 23.

Table 23. Results of studies of genotoxicity with BAM

Type of study	Organism/cell line	Dose range tested	Purity (%)	Result	Reference
In vitro					
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537	625–5 000 µg/plate (+S9 mix) 625–5 000 µg/plate (−S9 mix) (in DMSO)	100	Negative ±S9 mix	Koorn (1993)
Unscheduled DNA synthesis assay	Primary rat hepatocytes	3–1 000 µg/mL (+S9 mix) 3–1 000 µg/mL (−S9 mix) (in 1% DMSO)	100	Negative ±S9 mix	Van de Waart (1993a)
In vivo					
Mouse micronucleus	Male and female Swiss OF-1 mice (5/sex/dose)	250 mg/kg bw (gavage in corn oil)	100	Negative	Van de Waart (1993b)
Mouse micronucleus	Male and female Swiss OF-1 mice (3/sex/dose)	1 000, 2 000 and 4 000 mg/kg bw (M)[a] 100, 250 and 500 mg/kg bw, M/F (gavage in corn oil)	100	Negative	Van de Waart (1995)

bw: body weight; DMSO: dimethyl sulfoxide; DNA: deoxyribonucleic acid; F: female; M: male; S9: 9000 × *g* supernatant fraction from rat liver homogenate

[a] Treated animals in the 2000 and 4000 mg/kg bw dose groups died immediately after dosing, and animals in the 1000 mg/kg bw dose group died in 24 hours. No information on onset of clinical signs or individual animal data were presented.

[1] Note that the current reviewer calculated doses of 2.2 and 3.6 mg/kg bw per day for the dietary concentrations of 60 and 100 ppm.

Multigeneration studies

In a three-generation reproductive toxicity study, BAM (batch no. 195; purity 99.5%) was administered in the diet to Long-Evans rats (10 males per dose and 20 females per dose in the P, F_1 and F_2 generations) at a concentration of 0, 60, 100 or 180 ppm (equivalent to 0, 4.0, 6.7 and 12 mg/kg bw per day, respectively). Two litters were produced in each generation. The number of pups per litter was counted, and litters were weighed on PND 21. Litters were culled to 10 animals each on PND 5. P, F_1 and F_2 generation parental animals were weighed and examined for gross pathology on the day of termination (unspecified). Organ weights were measured, and histopathology was performed on select F_{3b} weanlings only.

No treatment-related mortality was observed in the P, F_{1b} or F_{2b} parental generations. A 6% ($P < 0.05$) decrease in mean parental terminal body weight was observed at 180 ppm in the F_{2b} generation; however, the decrease was not biologically significant and was not observed in P or F_{1b} parental animals. No treatment-related gross pathology was observed in P, F_{1b} or F_{2b} parental animals. Fertility and gestation indices were similar across treatment groups in P, F_1 and F_2 generation dams.

No treatment-related differences were observed in the mean number of pups per litter on PNDs 1, 5 (precull) or 21 in the F_1, F_2 or F_3 generations. Viability and lactation indices were similar across the treatment groups in F_1, F_2 and F_3 generation pups. Mean survival from birth to PND 5 (viability index) was slightly reduced (86.2%, $P < 0.01$; versus 95.7% in controls) at 180 ppm in the F_{3b} generation; however, this decrease was not observed in any other generation. In addition, mean survival from birth to weaning was similar across doses for each of the three generations of offspring. Hyperexcitability was observed in pups (number not reported) from four litters in the F_{1b} generation only.

Mean weanling weights (calculated as entire litter weight divided by number of pups per litter) at 180 ppm were decreased by 15% ($P < 0.05$), 12% ($P < 0.05$) and 14% ($P < 0.01$) in the F_{1b}, F_{3a} and F_{3b} generations, respectively; however, the decreases were not dose dependent, not observed in the F_2 generation and not calculated from individual pup body weights. No treatment-related differences were observed in mean absolute brain weights or mean brain weight to body weight ratios in F_{3b} generation weanling rats of either sex. A 12% increase in both mean absolute kidney weights and mean kidney weight to body weight ratios ($P < 0.01$) was observed in F_{3b} generation female weanlings at 180 ppm. A 10% increase in both mean absolute liver weights and mean liver weight to body weight ratios ($P < 0.05$) in males at 180 ppm and in females at and above 100 ppm was observed in this same generation. Changes in mean organ weights in F_{3b} generation weanlings were not considered toxicologically significant, however, because of a lack of histopathological correlates in the liver and kidney.

The NOAEL for parental systemic toxicity, reproductive toxicity and offspring toxicity was 180 ppm (equivalent to 12 mg/kg bw per day), the highest dose tested.

No individual animal data were provided. The study was completed in 1971, and the report was reissued in 1993 (Hine, Eisenlord & Loquvam, 1993).

Developmental toxicity

In a prenatal developmental toxicity study, BAM (batch no. FUX001000; purity 99.4%) was administered by gavage in 1% gum tragacanth to 16 pregnant New Zealand White rabbits per dose from GD 7 to GD 19 inclusive at a dose level of 0, 10, 30 or 90 mg/kg bw per day. Animals were checked for mortality and moribundity twice daily. Observations for clinical signs of toxicity were made once daily. Body weight and feed consumption data were recorded on GDs 0, 7, 13, 19, 23 and 28. On GD 28, all surviving animals were terminated, and litters were delivered by caesarean section. A gross pathological examination was conducted. Various fetal and litter parameters were evaluated.

Five (of 16) females treated at 90 mg/kg bw per day were terminated in extremis. Three of the five terminated high-dose dams had late abortions (GDs 19, 21 and 22, respectively). The incidence of abortion followed by termination at 0, 10 and 30 mg/kg bw per day was 1/16, 1/16 and 0/16, respectively. The other 2/5 high-dose animals were terminated moribund. Moribund condition

followed by termination was observed in 1/16, 0/16 and 2/16 animals at 0, 10 and 30 mg/kg bw per day, respectively. The incidence of thin appearance was increased at 90 mg/kg bw per day (10/16), whereas the incidences at 0, 10 and 30 mg/kg bw per day were 1/16, 0/16 and 2/16, respectively. A decrease (129%) in mean body weight gain, relative to controls, was observed during the dosing period (GDs 7–19) in does treated at 90 mg/kg bw per day. Similarly, feed consumption at 90 mg/kg bw per day was decreased during the dosing period by 49%, relative to controls. Body weight gain and feed consumption in high-dose animals rebounded above control levels during the post-dosing period (GDs 20–28). No treatment-related gross pathology was observed.

No treatment-related effects were observed on several developmental end-points, including the number of resorptions, post-implantation loss, litter size and sex ratio. Mean fetal body weight at 90 mg/kg bw per day (33.9 g) was decreased by 6%, relative to controls; however, the change was not statistically significant, and the mean value fell within the historical control range (27.7–39.4 g). No treatment-related changes in the incidences of external defects were observed.

An increase in the incidence of bipartite interparietal bone was observed at 90 mg/kg bw per day. Litter incidences were 1/14, 1/15, 2/14 and 3/11 at 0, 10, 30 and 90 mg/kg bw per day. Bipartite interparietal bone is considered a malformation in rats. Historical control data were not provided for the litter incidences of bipartite interparietal bone. However, the historical control data for fetal incidences were not statistically significantly different from those of concurrent controls, and the concurrent control fetal incidence (0.8%) exceeded that of the historical controls (0.3%). An increase in the incidence of postcaval lung lobe agenesis was also observed at 90 mg/kg bw per day. Litter incidences were 0/14, 0/15, 1/14 and 3/11 at 0, 10, 30 and 90 mg/kg bw per day. The fetal incidence of postcaval lung lobe agenesis at 90 mg/kg bw per day (3.2%) exceeded that of the historical controls (1.2%), whereas that at 30 mg/kg bw per day (0.9%) did not.

The NOAEL for maternal toxicity was 30 mg/kg bw per day, based on increased incidences of clinical signs (late abortion, thin appearance) and decreased (severe) body weight gain and feed consumption during the dosing period at 90 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 30 mg/kg bw per day, based on increased incidences of late abortion and skeletal (bipartite interparietal bone) and visceral (postcaval lung lobe agenesis) malformations at 90 mg/kg bw per day (McIntyre, 1987).

Olfactory response evaluations

BAM was evaluated for its olfactory toxicity. The nasal passages of C57Bl mice were examined following single intraperitoneal injections of BAM at doses of 25, 50 and 100 mg/kg bw. No morphological changes were observed in the nasal tissue following dosing at 25 and 50 mg/kg bw. At 100 mg/kg bw, BAM induced an extensive destruction of the olfactory region, similar to that observed with dichlobenil and chlorthiamid at 12 mg/kg bw and above. Necrosis of Bowman's glands was evident first, whereas degeneration and necrosis of the olfactory neuroepithelium developed less rapidly (Brittebo et al., 1991). This study suggest that BAM is less potent than dichlobenil and chlorthiamid in producing a nasal olfactory response.

In conclusion, both BAM and dichlobenil (see section 2.6(b)) produce harmful effects on nasal mucosa (olfactory response) via the intraperitoneal route. BAM is less potent than dichlobenil in eliciting an olfactory response. Dichlobenil is also shown to produce an olfactory response via dermal, inhalation and oral routes of exposure. If the level is exceeded, it has been shown that animals can recover from the harmful effects of dichlobenil on the nasal mucosa, and also there is a threshold for dichlobenil and BAM below which the effects are not observed.

Toxicity of metabolites

The current Meeting evaluated the following BAM metabolites found in plants or animal tissues: 2,6-dichloro-4-hydroxybenzonitrile, 6-chloro-3-hydroxy-2-thiobenzamide, 6-chloro-3-hydroxy-2-methylsulfinylbenzamide and 6-chloro-3-hydroxy-2-methylthiobenzamide. These chemicals were evaluated using the DEREK knowledge-based program to determine whether there are any structural alerts for toxicity. The DEREK analysis did not give any alerts for long-term

toxicity, similar to the findings for the parent chemical BAM. Based on a lack of any structural alerts and the fact that these compounds are the result of either hydroxylation or sulfate conjugation, the Meeting concluded that the above-mentioned BAM metabolites are likely to be of lower toxicity than the parent compound BAM.

3. Observations in humans

No information was provided.

Comments

Biochemical aspects

Following gavage dosing of rats at 5.6 mg/kg bw, dichlobenil was extensively absorbed; about 89% and 97% of the administered dose were absorbed in males and females, respectively, in 7 days, based on a comparison of urinary excretion following oral and intravenous administration. Oral absorption after repeated exposure was at least 60%, irrespective of dose or sex. Liver, kidney and kidney fat reached the highest concentrations of radioactivity; concentrations peaked 1–3 hours after administration in these organs and in plasma. The elimination of the radioactivity associated with [^{14}C]dichlobenil following oral administration was rapid, with most (about 80%) being eliminated within 24 hours at the low dose. About 60% was excreted in the urine and about 20% in the faeces, irrespective of sex or dose regimen, within 7–10 days after initiation of exposure. Approximately 79% of the administered dose was excreted in the bile within 24 hours in bile duct–cannulated rats, suggesting enterohepatic recirculation.

Dichlobenil was extensively metabolized, and no parent compound was detected in the urine. Most of the urinary metabolites were conjugates, such as sulfate, glutathione (and derivatives) and glucuronic acid conjugates. In faeces, three major metabolites (each representing at least 5% of the administered dose) were observed, as well as parent compound (dichlobenil). The proportion of conjugated metabolites decreased with increasing dose, whereas the amount of parent compound in faeces increased. Dichlobenil was metabolized in rats via two metabolic pathways: the first pathway involves hydroxylation at the 3 or 4 position, followed by glucuronidation or sulfation, and the second pathway involves substitution of one chlorine atom by glutathione.

Toxicological data

The acute oral and dermal LD_{50}s were greater than 2000 mg/kg bw in rats and rabbits, respectively. The acute inhalation LC_{50} in rats was greater than 3.2 mg/L. Dichlobenil was non-irritating to rabbit skin and eyes. It was not a skin sensitizer in guinea-pigs, as determined by the Magnusson and Kligman maximization test.

The liver was the primary target organ in mice, rats, hamsters and dogs in repeated-dose toxicity studies.

In a 90-day toxicity study in mice using dietary dichlobenil concentrations of 0, 25, 125, 625 and 3125 ppm (equal to 0, 3.8, 19, 91 and 447 mg/kg bw per day for males and 0, 4.8, 24, 114 and 512 mg/kg bw per day for females, respectively), the NOAEL was 625 ppm (equal to 91 mg/kg bw per day), based on transient clinical signs of toxicity, decreased body weight gains, decreased feed consumption and liver toxicity (increased liver weights, clinical chemistry, severity of centrilobular hypertrophy and glycogen storage) at 3125 ppm (equal to 447 mg/kg bw per day).

In a 90-day toxicity study in hamsters using dietary dichlobenil concentrations of 0, 41, 209, 1289 and 7500/4648 ppm (equivalent to 0, 3, 16, 79 and 395/263 mg/kg bw per day, respectively), the NOAEL was 41 ppm (equivalent to 3 mg/kg bw per day), based on decreased weight and mineralization of the prostate and decreased absolute seminal vesicle and testicular weights at 209 ppm (equivalent to 16 mg/kg bw per day).

In a 90-day toxicity study in rats, dichlobenil was administered in the diet at a concentration of 0, 100, 1000 or 3000 ppm (equivalent to 0, 10, 100 and 300 mg/kg bw per day, respectively); an additional group of males was fed 10 000 ppm (equivalent to 1000 mg/kg bw per day). The NOAEL was 100 ppm (equivalent to 10 mg/kg bw per day), based on hepatocellular necrosis and inflammation in males seen at 1000 ppm (equivalent to 100 mg/kg bw per day).

In a 90-day toxicity study in dogs using dietary dichlobenil concentrations of 0, 50, 150 and 450 ppm (equivalent to 0, 1.3, 3.8 and 11 mg/kg bw per day), the NOAEL was 150 ppm (equivalent to 3.8 mg/kg bw per day), based on increased alkaline phosphatase, ALT and liver and kidney weights at 450 ppm (equivalent to 11 mg/kg bw per day).

In a 52-week toxicity study in dogs administered dichlobenil by capsule at a dose of 0, 1, 6 or 36 mg/kg bw per day, the NOAEL was 1 mg/kg bw per day, based on increases in liver weights, serum cholesterol, triglycerides, phospholipids and alkaline phosphatase in both sexes and in gamma-glutamyltransferase and periportal hypertrophy of the hepatocytes in males at 6 mg/kg bw per day.

In a 2-year toxicity study in dogs using dietary dichlobenil concentrations of 0, 20, 50 and 350 ppm (equivalent to 0, 0.5, 1.3 and 8.8 mg/kg bw per day), the NOAEL was 50 ppm (equivalent to 1.3 mg/kg bw per day), based on increases in liver weight and serum alkaline phosphatase (in both sexes) and in serum ALT (females only) and liver histopathology (leukocytic infiltration around the central veins in both sexes and necrosis in males) seen at 350 ppm (equivalent to 8.8 mg/kg bw per day).

The overall NOAEL for the 1- and 2-year dog studies was 50 ppm (equivalent to 1.3 mg/kg bw per day), and the overall LOAEL was 6 mg/kg bw per day.

In a first toxicity and carcinogenicity study in hamsters (91 weeks for males and 78 weeks for females) using dietary dichlobenil concentrations of 0, 675, 1500 and 3375 ppm (equal to 0, 51, 117 and 277 mg/kg bw per day for males and 0, 55, 121 and 277 mg/kg bw per day for females, respectively), decreased body weight gain in males and females and increased relative liver weight in females were observed at all doses. No NOAEL could be identified. The NOAEL for carcinogenicity in male hamsters was 1500 ppm (equal to 117 mg/kg bw per day), based on an increased incidence of hepatocellular adenoma and carcinoma at 3375 ppm (equal to 277 mg/kg bw per day). No treatment-related tumours were observed in female hamsters.

In the second toxicity and carcinogenicity study in hamsters, dichlobenil was administered at a dietary concentration of 0, 5, 26, 132 or 675 ppm (equal to 0, 0.34, 1.69, 9.39 and 45.6 mg/kg bw per day for males and 0, 0.35, 1.78, 9.20 and 48.9 mg/kg bw per day for females, respectively) for 88 weeks (males) or 80 weeks (females). The NOAEL was 26 ppm (equal to 1.69 mg/kg bw per day), based on reduced secretion of the prostate and seminal vesicles at 132 ppm (equal to 9.39 mg/kg bw per day).

In a 2-year toxicity and carcinogenicity study in rats using dietary dichlobenil concentrations of 0, 50, 400 and 3200 ppm (equal to 0, 3.2, 29 and 241 mg/kg bw per day for males and 0, 3.2, 26 and 248 mg/kg bw per day for females, respectively), the NOAEL was 50 ppm (equal to 3.2 mg/kg bw per day), based on changes in clinical chemistry (increased BUN, cholesterol), gross pathology (enlarged liver and kidney) and histopathology (nephrosis, parathyroid hyperplasia) in males and enlarged liver with increased liver weight and polyploidy with hepatocytic swelling in the liver in females at 400 ppm (equal to 26 mg/kg bw per day). There was an increased incidence of hepatocellular tumours at 3200 ppm in both sexes, reaching statistical significance only in females. The NOAEL for carcinogenicity was 400 ppm (equal to 26 mg/kg bw per day).

The Meeting concluded that dichlobenil is carcinogenic in rats and hamsters.

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

The Meeting concluded that dichlobenil is unlikely to be genotoxic.

In view of the lack of genotoxicity and on the basis of other available toxicological information, the Meeting concluded that the mode of action for the increased incidences of hepatocellular adenomas and carcinomas in male and female rats and male hamsters, although not completely understood, is likely to involve a threshold. Therefore, the Meeting concluded that dichlobenil is unlikely to pose a carcinogenic risk to humans from the diet.

In a two-generation reproductive toxicity study in rats given diets containing dichlobenil at a concentration of 0, 60, 350 or 2000 ppm (equivalent to 0, 4, 23 and 130 mg/kg bw per day, respectively), the NOAEL for parental toxicity was 350 ppm (equivalent to 23 mg/kg bw per day), based on decreased body weight gains during premating (males and females) and gestation (females) in both generations, decreased feed consumption during premating in both generations (males and females) and a decreased number of implantations per dam in F_1 females at 2000 ppm (equivalent to 130 mg/kg bw per day). The NOAEL for offspring toxicity was 60 ppm (equivalent to 4 mg/kg bw per day), based on decreased body weight during weaning in both generations seen at 350 ppm (equivalent to 23 mg/kg bw per day). The NOAEL for reproductive toxicity was 350 ppm (equivalent to 23 mg/kg bw per day), based on a decreased number of implantations per dam in F_1 females seen at 2000 ppm (equivalent to 130 mg/kg bw per day).

In a prenatal developmental toxicity study in rats that used dichlobenil doses of 0, 20, 60 and 180 mg/kg bw per day, the NOAEL for maternal toxicity was 20 mg/kg bw per day, based on decreased body weight gain, feed consumption and feed efficiency during the dosing period seen at 60 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 180 mg/kg bw per day, the highest dose tested.

In a prenatal developmental toxicity study in rabbits that tested at dichlobenil doses of 0, 15, 45 and 135 mg/kg bw per day, the NOAEL for maternal toxicity was 45 mg/kg bw per day, based on decreased body weight gain and feed consumption during the dosing period at 135 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 45 mg/kg bw per day, based on an increased number of total resorptions per dam, increased post-implantation loss and increased incidences of external, visceral and skeletal malformations (e.g. open eyes, major fusion of sternebrae, cleft palate) seen at 135 mg/kg bw per day.

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

No evidence of immunotoxicity was observed in an immunotoxicity study in rats administered dichlobenil by gavage at a dose level of 0, 20, 60 or 180 mg/kg bw per day for 28 days.

The Meeting concluded that dichlobenil is not immunotoxic.

Dichlobenil produces harmful effects on nasal mucosa via dermal, inhalation and oral routes of exposure. These effects on nasal mucosa are reversible, and there is a threshold below which the effect was not observed.

Toxicological data on metabolites and/or degradates

The acute oral LD_{50}s for 2,6-dichlorobenzamide (BAM; a plant metabolite and soil degradate) were 1538 and 1144 mg/kg bw for male and female mice, respectively.

In a 13-week toxicity study in rats using dietary BAM concentrations of 0, 50, 180, 600 and 2300 ppm (equal to 0, 4, 14, 49 and 172 mg/kg bw per day, respectively), the NOAEL was 180 ppm (equal to 14 mg/kg bw per day), based on reduced skeletal muscle tone (males and females) and decreased body weight gain (females) seen at 600 ppm (equal to 49 mg/kg bw per day).

In a 2-year study of toxicity in dogs using dietary BAM concentrations of 0, 60, 100, 180 and 500 ppm (equivalent to 0, 1.5, 2.5, 4.5 and 12.5 mg/kg bw per day, respectively), the NOAEL was 180 ppm (equivalent to 4.5 mg/kg bw per day), based on decreased body weight and body weight gain seen at 500 ppm (equivalent to 12.5 mg/kg bw per day).

In a 2-year study of carcinogenicity in rats using dietary BAM concentrations of 0, 60, 100, 180 and 500 ppm (equal to 0, 2.2, 3.6, 6.5 and 19 mg/kg bw per day for males and 0, 2.8, 4.7, 8.5 and 25 mg/kg bw per day for females, respectively), the NOAEL was 100 ppm (equal to 4.7 mg/kg bw per

day), based on decreased body weight and body weight gain and an increased incidence of hepatocellular alteration (eosinophilic foci) seen in females at 180 ppm (equal to 8.5 mg/kg bw per day). The NOAEL for carcinogenicity was 180 ppm (equal to 8.5 mg/kg bw per day), based on a marginally significant increase in the incidence of hepatocellular adenomas seen in females at 500 ppm (equal to 25 mg/kg bw per day).

The Meeting concluded that BAM is carcinogenic in rats.

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

The Meeting concluded that BAM is unlikely to be genotoxic.

In view of the lack of genotoxicity and on the basis of other available toxicological information, the Meeting concluded that the mode of action for the increased incidence of hepatocellular adenomas in female rats, although not completely understood, is likely to involve a threshold. Therefore, the Meeting concluded that BAM is unlikely to pose a carcinogenic risk to humans from the diet.

In a three-generation reproductive toxicity study in rats given diets providing a BAM dose of 0, 60, 100 or 180 ppm (equivalent to 0, 4.0, 6.7 and 12 mg/kg bw per day, respectively), the NOAEL for parental toxicity, offspring toxicity and reproductive toxicity was 180 ppm (equivalent to 12 mg/kg bw per day), the highest dose tested.

In a prenatal developmental toxicity study in rabbits that administered BAM by gavage at a dose of 0, 10, 30 or 90 mg/kg bw per day, the NOAEL for maternal toxicity was 30 mg/kg bw per day, based on late abortion, thin appearance and severely decreased body weight gain and feed consumption during the dosing period at 90 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 30 mg/kg bw per day, based on increased incidences of late abortion and skeletal (bipartite interparietal bone) and visceral (postcaval lung lobe agenesis) malformations observed at 90 mg/kg bw per day.

The Meeting concluded that BAM is teratogenic in rabbits.

BAM produces harmful effects on nasal mucosa via the intraperitoneal route of exposure. The reversibility of these effects was not investigated. There is a threshold below which the effects were not observed.

Human data

No information on employees working in dichlobenil manufacturing plants was provided.

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

Toxicological evaluation

Dichlobenil

The Meeting established an acceptable daily intake (ADI) of 0–0.01 mg/kg bw on the basis of an overall NOAEL of 1.3 mg/kg bw per day in 1- and 2-year dietary studies of toxicity in dogs, on the basis of liver toxicity (increased liver weight, liver enzymes, cholesterol and triglycerides) at 6 mg/kg bw per day. A safety factor of 100 was applied. This ADI is supported by an overall NOAEL of 1.69 mg/kg bw per day in a dietary carcinogenicity study in hamsters, based on reduced secretion of the prostate and seminal vesicles, decreased body weight gain and hyperplasia of the adrenal cortex, small intestine and bone marrow observed at 9.39 mg/kg bw per day. The upper bound of the ADI provides a margin of exposure of at least 24 000 relative to the LOAEL for liver tumours in hamsters and rats.

An acute reference dose (ARfD) of 0.5 mg/kg bw was established on the basis of a NOAEL of 45 mg/kg bw per day in a study of developmental toxicity in rabbits, based on increased incidences of external, visceral and skeletal malformations (e.g. open eyes, major fusion of sternebrae, cleft palate) seen at 135 mg/kg bw per day. A safety factor of 100 was applied. This ARfD applies to women of childbearing age only. The Meeting concluded that it is not necessary to establish an ARfD for the remainder of the population in view of the low acute oral toxicity of dichlobenil and the absence of any other toxicological effects that would be likely to be elicited by a single dose.

2,6-Dichlorobenzamide (BAM)

Based on a re-evaluation of the data, the Meeting withdrew the ADI and ARfD for 2,6-dichlorobenzamide (BAM) established by the 2009 JMPR as part of the evaluation of fluopicolide.

The Meeting established an ADI of 0–0.05 mg/kg bw for 2,6-dichlorobenzamide (BAM) on the basis of a NOAEL of 4.5 mg/kg bw per day in a 2-year dietary study of toxicity in dogs, based on decreased body weight and body weight gain at 12.5 mg/kg bw per day. A safety factor of 100 was applied. This ADI is supported by a NOAEL of 4.7 mg/kg bw per day in a 2-year dietary carcinogenicity study in rats, based on decreased body weight and body weight gain and an increased incidence of hepatocellular alteration (eosinophilic foci) in females observed at 8.5 mg/kg bw per day. The upper bound of the ADI provides a margin of exposure of at least 500 relative to the LOAEL for liver tumours in rats and also a 240-fold margin of exposure relative to the highest dose tested in a three-generation reproductive toxicity study in rats, at which no effects were observed.

The Meeting established an ARfD of 0.3 mg/kg bw for 2,6-dichlorobenzamide (BAM) on the basis of a NOAEL of 30 mg/kg bw per day in a developmental toxicity study in rabbits, based on increased incidences of skeletal (bipartite interparietal bone) and visceral (postcaval lung lobe agenesis) malformations seen at 90 mg/kg bw per day. A safety factor of 100 was applied. This ARfD applies to women of childbearing age only. The Meeting concluded that it is not necessary to establish an ARfD for the remainder of the population in view of the low acute oral toxicity of BAM and the absence of any other toxicological effects that would be likely to be elicited by a single dose.

Levels relevant to risk assessment of dichlobenil

Species	Study	Effect	NOAEL	LOAEL
Hamster	Eighteen-month studies of toxicity and carcinogenicity[a,b]	Toxicity	26 ppm, equal to 1.69 mg/kg bw per day	132 ppm, equal to 9.39 mg/kg bw per day
		Carcinogenicity	1 500 ppm, equal to 117 mg/kg bw per day	3 375 ppm, equal to 277 mg/kg bw per day
Rat	Two-year study of toxicity and carcinogenicity[a]	Toxicity	50 ppm, equal to 3.2 mg/kg bw per day	400 ppm, equal to 26 mg/kg bw per day
		Carcinogenicity	400 ppm, equal to 26 mg/kg bw per day	3 200 ppm, equal to 241 mg/kg bw per day
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	350 ppm, equivalent to 23 mg/kg bw per day	2 000 ppm, equivalent to 130 mg/kg bw per day
		Parental toxicity	350 ppm, equivalent to 23 mg/kg bw per day	2 000 ppm, equivalent to 130 mg/kg bw per day
		Offspring toxicity	60 ppm, equivalent to 4 mg/kg bw per day	350 ppm, equivalent to 23 mg/kg bw per day
	Developmental toxicity study[c]	Maternal toxicity	20 mg/kg bw per day	60 mg/kg bw per day
		Embryo and fetal toxicity	180 mg/kg bw per day[d]	–

Species	Study	Effect	NOAEL	LOAEL
Rabbit	Developmental toxicity study[c]	Maternal toxicity	45 mg/kg bw per day	135 mg/kg bw per day
		Embryo and fetal toxicity	45 mg/kg bw per day	135 mg/kg bw per day
Dog	One-[c] and 2-year studies of toxicity[a,b]	Toxicity	50 ppm, equivalent to 1.3 mg/kg bw per day	6 mg/kg bw per day

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

Levels relevant to risk assessment of BAM

Species	Study	Effect	NOAEL	LOAEL
Rat	Two-year study of toxicity and carcinogenicity[a]	Toxicity	100 ppm, equal to 4.7 mg/kg bw per day	180 ppm, equal to 8.5 mg/kg bw per day
		Carcinogenicity	180 ppm, equal to 8.5 mg/kg bw per day	500 ppm, equal to 25 mg/kg bw per day
	Three-generation study of reproductive toxicity[a]	Reproductive toxicity	180 ppm, equivalent to 12 mg/kg bw per day[b]	–
		Parental toxicity	180 ppm, equivalent to 12 mg/kg bw per day[b]	–
		Offspring toxicity	180 ppm, equivalent to 12 mg/kg bw per day[b]	–
Rabbit	Developmental toxicity study[c]	Maternal toxicity	30 mg/kg bw per day	90 mg/kg bw per day
		Embryo and fetal toxicity	30 mg/kg bw per day	90 mg/kg bw per day
Dog	Two-year study of toxicity[a]	Toxicity	180 ppm, equivalent to 4.5 mg/kg bw per day	500 ppm, equivalent to 12.5 mg/kg bw per day

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

Estimate of acceptable daily intake (ADI)

0–0.01 mg/kg bw for dichlobenil

0–0.05 mg/kg bw for 2,6-dichlorobenzamide (BAM)

Estimate of acute reference dose (ARfD)

0.5 mg/kg bw for dichlobenil (applies to women of childbearing age only)

0.3 mg/kg bw for 2,6-dichlorobenzamide (BAM) (applies to women of childbearing age only)

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 dichlobenil

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapidly and completely absorbed from gastrointestinal tract (at least 89% in 7 days)
Dermal absorption	No data
Distribution	Rapidly distributed, highest concentrations in liver, kidney, kidney fat and brown fat; concentrations peaked in 1–3 hours
Potential for accumulation	No evidence of accumulation
Rate and extent of excretion	Rapid; about 80% excreted within first 24 hours following single low dose
Metabolism in animals	Extensive, 10 metabolites in urine (no parent) and four metabolites and parent compound in faeces
Toxicologically significant compounds in animals and plants	Dichlobenil and 2,6-dichlorobenzamide (BAM; plant metabolite)
Acute toxicity	
Rat, LD_{50}, oral	> 2 000 mg/kg bw
Rabbit, LD_{50}, dermal	> 2 000 mg/kg bw
Rat, LC_{50}, inhalation	> 3.2 mg/L (4 hours)
Rabbit, dermal irritation	Not irritating
Rabbit, ocular irritation	Not irritating
Guinea-pig, dermal sensitization	Not sensitizing (maximization test)
Short-term studies of toxicity	
Target/critical effect	Liver
Lowest relevant oral NOAEL	1.3 mg/kg bw per day (dog)
Lowest relevant dermal NOAEL	1 000 mg/kg bw per day, highest dose tested (rabbit)
Lowest relevant inhalation NOAEC	12 mg/m^3, highest concentration tested (rat)
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Liver and kidney (rat) and liver (hamster)
Lowest relevant oral NOAEL	1.69 mg/kg bw per day (hamster)
Carcinogenicity	Liver tumours (male hamster, male and female rats); unlikely to pose a carcinogenic risk to humans from the diet
Genotoxicity	
	Unlikely to be genotoxic
Reproductive toxicity	
Target/critical effect	Decreased body weights in adults and pups, decreased number of implantations per dam in F_1 females
Lowest relevant parental NOAEL	23 mg/kg bw per day
Lowest relevant offspring NOAEL	4 mg/kg bw per day
Lowest relevant reproductive NOAEL	23 mg/kg bw per day
Developmental toxicity	
Target/critical effect	Increased total resorptions, post-implantation loss and

	malformations (rabbit)
Lowest relevant maternal NOAEL	20 mg/kg bw per day (rat)
Lowest relevant embryo/fetal NOAEL	45 mg/kg bw per day (rabbit)
Neurotoxicity	
Acute neurotoxicity NOAEL	No data
Subchronic neurotoxicity NOAEL	No data
Developmental neurotoxicity NOAEL	No data
Other toxicological studies	
Immunotoxicity NOAEL	180 mg/kg bw per day, highest dose tested
Medical data	
	No data provided

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

Acute toxicity	
Mouse, LD_{50}, oral	1 140 mg/kg bw
Short-term studies of toxicity	
Target/critical effect	Body weight and body weight gain
Lowest relevant oral NOAEL	4.5 mg/kg bw per day (dog)
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Liver and body weights
Lowest relevant oral NOAEL	4.7 mg/kg bw per day (rat)
Carcinogenicity	Hepatocellular adenomas in female rats; unlikely to pose a carcinogenic risk to humans from the diet
Genotoxicity	
	Unlikely to be genotoxic
Reproductive toxicity	
Target/critical effect	None
Lowest relevant parental NOAEL	12 mg/kg bw per day, highest dose tested
Lowest relevant offspring NOAEL	12 mg/kg bw per day, highest dose tested
Lowest relevant reproductive NOAEL	12 mg/kg bw per day, highest dose tested
Developmental toxicity	
Target/critical effect	Increased incidences of skeletal (bipartite interparietal bone) and visceral (postcaval lung lobe agenesis) anomalies
Lowest relevant maternal NOAEL	30 mg/kg bw per day (rabbit)
Lowest relevant embryo/fetal NOAEL	30 mg/kg bw per day (rabbit)
Neurotoxicity	
Acute neurotoxicity NOAEL	No data
Subchronic neurotoxicity NOAEL	No data
Developmental neurotoxicity NOAEL	No data

Summary

	Value	Study	Safety factor
Dichlobenil			
ADI	0–0.01 mg/kg bw	One-year and 2-year studies of toxicity (dog)	100
ARfD[a]	0.5 mg/kg bw	Developmental toxicity study (rabbit)	100
2,6-Dichlorobenzamide (BAM)			
ADI	0–0.05 mg/kg bw	Two-year study of toxicity (dog)	100
ARfD[a]	0.3 mg/kg bw	Developmental toxicity study (rabbit)	100

[a] Applies to women of childbearing age only.

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FLUFENOXURON

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Explanation

Flufenoxuron is the International Organization for Standardization (ISO)–approved common name for *N*-{4-[2-chloro-4-(trifluoromethyl)phenoxy]-2-fluorophenyl}-*N*′-(2,6-difluorobenzoyl)urea (International Union of Pure and Applied Chemistry), which has the Chemical Abstracts Service number 101463-69-8. Flufenoxuron is a benzoylurea insecticide and acaricide that is used on fruits, vines and ornamentals to control insects and mites. The pesticidal mode of action is the inhibition of chitin synthesis.

Flufenoxuron 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). The Meeting considered that the database was adequate for the risk assessment.

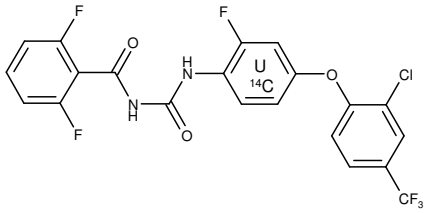
Evaluation for acceptable daily intake

1. Biochemical aspects

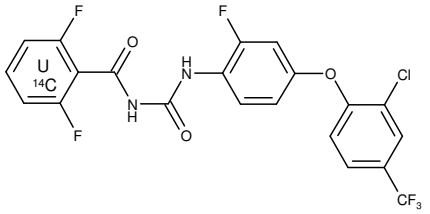
Two ^{14}C-radiolabelled flufenoxuron molecules were used for the metabolism and distribution studies (Fig. 1).

Fig. 1. Structure of flufenoxuron with positions of radiolabel

(fluoroaniline-U-^{14}C)-labelled flufenoxuron is designated as "aniline-labelled flufenoxuron" or "[^{14}C-aniline]flufenoxuron"



(benzoyl-U-^{14}C)-labelled flufenoxuron is designated as "benzoyl-labelled flufenoxuron" or "[^{14}C-benzoyl]flufenoxuron"



1.1 Absorption, distribution and excretion

Rats

The absorption, distribution and excretion of [^{14}C-aniline]flufenoxuron (radiochemical purity 99.2%; batch no. S0842 = 2453-81), dissolved in 30% dimethyl sulfoxide (DMSO) in Mulgofen EL719 castor oil, were studied in groups of five male and five female F344 rats dosed orally by gavage at a single dose of 350 mg/kg body weight (bw). The identification of flufenoxuron and its metabolites in tissues and excreta is described in section 1.2 (Huckle, 1987; Hutson, 1991a,b). Urine and faeces were collected at 24-hour intervals. Cage wash was performed on day 3. After 72 hours, the rats were killed, and blood and tissues were sampled and analysed for radioactivity. A preliminary study indicated that less than 0.1% of radioactivity was exhaled as $^{14}CO_2$.

Recovery was 93%. At 24 hours after dosing, 56% and 35% of the radioactivity were found in faeces of males and females, respectively. After 72 hours, 84–85% of the radioactivity was excreted in both sexes. At this time, a total of up to 0.6% of the radioactivity was found in urine, and 7.0–8.3% was found in organs and carcass. Faeces collected from 48 to 72 hours contained 2.5–5.2% of the administered dose. It is likely that the faecal excretion observed for the first 2 days is due to unabsorbed test substance at this high dose level, whereas radioactivity levels in faeces collected on the third day reflect excretion of absorbed test material. Radioactivity was widely distributed to the tissues, the highest concentrations being found in fat, gut wall, liver, bone marrow, skin and kidneys. Based on the levels in organs, carcass, and urine and faeces collected from 48 to 72 hours, it can be concluded that about 9.9–14.1% of the administered dose was absorbed. The ratio of concentration in fat to concentration in blood was about 57, indicating a high potential for accumulation. Tissue levels were similar in males and females (Huckle, 1987; Hutson, 1991a,b).

The absorption, distribution and excretion of [^{14}C-aniline]flufenoxuron (radiochemical purity 99.4%; batch no. 986-2 = 2712-038), dissolved in 10% DMSO, 10% Mulgofen EL719 castor oil and 80% saline, were studied in groups of five male and five female F344 rats dosed orally by gavage at a single dose of 3.5 mg/kg bw. The identification of flufenoxuron and its metabolites in tissues and excreta is described in section 1.2 (Huckle, 1987; Hutson, 1991c,d). Urine and faeces were collected at 24-hour intervals. Cage wash was performed on day 7. After 168 hours, the rats were killed, and blood and tissues were sampled and analysed for radioactivity. A preliminary study indicated that less than 0.01% of radioactivity was exhaled as $^{14}CO_2$.

Recovery was 93–96%. At 24 hours after dosing, about 9% of the radioactivity was found in faeces of both sexes. On the second day after treatment, faecal excretion was about 4%. Subsequently, daily excretion in faeces was about 2%. In urine, about 2% of the radioactivity was excreted during

the first 48 hours, and about 3% from 48 to 168 hours. At 168 hours, about 67% of the radioactivity was found in organs and carcass. Radioactivity was widely distributed to the tissues, the highest concentrations being found in fat, gut, skin, liver and kidneys. Tissue levels were similar in males and females. Based on the levels in urine, organs and carcass, it can be concluded that at least about 72% of the administered dose was absorbed. The ratio of radioactivity concentration in fat to radioactivity concentration in whole blood was about 53, indicating a high potential for bioaccumulation (Huckle, 1988; Hutson, 1991c,d).

The absorption, distribution and excretion of [^{14}C-aniline]flufenoxuron (radiochemical purity 99%; batch nos S842/4, S986/5 and S988/4), dissolved in 10% DMSO, 10% Mulgofen EL719 castor oil and 80% isotonic saline at 0.9%, following 28 daily oral gavage doses of 3.5 mg/kg bw were studied in female F344 rats. Groups of three females were killed on days 2, 8, 15, 22, 29, 30, 32, 35, 42, 56, 70, 95 and 205, and blood and tissues were sampled and analysed for radioactivity. The identification of flufenoxuron and its metabolites in tissues is described in section 1.2 (Morrison, 1989; Hutson, 1991e). One female was killed 1 day after the last dosing, and body sections were subjected to autoradiography.

The whole-body autoradiography performed in one female on day 29 indicated that the radioactivity was extensively distributed throughout the carcass.

Total radioactive residue (TRR) concentrations increased with the number of administered doses throughout the 28-day treatment period (Table 1). The increase was apparently linear with time for most tissues, whereas it tended to reach an equilibrium concentration (plateau, not yet clearly observable) in the skin, gastrointestinal tract and bone marrow.

Table 1. Quantitative distribution of radioactivity in the tissues of female rats administered 28 daily oral doses of [^{14}C-aniline]flufenoxuron

	TRR (µg equivalents/g tissue)								
Day	**Blood**	**Kidney**	**Liver**	**Gut**	**Ovary**	**Bone marrow**	**Skin**	**Carcass**	**Fat**
2	0.3	1.8	3.0	3.3	3.0	9.0	4.1	1.1	12.7
8	0.8	4.2	5.9	10.9	7.4	8.6	9.6	5.2	47.9
15	1.5	6.5	9.4	16.3	11.5	23.1	14.5	7.4	79.1
29	2.7	11.2	15.7	18.1	20.2	32.6	17.5	15.5	143.6
30	3.0	10.3	14.9	9.5	22.9	21.7	16.4	14.1	140.9
32	2.9	9.5	14.3	7.3	23.3	25.4	14.9	12.8	131.7
35	2.8	9.2	12.9	8.3	15.7	20.9	14.6	13.1	114.4
56	2.3	5.6	7.9	5.1	8.1	15.6	9.6	7.1	65.1
95	0.9	3.0	4.1	2.2	3.2	7.3	3.1	2.3	25.1
205	< 0.09	0.4	1.2	0.2	0.6	0.7	0.4	0.2	1.8
$t_{½}$ (days)	32	38	48	29	34	34	33	28	28

$t_{½}$: half-life

Source: Morrison & Huckle (1988); Hutson (1991e)

After termination of dosing, the tissue residues decreased with time. The mean half-life was 34 days (Morrison & Huckle, 1988; Hutson, 1991e).

Five groups of 10 male Fischer 344 rats were fed diets containing unlabelled flufenoxuron (purity 97.4%; batch no. 7016 = ST87/129) at 0, 0.005, 0.05, 0.5, 5, 50 or 500 parts per million (ppm) (equivalent to 0, 0.000 25, 0.0025, 0.025, 0.25, 2.5 and 25 mg/kg bw per day) for 100 days. Twenty animals received control diet. The rats were killed at the end of the study, and omental fat samples from each animal were analysed for flufenoxuron.

In the control and the 0.005 ppm groups, flufenoxuron was not detectable in fat (limit of quantification [LOQ] = 0.1 µg/g). In the 0.05, 0.5, 5 and 50 ppm treatment groups, the average concentrations found in the fat were 0.198, 1.67, 18.6 and 188 µg/g, respectively. These were 3.96, 3.34, 3.72 and 3.76 times the nominal dietary concentrations of the respective groups, indicating that there was a linear relationship between administered dietary concentrations and concentrations of the test item in the fat tissue. In the 500 ppm dose group, the average concentration in the fat (230 µg/g) was only 0.46 times that of the diet and only 22% higher than the average (188 µg/g) concentration detected in the fat tissue of animals fed 50 ppm flufenoxuron. This indicates that there is saturation of the absorption and/or storage of flufenoxuron in fat tissues (Baldwin, 1988).

The absorption, distribution and excretion of [^{14}C-benzoyl]flufenoxuron (radiochemical purity > 97%; batch nos S1101/3 and S1101/4), dissolved in an aqueous solution containing 10% DMSO and 10% Mulgofen EL719 castor oil, were studied in F344 rats. The identification of flufenoxuron and its metabolites in tissues is described in section 1.2 (Mayo, 1992). In study A, groups of three males and three females received a single dose of 3.5 or 350 mg/kg bw and were placed in metabolism cages. Urine was collected for 0–6 and 6–24 hours and then at 24-hour intervals up to 168 hours. Faeces were collected during 24-hour intervals up to 168 hours. Expired air was trapped for 0–24 and 24–48 hours after dosing. After 7 days, the rats were killed, and radioactivity levels in the gastrointestinal tract and carcass were measured. In study B, groups of six male and six female rats received a single dose of 3.5 or 350 mg/kg bw. Blood samples were taken from each animal predosing, at 0.25, 0.5, 1, 2, 3, 4, 6, 9 and 24 hours after dosing and then at 1- to 3-day intervals until 336 hours after dosing. The samples from three male and three female rats were used for direct measurement of radioactivity content. The other animals were used for the measurement of radioactivity content in the plasma after removal of the cells by centrifugation. In study C, three males and three females received a single dose of 3.5 mg/kg bw after cannulation of the bile duct. Urine and faeces were collected for 0–24 and 24–48 hours for measurement of the radioactivity. Bile was collected at 3-hour intervals for 48 hours post-dosing. After 48 hours, the rats were killed, and the levels of radioactivity were measured in the gastrointestinal tract and carcass. In study D, one male and one female rat received a single oral dose of 3.5 mg/kg bw. After 4 hours, the rats were killed, and whole-body autoradiography was performed. In study E, two groups of nine males and nine females received a single dose of 3.5 or 350 mg/kg bw. Three male and three female rats per dose were killed at 4, 20 or 168 hours after dosing. Blood samples, organs, tissues and carcasses were subjected to radioactivity measurement. For all studies in which samples were analysed, radioactivity measurements were performed on faeces, blood, gastrointestinal tract, liver and spleen using liquid scintillation counting (LSC) on oxygen combustion products. Direct LSC was used for other sample types, including cage wash and carbon dioxide traps.

Study A: At 3.5 mg/kg bw, after 7 days, urine contained 30% of the administered dose in males and 24% in females, including cage washes. In faeces, 19% (males) and 12% (females) of the administered dose were excreted after 7 days. Faeces collected from 48 to 168 hours contained 3.4% and 2.4% of the administered dose in males and females, respectively. It is likely that the radioactivity levels in faeces collected from the third day onwards reflect excretion of absorbed test material. At 168 hours, radioactivity in the remaining carcasses accounted for 46% (males) and 59% (females) of

the administered dose. The gastrointestinal tracts including contents contained 1–2% of the dose. At 350 mg/kg bw, the radioactivity was mainly excreted in the faeces for the 7 days after dosing (93% and 100% in males and females, respectively). Faeces collected from 48 to 168 hours and the content of the gastrointestinal tract contained 2.4% and 10.9% of the administered dose in males and females, respectively. Excretion in urine was 0.7% in males and 0.5% in females. After 168 hours, radioactivity in the remaining carcasses and the gastrointestinal tracts (including contents) accounted for less than 1% of the administered dose (male and female). Based on the levels in urine, faeces, gastrointestinal tract and carcass after a dose of 3.5 mg/kg bw, the absorption was at least 80% in males and 87% in females. At the high dose, absorption was about 3% in males and 12% in females.

Study B: The kinetic parameters of flufenoxuron residues in blood and plasma are summarized in Table 2.

Table 2. Toxicokinetic parameters of radioactive residues of flufenoxuron after a single oral administration of benzoyl-labelled flufenoxuron to rats

	3.5 mg/kg bw				350 mg/kg bw			
	Blood		Plasma		Blood		Plasma	
Parameter	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
C_{max} (µg/g)	0.42	0.60	0.27	0.39	0.75	1.02	0.77	1.10
T_{max} (h)	3	3	6	6	3	3	4	6
$t_{½\ elim}$ (h)	178	327	155	428	NC	NC	NC	NC
$AUC_{0-\infty}$ (µg × h/mL)	38.6	62.3	25.4	62.5	NC	NC	NC	NC
$AUC_{0.25-24\ h}$ (µg × h/mL)	7.0	8.1	4.8	6.3	13.5	13.7	13.3	16.1

$AUC_{0-\infty}$: area under the concentration–time curve from time 0 to infinity; $AUC_{0.25-24\ h}$: area under the concentration–time curve from 0.25 to 24 hours; bw: body weight; C_{max}: maximum concentration in blood or plasma; F: female; M: male; NC: could not be reliably calculated; $t_{½\ elim}$: elimination half-life; T_{max}: time to reach C_{max}

Source: Hawkins et al. (1992)

Comparison of the $AUC_{0.25-24\ h}$ values indicates that a 100 times higher dose level resulted in only a 1.65 times higher plasma exposure and a 1.8 times higher blood exposure (means on combined sexes), showing that there was a saturation of the absorption at the high dose level of 350 mg/kg bw.

Study C: Within 48 hours after a flufenoxuron dose of 3.5 mg/kg bw, 4–5% of the radioactivity was excreted in bile (both sexes), 14% (males) and 9% (females) were excreted in urine and 11% (males) and 4% (females) were excreted in faeces. At 48 hours, 61% (males) and 78% (females) of radioactivity were found in the carcass, and 3–4% (both sexes) remained in the gastrointestinal tract. Based on the amounts recovered from urine, bile and carcass, absorption was at least 79% in males and 92% in females.

Study D: Autoradiography of rats killed 4 hours after dosing with flufenoxuron at 3.5 mg/kg bw showed high concentrations of radioactivity in stomach contents, brown fat, small intestine contents and the preputial and clitoral glands. Intermediate concentrations were found in kidney, fat, exorbital lacrimal gland, spleen, liver, muscle, infra-orbital lacrimal gland, lung, pancreas, bone marrow, adrenals, Harderian gland, salivary gland, myocardium, brain, thyroid, thymus and spinal cord. In the female rat, intermediate levels were also observed in the uterine wall and the ovary.

Study E: At 4 hours after a single oral dose of 3.5 mg/kg bw, the highest concentrations of radioactivity were found in the adrenals, gastrointestinal tract, liver, ovaries, thyroid and bone marrow (8–28 µg/g). Tissues with intermediate concentrations were the kidneys, lungs, pancreas, and perirenal and subcutaneous fat (4–7 µg/g). Concentrations in other tissues were below 4 µg/g. At 20

hours, the highest concentrations were found in the perirenal and subcutaneous fat (15–17 µg/g). Intermediate concentrations were observed in the adrenals, bone marrow, ovaries, pancreas and thyroid (4–8 µg/g). Concentrations in other tissues were below 2 µg/g. At 168 hours, highest concentrations were present in the perirenal and subcutaneous fat (9–11 µg/g). Intermediate concentrations were observed in the adrenals, bone marrow, pancreas and thyroid (2–3 µg/g). Concentrations in other tissues were in the range of 0.05–1 µg/g.

At 4 hours after an oral dose of 350 mg/kg bw, high concentrations of radioactive compounds were found in the gastrointestinal tract (4000–5000 µg/g). Intermediate concentrations were observed in the adrenals, bone marrow, kidney, liver, ovaries and pancreas (4–8 µg/g). Concentrations were lower in the perirenal and subcutaneous fat (2–3 µg/g). In all other tissues, the concentrations were 1–3 µg/g. At 20 hours, high concentrations were found in the gastrointestinal tract (270–670 µg/g) and in the perirenal and subcutaneous fat (6–14 µg/g). Intermediate concentrations were observed in the adrenals, bone marrow and pancreas (2–7 µg/g). Concentrations in the other tissues were either below the limit of detection or in the range of 1–3 µg/g. At 168 hours, concentrations of radioactivity in most tissues were below the limit of reliable measurement. Concentrations were highest in the perirenal and subcutaneous fat (9 µg/g). The other tissues with any detectable radioactivity were the gastrointestinal tract, liver, adrenals (females only) and pancreas (all 1–3 µg/g) (Hawkins et al., 1992).

The absorption, distribution and excretion of [^{14}C-aniline]flufenoxuron (radiochemical purity > 98%; batch no. S1094/3), dissolved in a solution containing 10% DMSO, 10% Mulgofen EL719 castor oil and 80% saline, were studied in F344 rats. Groups of three males and three females received a single dose of 3.5 mg/kg bw by gavage after cannulation of the bile duct. Urine, faeces and bile were collected for 0–24 and 24–48 hours. After 48 hours, the rats were killed, and radioactivity levels in the gastrointestinal tract and carcass were measured. The identification of flufenoxuron and its metabolites in bile is described in section 1.2 (Kirkpatrick, 1992).

Total recovery was 90%. Over the 48 hours, excretion of radioactivity was 19.7% (males) and 6.7% (females) in bile, 2.6% (males) and 1.6% (females) in urine (including cage wash) and 4.0% (males) and 30.2% (females) in faeces. At termination, 5.0% (male) and 4.4% (female) of the dose were recovered from the gastrointestinal tract. In the carcass, 59.1% (male) and 47.3% (female) of the administered dose were found. Based on the radioactivity found in urine, cage wash, bile and carcass, total absorption over 48 hours was 81.4% for male rats and 55.6% for female rats. As the animals were cannulated, these values do not take into account possible enhanced absorption enabled by bile emulsions (the test item being known to be lipophilic) (Kirkpatrick, 1992).

Dogs

The absorption, distribution and excretion of [^{14}C-aniline]flufenoxuron (radiochemical purity > 98%; batch no. S1022/2), dissolved in DMSO and Mulgofen EL719 in saline, were studied in two male and two female Beagle dogs dosed orally by gavage at a single dose of 3.5 mg/kg bw. The identification of flufenoxuron and its metabolites in tissues and excreta is described in section 1.2 (Hawkins, Elsom & Girkin, 1988; Elsom, 1991a,b). Urine was collected from 0 to 6 hours and from 6 to 24 hours and at 24-hour intervals thereafter. Faeces was collected at 24-hour intervals. Cage wash was performed at each 24-hour interval. Blood samples for haematocrit and radioactivity measurements on whole blood were taken predosing, 1, 4, 8 and 24 hours post-dosing and then at 24-hour intervals thereafter. A part of these samples and additional samples taken at 0.5, 1.5, 2, 6 and 12 hours post-dosing were analysed for radioactivity concentrations in the plasma and in blood cells. After 168 hours, the dogs were killed, and tissues were sampled and analysed for radioactivity.

Recovery was 85%. At 24 hours after dosing, 46–62% of the radioactivity was excreted in faeces. It is noted that three dogs had diarrhoea on the first day of the study, resulting in the excretion of 13–51% of the dose within the first hour after dosing. After 7 days, 56–72% of the radioactivity was excreted in faeces. Excretion in urine was less than 0.7% per day. The finding of 13% of the

radioactivity in the urine of one male dog on day 1 was attributed to contamination of the urine by faeces and/or vomit. After 7 days, 2–4% of the radioactivity was excreted in the urine. Seven days after dosing, 12–23% of the radioactivity was found in tissues. Higher levels were found in fat, bone marrow, adrenals, liver and kidneys. Most tissues apart from the brain contained concentrations of radioactivity equal to or greater than those in plasma. Based on the radioactivity found in urine and tissues, the absorption in the individual dogs was at least 14–27%. In whole blood, an initial peak concentration of 0.264 µg/g was found at 4 hours. Subsequently, the concentration declined to 0.232 µg/g at 8 hours, after which the concentrations slowly continued to rise to 296 µg/g at 168 hours. In plasma, a peak concentration of 0.407 µg/mL was observed at 4 hours. Subsequently, plasma concentrations slowly declined to 0.179 µg/mL at 168 hours. In blood cells, radioactivity concentrations rose from 106 µg/g at 4 hours to 472 µg/g at 168 hours. The ratio of the concentration in fat to the concentration in whole blood was about 12 at 168 hours (Hawkins, Elsom & Girkin, 1988; Elsom, 1991a,b).

The absorption, distribution and excretion of flufenoxuron (purity 97.9%; batch no. 7005) administered in the diet at 500 ppm (equal to 8.6–12.2 mg/kg bw per day) for 19 weeks were studied in seven obese female Beagle dogs (4–7 years old, weighing 16–23 kg). Blood samples were collected from the jugular vein pretreatment, on days 2, 3, 4 and 7 and weekly thereafter until day 56. Additional samples were taken on days 77, 98, 119 and 133 and biweekly thereafter until day 189. Subcutaneous fat samples (approximately 3 g) from all animals were removed by biopsy from the flanks under local anaesthesia at the same time as blood sampling. Twenty-four hours after the last dietary administration, three treated dogs were killed. Two dogs were killed after a 4-week treatment-free period. The remaining two dogs were killed after an 8-week treatment-free period. Tissue samples (liver, kidney, muscle, fat and bone marrow) were taken for analysis of flufenoxuron levels.

No treatment-related clinical signs were observed. All dogs showed a gradual weight loss (0.6–2.9 kg) over the 19 weeks of treatment, which was attributed to the daily rationing of feed to 400 g. Feed consumption was not affected. Individual and mean values of flufenoxuron concentrations in blood of treated animals are presented in Table 3. The mean results show a continuous rise in blood concentration of flufenoxuron until the end of treatment at day 133. In the recovery phase (from day 134 onward), there was, inversely, an approximately exponential decline in flufenoxuron concentrations in blood. An elimination half-life of 33 days was calculated. However, this value is only a rough estimation in view of the considerable individual variation in blood concentrations at each time point.

Individual and mean values of flufenoxuron concentrations in fat sampled from treated animals are presented in Table 4. The mean results show an approximately parabolic rise in flufenoxuron residues in fat during the treatment period. In the recovery phase (from day 134 onward), an approximately linear decline in flufenoxuron levels was observed. There was considerable variation in individual animal results at each time point. The concentration curve is similar to that obtained for blood samples. The fat and the bone marrow contained the highest concentrations of flufenoxuron (respectively: 3.9–43.2 and 3.6–47.1 µg/g) (Greenough, Goburdhun & Parkinson, 1988).

1.2 Biotransformation

Rats

The metabolism of [^{14}C-aniline]flufenoxuron (radiochemical purity 99.2%; batch no. S0842 = 2453-81), dissolved in 30% DMSO in Mulgofen EL719 castor oil, was studied in groups of five male and five female F344 rats dosed orally by gavage at a single dose of 350 mg/kg bw. The study design and toxicokinetics are described in section 1.1 (Huckle, 1987; Hutson, 1991a,b). Identification of metabolites in faecal samples was performed using mass spectrometry. Identification of metabolites in fat and carcass samples was performed using high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC). Unextractable radioactivity in faeces was quantified by LSC.

Table 3. Concentrations of flufenoxuron in blood of dogs (Nos 3–8) given flufenoxuron daily in the diet at a concentration of 500 ppm

		Concentrations in blood (µg/mL)							
Day of study		**No. 2**	**No. 3**	**No. 4**	**No. 5**	**No. 6**	**No. 7**	**No. 8**	**Mean**
Mean daily dose (mg/kg bw)		*11.52*	*9.31*	*8.63*	*12.17*	*9.89*	*11.99*	*10.21*	*10.53*
Pretrial									
	Day –1	0	0	0	0	0	0	0	0
Treatment period									
	Day 2	0.094	0.000	0.000	0.031	0.148	0.076	0.000	0.050
	Day 7	0.061	0.000	0.042	0.044	0.082	0.082	0.031	0.049
	Day 14	0.153	0.039	0.080	0.069	0.072	0.134	0.082	0.090
	Day 21	0.250	0.203	0.144	0.157	0.129	0.152	0.089	0.156
	Day 35	0.353	0.226	0.161	0.253	0.218	0.180	0.156	0.221
	Day 49	0.389	0.391	0.336	0.396	0.312	0.342	0.293	0.351
	Day 56	0.447	0.259	0.330	0.359	0.327	0.340	0.283	0.335
	Day 98	0.518	0.180	0.400	0.375	0.409	0.299	0.344	0.361
	Day 133	0.534	0.348	0.431	0.389	0.617	0.306	0.441	0.438
Recovery period									
	Day 147	–	–	–	0.262	0.412	0.165	0.317	0.289
	Day 161	–	–	–	0.150	0.255	0.122	0.247	0.194
	Day 175	–	–	–	–	–	0.095	0.160	0.128
	Day 189	–	–	–	–	–	0.038	0.144	0.076

bw: body weight; ppm: parts per million

Source: Greenough, Goburdhun & Parkinson (1988)

In faeces, only unchanged parent compound was detected. About 97% of the radioactivity in fat was identified as unchanged aniline-labelled flufenoxuron; the remainder of the radioactivity (~3%) was not characterized. Unchanged aniline-labelled flufenoxuron was the main component in the carcass extract. The combined total of all other minor (non-discrete) radioactive products (< 0.2% of the administered dose) was not characterized. No attempts were made to investigate the profile of aniline-labelled flufenoxuron degradation products present in the urine, liver, kidney or muscle, as they represented less than 5% of the administered radioactive dose (Huckle, 1987; Hutson, 1991a,b).

The metabolism of [^{14}C-aniline]flufenoxuron (radiochemical purity 99.4%; batch no. 986-2 = 2712-038), dissolved in 10% DMSO, 10% Mulgofen EL719 castor oil and 80% saline, was studied in groups of five male and five female F344 rats dosed orally by gavage at a single dose of 3.5 mg/kg bw. The study design and toxicokinetics are described in section 1.1 (Huckle, 1988; Hutson, 1991c,d). In faecal, organ and tissue samples, metabolites were identified by TLC; in urine samples, metabolites were analysed by TLC and mass spectrometry.

Table 4. Concentrations of flufenoxuron in fat samples of dogs (Nos 2–8) given flufenoxuron daily in the diet at a concentration of 500 ppm

	Concentration in fat (µg/g)							
Day of study	**No. 2**	**No. 3**	**No. 4**	**No. 5**	**No. 6**	**No. 7**	**No. 8**	**Mean**
Mean daily dose (mg/kg bw)	*11.52*	*9.31*	*8.63*	*12.17*	*9.89*	*11.99*	*10.21*	*10.53*
Treatment period								
Day 14	6.65	7.09	5.82	6.43	6.77	5.94	5.80	6.36
Day 28	14.57	5.13	11.86	12.11	12.69	8.69	12.69	11.11
Day 42	27.79	12.99	18.73	16.75	19.59	17.24	15.84	18.42
Day 56	30.27	20.85	26.95	27.46	30.47	30.41	21.37	26.83
Day 77	37.15	20.60	31.89	29.94	33.75	28.13	30.66	30.30
Day 98	44.21	24.74	33.53	35.21	33.22	22.92	28.91	30.39
Day 119	43.83	22.01	29.35	23.82	29.82	32.87	35.30	31.00
Day 134	37.56	23.05	37.45	31.47	18.04	70.86	35.40	36.26
Recovery period								
Day 147	–	–	–	23.48	30.65	18.72	26.05	24.73
Day 161	–	–	–	13.68	23.44	13.55	16.28	16.74
Day 175	–	–	–	–	–	4.85	12.80	8.83
Day 189	–	–	–	–	–	2.11	0.56	1.34

bw: body weight; ppm: parts per million

Source: Greenough, Goburdhun & Parkinson (1988)

In faeces, unchanged aniline-labelled flufenoxuron accounted for 6%, 0.4% and 0.3% of the administered dose on days 1, 3 and 7, respectively (both sexes). Minor metabolites, less than 1% of the administered dose, were Reg. No. 4110959 (WL 132612, i.e. 2-amino-5-(2-chloro-4-(trifluoromethyl)phenoxy)-3-fluorophenol), Reg. No. 4064702 (WL 129183, i.e. *N*-[4-[2-chloro-4-(trifluoromethyl) phenoxy]-2-fluorophenyl] urea) and Reg. No. 241208 (WL 115096, i.e. 4-[2-chloro-4-(trifluoromethyl)phenoxy-2-fluorobenzenamine). Four per cent of the administered dose in faeces could not be identified. In all organs, the majority of the radioactivity was identified by TLC as unchanged flufenoxuron. In liver extracts, there were at least three other minor components, which were not identified. No metabolite, besides the unchanged compound, was identified in any tissue sample. Metabolite profiles in extracts from males and females appeared to be qualitatively similar. These results indicated that the absorbed flufenoxuron was metabolized by cleavage of the bond adjacent to the 2,6-difluorobenzoyl moiety (Huckle, 1988; Hutson, 1991c,d).

The metabolism of [^{14}C-aniline]flufenoxuron (radiochemical purity 99%; batch nos S842/4, S986/5 and S988/4), dissolved in 10% DMSO, 10% Mulgofen EL719 castor oil and 80% isotonic saline at 0.9%, following 28 oral gavage doses of 3.5 mg/kg bw per day was studied in groups of female F344 rats. Groups of three females were killed on days 2, 8, 15, 22, 29, 30, 32, 35, 42, 56, 70, 95 and 205, and blood and tissues were sampled and analysed for radioactivity. Radioactive compounds in abdominal fat were identified using TLC in comparison with the reference test item and by HPLC. The study design and toxicokinetics are described in section 1.1 (Morrison & Huckle, 1988; Hutson, 1991a,b).

Following extraction of the abdominal fat samples from days 29, 56, 70 and 95, TLC analysis showed a single radioactive product with chromatographic properties indistinguishable from those of flufenoxuron. Following quantification of plates, the parent compound accounted for 97.8% (97.2–98.4%) of the radioactivity applied to the TLC plates (Morrison, 1989; Hutson, 1991f).

The metabolism of [^{14}C-aniline]flufenoxuron (radiochemical purity > 98%; batch no. S1094/3), dissolved in a solution containing 10% DMSO, 10% Mulgofen EL719 castor oil and 80% saline, was studied in groups of three male and three female F344 rats dosed orally by gavage at a single dose of 3.5 mg/kg bw after cannulation of the bile duct. The study design and toxicokinetics are described in section 1.1 (Kirkpatrick, 1992). Samples of unhydrolysed and acid-hydrolysed bile were analysed in comparison with reference compounds by TLC and HPLC.

The bile contained a high proportion of polar unresolved metabolites, which accounted for 74–79% of the radioactivity. The remaining major component co-eluted with unchanged aniline-labelled flufenoxuron and accounted for 16–21% of the radioactivity. A minor fraction of the radioactivity (0.6–0.9%) resembled Reg. No. 241208 (WL 115096). After acid hydrolysis of bile samples, the proportions of unchanged compound in males and females were similar to those found before acid hydrolysis. The radioactivity co-eluting with Reg. No. 241208 (WL 115096) increased to 5.9% (male) and 6.5% (female) of the sample radioactivity. The fraction of unidentified, unresolved components increased when compared with unhydrolysed samples. This indicated that some polar material had been hydrolysed (Kirkpatrick, 1992).

The metabolism of [^{14}C-benzoyl]flufenoxuron (radiochemical purity > 97%; batch nos S1101/3 and S1101/4), dissolved in an aqueous solution containing 10% DMSO and 10% Mulgofen EL719 castor oil, was studied in F344 rats. The study design and toxicokinetics of flufenoxuron are described in section 1.1 (Hawkins et al., 1992; Mayo, 1992). Metabolites were identified by TLC in urine (low dose only) and faeces (low and high doses) of rats receiving flufenoxuron at a single dose of 3.5 or 350 mg/kg bw. The fat from rats treated with 3.5 mg/kg bw and killed at 20 hours post-dosing was sampled, and radioactive compounds were identified using direct TLC in comparison with reference compounds.

In urine of rats treated at the low dose of 3.5 mg/kg bw, there was a major metabolite co-chromatographing with Reg. No. 206925 (2,6-difluorobenzoic acid) and accounting for 12.1% (males) or 10.1% (females) of the dose in 0–48 hours (the amount in 0- to 24-hour urine was also > 10% once corrected for oral absorption of the dose). A second metabolite was found to co-chromatograph with Reg. No. 102719 (2,6-difluorobenzamide) and accounted for 0.3% of the dose during 0–48 hours. Three further polar metabolites (not further identified) were separated in a more polar TLC system; these accounted for 0.4–0.8%, 0.2–0.4% and 0.1–0.3% of the dose. No parent was detected in urine.

Primarily unchanged parent flufenoxuron was detected in the 0- to 48-hour faecal samples from animals treated with benzoyl-labelled flufenoxuron at 3.5 mg/kg bw (9–14% of the administered dose) and 350 mg/kg bw (90–91% of the administered dose).

Only one component was identified in extracts of subcutaneous fat taken 20 hours after low-level dosing. This was shown to co-chromatograph with the parent compound and accounted for approximately all radioactivity found in fat (Hawkins et al., 1992; Mayo, 1992).

Dogs

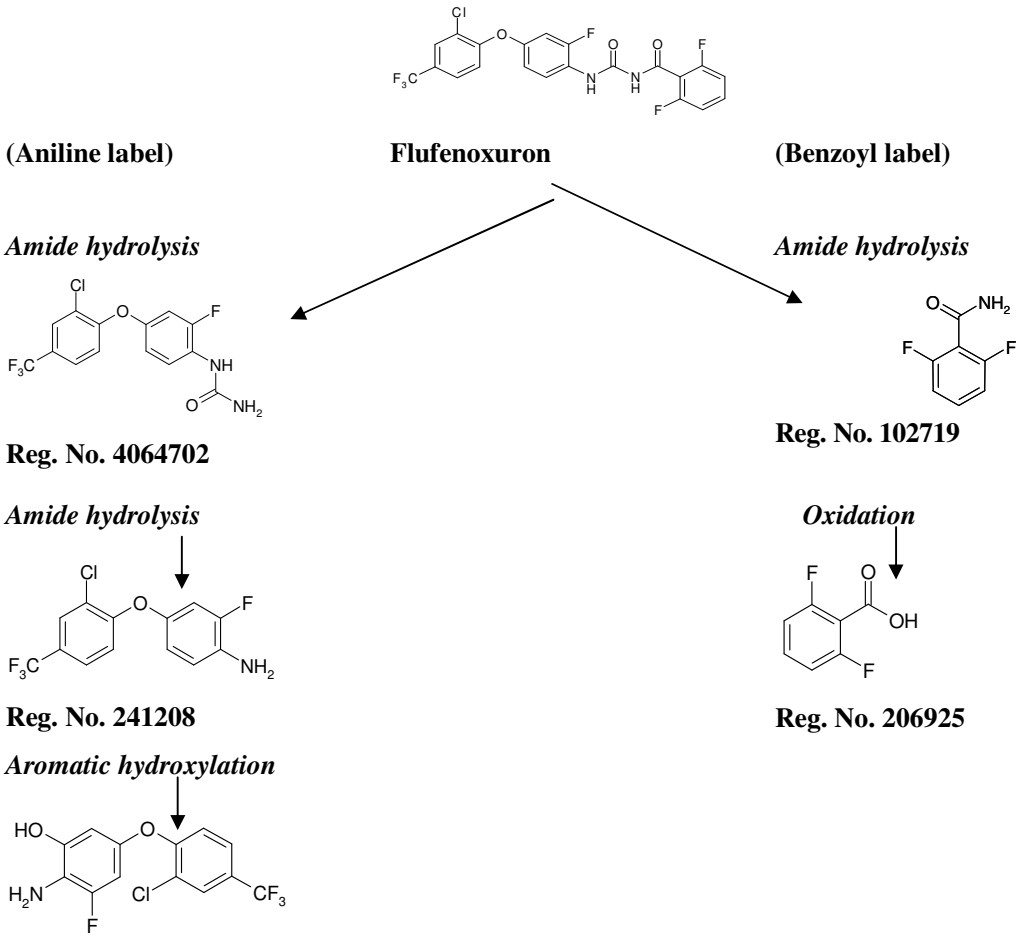
The metabolism of [^{14}C-aniline]flufenoxuron (radiochemical purity > 98%; batch no. S1022/2), dissolved in DMSO and Mulgofen EL719 castor oil in saline, was studied in two male and two female Beagle dogs dosed orally by gavage at a single dose of 3.5 mg/kg bw. The study design and toxicokinetics of flufenoxuron are described in section 1.1 (Hawkins, Elsom & Girkin, 1988;

Elsom, 1991a,b). Metabolites were identified by TLC in urine and faeces by comparison with reference compounds.

Faeces and urine contained virtually only unchanged parent compound. One minor metabolite, co-eluting with Reg. No. 241208 (WL 115096), was present in 24- to 48-hour faecal samples (Hawkins, Elsom & Girkin, 1988; Elsom, 1991a,b).

The proposed metabolic pathway of flufenoxuron in rats is shown in Fig. 2.

Fig. 2. Proposed metabolic pathway of flufenoxuron in rats



Reg. No. 4064702: *N*-[4-[2-Chloro-4-(trifluoromethyl) phenoxy]-2-fluorophenyl] urea
Reg. No. 241208: 4-[2-Chloro-4-(trifluoromethyl)phenoxy-2-fluorobenzenamine
Reg. No. 4110959: 2-Amino-5-(2-chloro-4-(trifluoromethyl)phenoxy)-3-fluorophenol
Reg. No. 102719: 2,6-Difluorobenzamide
Reg. No. 206925: 2,6-Difluorobenzoic acid

2. Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

The results of studies of acute toxicity with flufenoxuron are summarized in Table 5.

Table 5. Results of studies of acute toxicity with flufenoxuron

Species	Strain	Sex	Route	Vehicle	Purity (%)	LD_{50} / LC_{50}	Reference
Rat	Fischer 344	M/F	Oral	Aqueous carboxymethyl cellulose	97.6	> 5 000 mg/kg bw (M/F)	Gardner (1989)[a]
Rat	Fischer 344	M/F	Oral	DMSO	99.0	> 3 000 mg/kg bw (M/F)	Price (1986, 1991)[b]
Rat	Fischer 344	M/F	Dermal	Water	99.0	> 2 000 mg/kg bw (M/F)	Price (1986, 1991)[c]
Rat	Sprague-Dawley	M/F	Inhalation	—	98	5.1 mg/L (M/F)	McDonald (1986, 1991)[d]

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

[a] Performed according to Organisation for Economic Co-operation and Development (OECD) Test Guideline 401. The rats were given a dose of 5000 mg/kg bw. No mortality or clinical signs were observed. Necropsy revealed no abnormalities. Batch no. 7003 = WB 16867.

[b] Study design resembles OECD Test Guideline 401. The rats were given a dose of 3000 mg/kg bw. One female rat died on day 2 of the study. In this rat, the stomach contained compacted powder and showed haemorrhagic petechiae. All rats showed clinical signs 6 hours following dosing that consisted of chromodacryorrhoea (both sexes), abnormal gait (males only), lethargy (females only), increased lacrimation (females only) and blood-tinged urine (females only). These signs resolved in all survivors by study day 2. Batch no. 13 = ST86/022.

[c] Performed according to OECD Test Guideline 402. The rats were given a dose of 2000 mg/kg bw. No mortality or clinical signs were observed. Batch no. 13 = ST86/022.

[d] Study design resembles OECD Test Guideline 403. Rats were exposed nose only to an actual flufenoxuron concentration of 5.1 mg/L. Another group of five male and five female rats was exposed to air only. No mortality was observed. All rats displayed subdued behaviour immediately following cessation of exposure. In blood samples obtained 1 hour after the end of the exposure, no effect of flufenoxuron exposure on methaemoglobin levels was observed. Necropsy showed no abnormalities. Mass median aerodynamic diameter was 3.6 (± 2.0) µm. Batch no. 16.

(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 three male and three female New Zealand White rabbits was exposed for 4 hours under semi-occlusion to 0.5 g flufenoxuron (purity 99%; batch no. 13) moistened with water. Dermal irritation was scored according to the Draize system at 0.5, 24, 48 and 72 hours after patch removal.

No signs of dermal irritation were observed. Flufenoxuron was considered not irritating to rabbit skin (Price, 1986, 1991).

(c) Ocular irritation

In an acute eye irritation study, 25 mg of flufenoxuron (purity 99%; batch no. 13) was instilled into the conjunctival sac of one eye of each of three male and three female New Zealand White rabbits. The untreated eye served as a control. The eyes were examined macroscopically for signs of irritation according to the Draize system at 1, 24, 48 and 72 hours and at day 7 post-

instillation. The study design resembles OECD Test Guideline 405; however, the applied dose of 25 mg is low compared with the 100 mg or 0.1 mL of solid required by the test guideline.

Slight congestion of the conjunctival blood vessels was present in all animals at 1 hour after instillation and in one animal at 24 hours. At 1 hour, minimal chemosis was observed in two animals. The untreated eyes of all rabbits were overtly normal at all examinations. In the present test, flufenoxuron was not irritating to the eye; however, the dose used was considerably lower than that required by the test guideline (Price, 1986, 1991).

(d) Dermal sensitization

In a dermal sensitization study using the Magnusson and Kligman maximization test, performed in accordance with OECD Test Guideline 406, flufenoxuron (purity 99.1%; batch no. COD-000444) was tested in 20 female Dunkin-Hartley guinea-pigs. The vehicle control group consisted of five animals. The laboratory maintains periodic testing of this animal strain with a positive control substance (α-cinnamaldehyde).

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. Under the conditions of this study, flufenoxuron was not a skin sensitizer (Gamer & Leibold, 2005).

2.2 Short-term studies of toxicity

Mice

In a 28-day dietary study, flufenoxuron (purity 99%; batch no. 13 = ST86/019) was administered to groups of seven male and seven female C57/C3H F_1 hybrid mice at 0, 50, 500, 5000, 10 000 or 50 000 ppm (equivalent to 0, 7.1, 71, 710, 1400 and 7100 mg/kg bw per day, respectively). A concurrent control group of 14 males and 14 females was fed basal diet. Mice were observed daily for mortality and clinical signs. Body weight and feed consumption were measured weekly. Prior to termination, blood samples were taken from all animals for haematology, including blood smear examination, and blood biochemistry was performed on cardiac blood taken after the mice were killed. All mice underwent complete necropsy. Testes, liver, heart (males only), brain, kidneys and spleen (both sexes) were weighed. A wide range of organs of all control and high-dose animals and some mice from the other dose groups was examined microscopically. The study design resembles OECD Test Guideline 407.

Three female mice were killed in a moribund state (two at 10 000 ppm on days 26 and 27 and one at 50 000 ppm on day 14). Histological examination of these three mice revealed necrosis of the lymphoid tissue. The cause of death could not be established. At 50 000 ppm, pale faeces were observed in all mice, which was attributed to unabsorbed test material. Reduced feed consumption (22–23%) was observed in females at 50 000 ppm during weeks 2 and 4 (data for week 3 are missing). In these females, a slight reduction in body weight was observed at week 2. Slight changes in haematological parameters were considered not toxicologically relevant. Relative heart weight was slightly increased (12%) in females at 50 000 ppm. Histopathological examination revealed no changes in heart tissue. The study author considered the increased relative heart weight not to be treatment related. No changes in clinical chemistry parameters or histopathology were observed.

The no-observed-adverse-effect level (NOAEL) was 10 000 ppm (equivalent to 1400 mg/kg bw per day), based on reduced feed consumption in females only observed at 50 000 ppm (equivalent to 7100 mg/kg bw per day) (Esdaile, 1991a).

Flufenoxuron (purity 96.6%; batch no. 16 = ST86/165) was administered via the diet to groups of 10 male and 10 female C57/C3H F_1 hybrid mice for 13 weeks at a dietary level of 50, 500, 5000, 10 000 or 50 000 ppm (equal to 10, 103, 1069, 2139 and 11 071 mg/kg bw per day for males and 12, 124, 1247, 2482 and 12 619 mg/kg bw per day for females, respectively). A concurrent

control group of 20 males and 20 females was fed basal diet. Each of these groups was doubled using an equal number of additional animals (treated or controls, named "satellites"), which were subjected only to blood sampling for clinical chemistry analysis. The mice were examined daily for mortality and clinical signs. Feed consumption and body weights were recorded weekly. Ophthalmological examinations were performed prior to treatment and during week 13. Haematology, including assessment of methaemoglobin, clinical chemistry and urine analysis were performed at the end of the treatment period. All mice were necropsied, and weights of liver, spleen, kidneys, testes, heart, brain, uterus and adrenal glands were recorded. A wide range of tissues, including femoral bone marrow smears of mice of the control and 50 000 ppm groups and the lung, liver, kidneys and gross lesions from mice of the other groups, was examined microscopically.

Two females from the 5000 ppm group, two males from the 10 000 ppm group and one female from the 50 000 ppm group either died or were killed when moribund during the study. The cause of death could not be established. In view of the absence of specific pathological findings and the lack of a dose–response relationship, these deaths were not considered to be test substance related. Pale faeces observed in males and females at 50 000 ppm were attributed to unabsorbed test material. Feed consumption was generally comparable for all groups. In males at 10 000 and 50 000 ppm, body weight gain was lower (up to 22%) than in control males.

Results of haematological and blood biochemical examinations are summarized in Table 6. Haematological evaluations revealed minimal but statistically significant decreases in erythrocyte count, haemoglobin and haematocrit for males at 50 000 ppm. Males and females showed a statistically significant and generally dose-related increase in serum bilirubin from 500 to 50 000 ppm (increase of 26–117%). These haematological and blood biochemical changes were indicative of a slight anaemia. Methaemoglobin levels were not affected. Triglyceride levels were statistically significantly decreased in males at 10 000 and 50 000 ppm. Statistically significant decreases in blood urea nitrogen were found in males at 5000 and 50 000 ppm, but not at 10 000 ppm, and in females at 10 000 and 50 000 ppm.

Table 6. Haematological and blood biochemical findings in the 90-day dietary mouse study with flufenoxuron

	Sex	0 ppm	50 ppm	500 ppm	5 000 ppm	10 000 ppm	50 000 ppm
RBCs (10^{12}/L)	M	10.12	10.10	10.13	10.00	10.02	9.68**
	F	10.31	10.28	10.18	9.97	10.38	10.21
Haemoglobin (g/dL)	M	15.7	15.6	15.6	15.5	15.5	15.1**
	F	16.1	15.9	15.9	15.8	16.2	16.0
Haematocrit (ratio)	M	0.453	0.455	0.455	0.448	0.451	0.435**
	F	0.459	0.456	0.459	0.450	0.465	0.454
Methaemoglobin (%)	M	0.4	0.3	0.3	0.3	1.2	0.2
	F	0.3	0.3	0.2	0.1	0.6	0.5
Bilirubin	M	2.9	3.3	4.4**	5.5**	6.5**	6.3**
	F	4.2	4.5	5.3**	5.8**	5.9**	6.7**
Triglyceride	M	2.15	1.99	2.12	1.86	1.51**	1.59**
	F	1.64	1.64	1.57	1.53	1.50	1.31
Urea nitrogen	M	11.7	11.3	10.7	10.3*	10.5	9.9**
	F	10.9	10.2	10.7	10.5	9.7*	9.1**

F: female; M: male; ppm: parts per million; RBCs: red blood cells; *: $P < 0.05$; **: $P < 0.01$ (William's test)

Source: Esdaile (1988, 1991b); Berry (1992a)

There were no treatment-related changes in organ weight, gross pathology or histopathology.

The NOAEL was 50 ppm (equal to 10 mg/kg bw per day), based on increased bilirubin concentrations in males and females at 500 ppm (equal to 103 mg/kg bw per day) (Esdaile, 1988, 1991b; Berry, 1992a).

Rats

In a 28-day dietary toxicity study, performed in accordance with OECD Test Guideline 407, flufenoxuron (purity 99%; batch no. 13) was administered to groups of seven male and seven female Fischer 344 rats at 50, 500, 5000, 10 000 or 50 000 ppm (equal to 4.8, 49, 475, 997 and 5147 mg/kg bw per day for males and 5.3, 53, 534, 1067 and 5432 mg/kg bw per day for females, respectively). Groups of 14 male and 14 female rats received basal diet. Animals were checked 6 times per week for clinical signs of toxicity. Body weights and feed consumption were measured weekly. Blood was sampled for haematology and clinical biochemistry at termination. Generation of methaemoglobin was assessed using the nonspecific CO-oximeter method. Urine was sampled during week 13 for analysis. All rats were necropsied, and weights of liver, spleen, kidneys, testes, heart, brain and adrenals were recorded. A wide range of tissues, including femoral blood smears, of rats of the control and high-dose rats and any macroscopic lesions in the other dose groups were examined microscopically.

No deaths were observed. At 50 000 ppm, pale faeces, which were attributed to unabsorbed test material, were observed in all rats. Feed consumption and body weight gains for all treated males and females at 50, 500, 5000 and 10 000 ppm and females at 50 000 ppm were comparable to those of controls. Males at 50 000 ppm had an increased feed consumption from weeks 2 to 4 (9–15%), resulting in a 5% higher body weight than controls at the end of the study. Males at 50 000 ppm had statistically significantly increased relative heart and spleen weights. However, as the increases were only 5% and 7%, respectively, they were not considered toxicologically relevant. No toxicologically relevant changes in haematology, macropathology or histopathology were observed. Apparent moderate increases in methaemoglobin levels were considered false-positive findings, as the increases were not dose dependent and were below the level of accuracy of the test method. Cholesterol levels showed some variations of statistical significance, but no biological relevance. Triglycerides were significantly lower in males (17–22%) treated at 5000–50 000 ppm compared with controls, and this was considered to be treatment related. However, the toxicological relevance of the mild reductions in triglyceride levels is not clear. In high-dose females, a slight reduction in β-globulin levels (−11%) was observed. Other small but statistically significant changes in clinical chemistry parameters were considered not toxicologically relevant.

The NOAEL was 50 000 ppm (equal to 5147 mg/kg bw per day), the highest dose tested (Esdaile, 1986a; Volger, 1991).

In a 13-week dietary toxicity study, flufenoxuron (purity 96.6%; batch no. 16 = ST86/165) was administered to groups of 10 male and 10 female Fischer 344 rats at 50, 500, 5000, 10 000 or 50 000 ppm (equal to 3.5, 35, 351, 689 and 3637 mg/kg bw per day for males and 4.1, 41, 399, 820 and 4151 mg/kg bw per day for females, respectively). A concurrent control group of 20 males and 20 females was fed basal diet. Animals were checked daily for clinical signs of toxicity. Body weights and feed consumption were measured weekly. Ophthalmological examinations were carried out on five rats of each sex from the control and high-dose groups before dosing and at week 13. Blood was sampled for haematology and clinical biochemistry at termination. Generation of methaemoglobin was assessed using the nonspecific CO-oximeter method. Urine analysis was performed on five animals of each sex from each of the treated groups and from 10 controls of each sex during week 13. All rats were necropsied, and weights of liver, spleen, kidneys, testes/uterus, heart, brain and adrenals were recorded. A wide range of tissues of rats of the control and 50 000 ppm groups and the lung, liver and kidneys from all groups were examined microscopically.

There were no mortalities. Pale faeces, which were attributed to unabsorbed test material, were observed in all rats at 50 000 ppm and in a few rats at 10 000 ppm. Feed consumption and body weight gains for all treated males and females at 50, 500, 5000 and 10 000 ppm and for females at 50 000 ppm were comparable with those of controls. Males at 50 000 ppm had a mildly increased feed consumption (up to 8%) from week 7 to termination, which was attributed to the high level of test substance in the feed. No effect on body weight gain was observed. At 5000 ppm and above, females exhibited haematological changes indicative of a mild anaemia, with statistically significant decreases in haemoglobin (4–7%), erythrocyte count (5–6%) and haematocrit (5–7%). In bone marrow smears of females at 50 000 ppm, a statistically significant decrease in myeloid/erythroid ratio was observed (control females, 1.8; 50 000 ppm females, 1.0), which indicates a slight compensatory increase in erythropoiesis. In males, there were no relevant indications of anaemia from assessment of blood samples, although bone marrow smears from 50 000 ppm males indicated an increase in erythropoiesis (myeloid/erythroid ratio in control males, 1.9; in 50 000 ppm males, 0.8). Small but statistically significant increases in methaemoglobin levels were observed in all treatment groups (from 0.8%/0.5% in control males/females to 1.6%/1.5% in high-dose males/females). However, as the increases were not clearly dose dependent and were below the level of accuracy of the test method, the finding is not considered toxicologically relevant. Several statistically significant biochemical findings were observed; however, the changes were small and not considered toxicologically relevant. There were no treatment-related effects on urinary or ophthalmological parameters.

At 5000 ppm and above, absolute and relative spleen weights were slightly but statistically significantly increased in females (12–17%). These increases are probably related to the haematological effects observed in females. No treatment-related macroscopic or histopathological changes were observed.

The NOAEL was 500 ppm (equal to 41 mg/kg bw per day), based on slightly higher spleen weights and haematological changes indicative of a mild anaemia observed in females at 5000 ppm (equal to 399 mg/kg bw per day) (Esdaile, 1987, 1991c; Berry, 1992b).

Dogs

In a 90-day toxicity study, four male and four female Beagle dogs per dose group received flufenoxuron (purity 96.6%; batch no. 6001 = ST86/176) at a dietary concentration of 0, 500, 5000 or 50 000 ppm (equal to 0, 18, 163 and 1961 mg/kg bw per day for males and 0, 21, 182 and 2039 mg/kg bw per day for females, respectively). As a diet formulation error resulted in underdosing of the 5000 ppm animals for the first 2 weeks of treatment (1000 ppm instead of 5000 ppm), the duration of dietary test substance administration was extended from 13 to 15 weeks for all groups. Animals were checked daily for clinical signs. Feed consumption was measured daily, and body weights were measured weekly. Water consumption over a 24-hour period and ophthalmological and electrocardiographic examinations were performed pretreatment and in the week before termination. Haematology and clinical chemistry were performed pretrial and during weeks 9 and 15 of the treatment period. Methaemoglobin and sulfhaemoglobin were determined using the specific method of Evelyn and Malloy. Additional haematological assays were also undertaken during week 2 (all animals), week 4 (controls and mid-dose group) and week 12 (all animals) of the treatment period. All dogs were necropsied, and weights of brain, thyroid glands with parathyroids, heart, liver with gallbladder, adrenals, kidneys, testes and ovaries were recorded. Histology was performed on a large selection of organs, including bone marrow smears from all dogs. The fat samples were also analysed for test item levels.

Dietary concentrations were close to the target values, except that during the first 2 weeks of treatment, the 5000 ppm diet was formulated, in error, to be 1000 ppm.

In control group dogs, flufenoxuron concentrations in renal fat ranged from 11 to 69 ppm (investigation by the sponsor), suggesting that the control group dogs were contaminated with

flufenoxuron during either the treatment period or the sampling of the renal fat specimens at necropsy. Therefore, the validity of the data collected for the control group is questionable. In the treatment groups, a 10-fold increase in the concentration of flufenoxuron resulted in an approximately 2-fold increase in flufenoxuron residues in renal fat. At 50 000 ppm, pallor of the gums and/or sclera was observed in several male and female dogs during weeks 13, 14 and 15. Examination of feed and water consumption, body weight gain and ophthalmology revealed no treatment-related effects. Electrocardiograms revealed a slightly prolonged Q-T interval for all females at 50 000 ppm compared with controls (0.21 versus 0.18 second: 17% higher) and with the pretest values in these treated females (0.21 versus 0.17 second: 24% higher).

At week 9, increases in reticulocyte counts (statistically significant at 5000 and 50 000 ppm) were found in treated male dogs. In males of all treatment groups, mild but statistically significant reductions in erythrocyte counts, haemoglobin and haematocrit were observed (Table 7). These findings are indicative of mild anemia. At week 9 in females, a similar trend in these parameters was observed; however, the changes did not reach statistical significance. At week 9, mean corpuscular volume (MCV) was increased in males and females at 5000 and 50 000 ppm, and mean corpuscular haemoglobin concentration (MCHC) was decreased in males of all treatment groups. Haematological examination at weeks 12 and 15 also showed similar trends in these parameters in male and female dogs, although the effects were less clear and only occasionally statistically significant (Table 8).

Measurement of methaemoglobin revealed dose-related increases in both sexes, which were statistically significant at 5000 ppm and above after 9 and 15 weeks of treatment (at week 9 also at 500 ppm in females) (Table 9). The presence of methaemoglobin was accompanied by small, statistically significant and dose-related increases in sulfhaemoglobin in males at 5000 ppm and in both sexes at 50 000 ppm.

Table 7. Haematological findings in dogs (pretrial and week 9)

		Pretrial				Week 9			
Test parameter	**Sex**	**0 ppm**	**500 ppm**	**5 000 ppm[a]**	**50 000 ppm**	**0 ppm**	**500 ppm**	**5 000 ppm[a]**	**50 000 ppm**
RBCs (10^{12}/L)	M	6.33	6.03	5.99	6.00	6.75	5.97**	5.80***	5.49***
	F	6.05	6.51	6.19	6.27	6.89	6.27	6.05	5.90
Haemoglobin (g/dL)	M	14.3	13.3	13.4	13.6	15.8	13.5**	13.4***	13.1***
	F	13.7	14.8	13.9	14.4	15.6	14.7	14.2	14.3
Haematocrit (L/L)	M	0.434	0.401	0.407	0.415	0.467	0.417**	0.420**	0.410**
	F	0.411	0.442	0.418	0.431	0.472	0.447	0.441	0.437
MCV (fL)	M	71	68	70	71	69	69	72*	75**
	F	70	70	69	71	69	71	72*	74**
MCHC (g/dL)	M	31.4	31.4	31.3	31.3	34.7	33.3**	32.5***	32.6***
	F	31.8	31.8	31.7	32.0	33.6	33.7	32.9	33.3
Reticulocytes (%)	M	0.9	0.9	1.1	0.9	0.6	1.0	1.8**	1.6**
	F	1.3	1.3	1.5	1.5	0.8	1.1	2.0	1.6

F: female; M: male; MCHC: mean corpuscular haemoglobin concentration; MCV: mean corpuscular volume; ppm: parts per million; RBCs: red blood cells; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ (F-max test; ANOVA; Student's *t*-test)

[a] Dosed with 1000 ppm for the first 2 treatment weeks.

Source: Greenough, Goburdhun & Duffen (1987); Clark (1988a,b); Greenough (1991a)

Table 8. Haematological findings in dogs (weeks 12 and 15)

		Week 12									
		Historical controls[b]		Week 12				Week 15			
Test parameter	**Sex**	**Range**	**Mean**	**0 ppm**	**500 ppm**	**5 000 ppm[a]**	**50 000 ppm**	**0 ppm**	**500 ppm**	**5 000 ppm[a]**	**50 000 ppm**
RBCs (10^{12}/L)	M	5.66–7.73	6.70	6.60	6.30	6.14	5.45***	6.53	6.21	6.04	5.41**
	F	5.72–7.89	6.81	6.81	6.21	6.22	6.14	6.82	6.55	6.12	6.33
Haemoglobin (g/dL)	M	12.6–17.6	15.1	15.1	14.4	14.2	13.0***	15.0	13.9	13.7	12.9
	F	12.9–18.1	15.5	15.6	14.5	14.7	14.8	15.6	15.1	14.1	15.3
Haematocrit (L/L)	M	0.39–0.53	0.458	0.461	0.438	0.437	0.406	0.449	0.423	0.421	0.401
	F	0.39–0.55	0.468	0.470	0.442	0.448	0.452	0.470	0.454	0.432	0.460
MCV (fL)	M	63–73	68	69	69	70	74	68	67	68	73*
	F	64–72	68	68	70	71	73	68	68	70	71
MCHC (g/dL)	M	32.2–35.9	34.0	34.8	34.3	34.1	33.4**	34.1	33.5	33.2	33.0
	F	32.6–35.6	34.1	34.6	34.3	34.3	34.2	33.9	34.0	33.2*	34.0
Reticulocytes (%)	M	0.2–1.3	0.7	ND	ND	ND	ND	0.4	1.5	1.0	1.5
	F	0.2–1.8	0.8	ND	ND	ND	ND	1.0	0.7	1.0	1.3

F: female; M: male; MCHC: mean corpuscular haemoglobin concentration; MCV: mean corpuscular volume; ppm: parts per million; ND: not determined; RBCs: red blood cells; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ (F-max test; ANOVA; Student's *t*-test)

[a] Dosed with 1000 ppm for the first 2 treatment weeks.

[b] Historical control data from Inveresk Research International (included in the report, approximately 200 dogs of each sex).

Source: Greenough, Goburdhun & Duffen (1987); Clark (1988a,b); Greenough (1991a)

Table 9. Methaemoglobin and sulfhaemoglobin determinations in dogs (weeks 9 and 15)

		Week 9				Week 15			
Test parameter	**Sex**	**0 ppm**	**500 ppm**	**5 000 ppm[a]**	**50 000 ppm**	**0 ppm**	**500 ppm**	**5 000 ppm[a]**	**50 000 ppm**
Methaemoglobin (%)	M	0.80	1.07	1.42***	1.82***	0.61	0.99	1.46***	1.88***
	F	0.79	1.10**	1.30***	1.80***	0.65	0.87	1.23***	1.69***
Sulfhaemoglobin (%)	M	0.12	0.23	0.33*	0.46***	0.10	0.16	0.32***	0.39***
	F	0.28	0.14	0.23	0.35	0.12	0.15	0.25	0.43*

F: female; M: male; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ (F-max test; ANOVA; Student's *t*-test)

[a] Dosed with 1000 ppm for the first 2 treatment weeks.

Source: Greenough, Goburdhun & Duffen (1987); Clark (1988a,b); Greenough (1991a)

Statistically significant increases in serum cholesterol values were observed during week 9 in males and females at 50 000 ppm (4.7 and 3.9 mmol/L versus 3.4 and 3.3 mmol/L in controls, respectively) and during week 15 in males at 5000 and 50 000 ppm (4.6 and 5.0 mmol/L versus 3.0 mmol/L in controls). Absolute and relative liver weights were significantly increased in males treated at 5000 ppm (absolute +34%, relative +28%) and 50 000 ppm (absolute +28%, relative +32%). There were no treatment-related macroscopic changes. Histopathological examination revealed higher frequencies of hyperplasia in the bone marrow of all treated groups (Table 10). Yellow pigment deposition in the bone marrow, considered by the study author to be haemosiderin associated with red blood cell breakdown and production, was observed at 5000 ppm (females only) and 50 000 ppm (both sexes). These bone marrow changes were considered to reflect a compensatory response to the slight anaemia observed at all dietary concentrations. It is stated that bone marrow smears prepared from high-dose animals showed hyperplasia, predominance of red cell precursors and a shift in myeloid/erythroid ratio towards red cell production (no data presented in the report). Increased yellow pigment deposition was also observed in the proximal tubules of the kidney in two male dogs at 50 000 ppm. In the liver, increased Kupffer cell pigmentation, considered indicative of mild anaemia, was noted in male and female dogs at 5000 and 50 000 ppm and in one female at 500 ppm. Although these yellow pigments were never characterized, they were attributed to haemosiderin deposits (this was confirmed for bone marrow and kidneys by negative Prussian blue staining in the 52-week dog study; see below). Haemosiderin deposits were also detected in the spleen of one high-dose male and female.

A NOAEL could not be established. The lowest-observed-adverse-effect level (LOAEL) was 500 ppm (equal to 18 mg/kg bw per day), based on reductions in erythrocyte counts, haemoglobin and haematocrit in males, increased Kupffer cell pigmentation in the liver of one female and higher frequencies of hyperplasia in the bone marrow in males and females (Greenough, Goburdhun & Duffen, 1987; Clark, 1988a,b; Greenough, 1991a).

In a 1-year toxicity study, four male and four female Beagle dogs per dose group received flufenoxuron (purity 98%; batch no. 7005) in the diet at 0, 10, 100, 500 or 50 000 ppm (equal to 0, 0.36, 3.5, 19 and 1898 mg/kg bw per day for males and 0, 0.36, 3.8, 19 and 1879 mg/kg bw per day for females, respectively). Animals were examined daily for clinical signs of toxicity. Feed consumption was measured daily. Body weights were recorded weekly for the first 13 weeks and monthly thereafter. Water consumption over a 24-hour period was measured pretrial and during weeks 12, 25, 38 and 50 of the treatment period. Ophthalmological examinations were performed pretreatment and in week 51. Electrocardiographic measurements were carried out pretrial and during weeks 26 and 51 of the treatment period. Haematology, clinical chemistry and urine analysis were performed pretest and in weeks 27 and 52. At the same time, faecal samples were tested for occult blood. Additional haematological investigations were also undertaken in weeks 5, 13 and 40.

Table 10. Histopathological changes in dogs (week 15)

	Sex	0 ppm	500 ppm	5 000 ppm	50 000 ppm
Liver, increased Kupffer cell pigmentation	M	0/4	0/4	4/4*	4/4*
	F	0/4	1/4	3/4	4/4*
Kidney, increased yellow pigment deposition in proximal tubules	M	0/4	0/4	0/4	2/4
	F	0/4	0/4	0/4	0/4
Spleen, increased haemosiderin	M	0/4	0/4	0/4	1/4
	F	0/4	0/4	0/4	1/4
Bone marrow, hyperplasia	M	0/4	3/4	4/4*	4/4*
	F	0/4	2/4	4/4*	4/4*
Bone marrow, increased yellow pigment deposition	M	0/4	0/4	0/4	4/4*
	F	0/4	0/4	3/4	3/4

F: female; M: male; ppm: parts per million; *: $P < 0.05$ (Fisher exact test)

Source: Greenough, Goburdhun & Duffen (1987); Clark (1988a,b); Greenough (1991a)

Furthermore, blood samples were taken at weeks 13, 29 and 40 (high dose only) and at termination to monitor flufenoxuron levels in blood. All dogs were necropsied, and weights of spleen, liver with gallbladder, kidneys, testes/ovaries, heart, brain, thyroids with parathyroids, and adrenal glands were recorded. Histology was performed on a large selection of organs, including bone marrow smears from all dogs. The fat samples were also analysed for flufenoxuron levels.

There were no treatment-related effects on mortality, clinical signs, feed or water consumption, body weight gain or ophthalmological or electrocardiographic parameters. Haematological examinations showed mild anaemia in dogs at 500 and 50 000 ppm. At 5 weeks at 50 000 ppm, statistically significant reductions in haemoglobin (−16%), red blood cell count (−17%) and MCHC (−5%) in males and increases in number of reticulocytes (230–250%), methaemoglobin levels (160%) and sulfhaemoglobin levels (325–580%) in both sexes were observed. From 13 weeks onward, mild anaemia was also generally visible at 500 ppm. The effects were more prominent in males. Data from the 13- and 52-week examinations are presented in Table 11. Red blood cells often showed morphological alterations (i.e. polychromasia and Howell-Jolly bodies) in high-dose animals. In addition, platelet counts were statistically significantly increased in males at 50 000 ppm from week 13 onwards and at 500 ppm from week 27 onwards. Platelet counts of females were increased at the high-dose level, albeit not statistically significantly.

Methaemoglobin levels are presented in Table 12. The slight but statistically significant increase in females at 100 ppm was considered not toxicologically relevant.

There were no significant treatment-related adverse effects on clinical chemistry or urine analysis parameters or positive faecal occult blood tests. Absolute and relative liver weights adjusted for terminal body weights were statistically significantly increased in males at 500 ppm (19% and 18%, respectively) and 50 000 ppm (36% and 24%, respectively) when compared with controls. In females at 50 000 ppm, these increases in liver weights (24% and 21%, respectively) were not statistically significant. No treatment-related macroscopic changes were observed. Histopathological changes included bone marrow hyperplasia, increased cellularity, increased numbers of erythrocyte precursors (normoblasts) and increased numbers of macrophages in bone marrow for all dogs at 50 000 ppm. One female at 500 ppm also showed bone marrow hyperplasia and increased cellularity. These changes in bone marrow were indicative of compensatory erythropoiesis and elevated erythrocyte turnover at 500 and 50 000 ppm. This was accompanied by increased deposition of

Table 11. Haematological findings in dogs (weeks 13 and 52)

		Week 13					Week 52				
Test parameters	**Sex**	**0 ppm**	**10 ppm**	**100 ppm**	**500 ppm**	**50 000 ppm**	**0 ppm**	**10 ppm**	**100 ppm**	**500 ppm**	**50 000 ppm**
RBCs (10^{12}/L)	M	7.33	6.63*	7.19	6.59*	6.08**	7.61	7.13	7.50	6.80*	6.47**
	F	7.39	7.08	7.30	7.05	6.39**	6.91	7.07	7.19	7.16	6.35
Haemoglobin (g/dL)	M	16.6	15.6	16.2	15.5	14.5	17.3	16.5	16.9	15.6	15.1
	F	17.5	16.6	17.1	17.0	15.3**	16.1	16.7	16.5	17.3	15.0
Haematocrit (L/L)	M	0.476	0.448	0.468	0.446	0.427	0.502	0.487	0.494	0.467	0.456
	F	0.499	0.475	0.485	0.489	0.454	0.474	0.492	0.484	0.510	0.454
MCV (fL)	M	65	67	65	68	70**	65	67	65	68	69
	F	67	67	66	69	70*	68	69	67	70	71
MCHC (g/dL)	M	35.3	35.3	35.2	35.2	34.3**	34.3	33.8	34.0	33.3**	33.0**
	F	35.6	35.4	35.7	35.2	34.2***	34.0	33.8	34.1	33.7	32.8
Reticulocytes (%)	M	0.6	0.4	0.3	0.9	1.3**	0.8	0.8	0.6	0.9	1.7*
	F	0.3	0.3	0.6	0.8	1.6**	0.3	0.5	0.7	1.5	1.7
Platelets (10^{-9}/L)	M	233	233	290	289	425**	286	272	274	431**	449*
	F	265	259	210	242	380	347	341	264	247	463
Sulfhaemoglobin (%)	M	0.05	0.05	0.07	0.08	0.28**	0.14	0.13	0.10	0.22	0.30**
	F	0.03	0.05	0.06*	0.09**	0.38***	0.09	0.23	0.14	0.33**	0.41***

F: female; M: male; MCHC: mean corpuscular haemoglobin concentration; MCV: mean corpuscular volume; ppm: parts per million; RBCs: red blood cells; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ (F-max test; ANOVA; Student's *t*-test)

Source: Goburdhun, Parkinson & Greenough (1988); Greenough (1991b)

Table 12. Methaemoglobin levels in dogs

	Methaemoglobin level (% of total haemoglobin)									
	Males					Females				
	0 ppm	10 ppm	100 ppm	500 ppm	50 000 ppm	0 ppm	10 ppm	100 ppm	500 ppm	50 000 ppm
Pretrial	0.48	0.54	0.52	0.50	0.54	0.64	0.58	0.59	0.56	0.57
Week 5	0.75	0.58	0.69	0.90	1.96***	0.66	0.97	0.58	0.87	1.48***
Week 13	0.73	0.77	0.76	0.99	1.52**	0.63	0.88	0.97	0.99	1.61
Week 27	0.54	0.63	0.53	0.86*	1.60***	0.56	0.68	0.68	0.84	1.96***
Week 40	0.75	0.71	0.75	1.05	1.87***	0.69	0.75	0.80**	1.53***	2.18***
Week 52	1.16	1.27	1.04	1.14	1.95	0.71	1.08	0.68	0.98	2.39***

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ (F-max test; ANOVA; Student's *t*-test)

Source: Goburdhun, Parkinson & Greenough (1988); Greenough (1991b)

yellow/brown pigment (not haemosiderin, as it was free of Fe^{3+} when stained) in the bone marrow of all dogs in the 50 000 ppm group. Brown pigmentation (free of Fe^{3+}) was also noted in the proximal tubular cells of the kidneys in four males and one female at 50 000 ppm and in one female at 500 ppm. Fat-like vacuoles in hepatocytes were found in all dogs receiving 50 000 ppm. In addition, increased Kupffer cell pigmentation, which was identified as haemosiderin, was observed in all 50 000 ppm animals and in three dogs (two females and one male) at 500 ppm. Increased haemosiderin deposition was also noted in the spleens of two males and three females at 50 000 ppm and one female at 500 ppm (with associated extramedullary haematopoiesis). The histopathological findings in the bone marrow, kidney and liver were likely related to the anaemia observed at 50 000 ppm and to a lesser degree at 500 ppm. Haemosiderin deposition in the spleen was noted for one male at 100 ppm and for one male at 10 ppm. The study pathologist concluded that the isolated findings of slightly increased splenic haemosiderin in one male dog in both the 100 and 10 ppm groups were not treatment related, because of the lack of a dose–response relationship and consistent corresponding findings of anaemia at these dietary concentrations. With increasing dose level, an increase in flufenoxuron concentration in fat was observed (from about 5 mg/kg at the low dose to about 900 mg/kg at the high dose). There was no proportionality. A low level of flufenoxuron was also detected in the fat of control group animals.

The NOAEL was 100 ppm (equal to 3.5 mg/kg bw per day), based on mild anaemia (reductions in red blood cell count and MCHC), increased absolute and relative liver weights and increased platelet count in males; increased sulfhaemoglobin levels, bone marrow hyperplasia and increased bone marrow cellularity, brown pigmentation (free of Fe^{3+}) in the proximal tubular cells of the kidneys and increased haemosiderin deposition with associated extramedullary haematopoiesis in the spleen in one or more females; and increased methaemoglobin levels and increased Kupffer cell pigmentation identified as haemosiderin in both sexes at 500 ppm (equal to 19 mg/kg bw per day) (Goburdhun, Parkinson & Greenough, 1988; Greenough, 1991b).

2.3 Long-term studies of toxicity and carcinogenicity

Mice

In a 2-year dietary carcinogenicity study, performed according to OECD Test Guideline 451, flufenoxuron (purity 98.1%; batch nos 7003 and 7033) was administered to groups of 50 male and 50 female B6C3F1 mice at 0, 100, 1000 or 10 000 ppm (equal to 0, 15.3, 152 and 1592 mg/kg bw per day for males and 0, 17.4, 187 and 1890 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. Body weight was recorded weekly during weeks 1–14 and biweekly thereafter. Feed consumption was recorded weekly. Feed conversion efficiency was calculated every week for the first 14 weeks. Blood samples collected at weeks 50, 76 and 102 and blood smears of control and high-dose mice were examined. Animals found dead, killed prematurely or killed at the end of the treatment period were weighed and necropsied. Organ weights (adrenals, brain, heart, liver, kidneys, lungs, spleen and ovaries/testes) were recorded. A wide range of tissues was examined microscopically from all control and high-dose animals. In other groups, only gross lesions (all animals) and kidneys, liver and lungs (after scheduled death only) were examined.

Mortality at 24 months was not affected by treatment in males, whereas it tended to be higher in females at 1000 or 10 000 ppm (36% mortality in both groups versus 22% mortality in controls); however, no statistical significance was reached. Pathological examination revealed no dose-related cause of death. The frequency of animals bearing palpable masses was not significantly dose related in either sex. Body weight development was unaffected in males. In high-dose females at the end of the study, a statistically significant reduction in body weight gain of about 16% was observed. Feed intake and feed conversion efficiency were not affected by treatment. Blood smear examination did not reveal an effect of treatment on relative percentages of white blood cells or on blood cell morphology. No effect of treatment on organ weight was observed. Macroscopic examination showed a higher frequency of uterine distension by a fluid in females treated at 1000 or 10 000 ppm, whereas uterine non-fluid distension was inversely dose related. When both types of uterine distension are combined, the incidences were not dose related. In addition, no change in the type or frequency of any uterine lesion was observed at histology. The incidence of neoplastic findings was comparable between all groups. Occasionally observed non-neoplastic microscopic changes were considered chance findings and not treatment related.

The NOAEL was 1000 ppm (equal to 187 mg/kg bw per day), based on decreased body weight gain in females at 10 000 ppm (equal to 1890 mg/kg bw per day). Flufenoxuron was not carcinogenic in B6C3F1 mice under the conditions of the study (Broadmeadow, 1996).

In a 2-year dietary carcinogenicity study, performed according to OECD Test Guideline 451, flufenoxuron (purity 97.6–98.1%; batch no. WB16867 = 7003 = ST87/002) was administered to groups of 50 male and 50 female C57/C3H F_1 hybrid mice (= B6C3F1) at 0, 500, 5000 or 50 000 ppm (equal to 0, 56, 559 and 7356 mg/kg bw per day for males and 0, 73, 739 and 7780 mg/kg bw per day for females, respectively). Additional groups of 10 male and 10 female mice were treated for 12 months for interim examination. The mice were checked daily for mortality and clinical signs. Body weight and feed consumption were recorded weekly during weeks 1–13 and once every 4 weeks thereafter. At the same time points, all animals were palpated for masses. Blood samples were collected at 12, 18 and 24 months for blood smear examination. Animals found dead, killed prematurely or killed at the end of the treatment period were weighed and necropsied. Organ weights (brain, heart, liver, kidneys, spleen and testes) were recorded. A wide range of tissues was examined microscopically from all control and high-dose animals. In other groups, only gross lesions (all animals) and kidneys, liver and lungs (after scheduled death only) were examined.

Mortality tended to be higher in mid- and high-dose females (15, 15, 19 and 20 at 0, 500, 5000 and 50 000 ppm, respectively), but the increases were not statistically significant. Feed spillage was higher in treated groups than in controls. In high-dose females, a slightly higher incidence of eye damage, caused by abrasion on the feed hopper, was observed. This was considered to be secondary to the increased incidence of feed spillage, as these animals may have been pushing their heads further down into the feed hopper. An increased incidence of lordotic episodes was observed in females at 5000 and 50 000 ppm (20/50 and 36/50 versus 8/50 in controls) and tended to be present in high-dose males (45/50 versus 32/50 in controls). In addition, at termination, a slight increase of focal alopecia in females at 5000 ppm was observed, which was considered to result from hair plucking. Experience from the test laboratory indicates that the lordotic behaviour and hair plucking are related to stress.

Terminal body weight gain was statistically significantly lower in high-dose males (−21%) and females (−30%) and in females at 5000 ppm (−14%). Feed intake was not affected. It is noted that due to feed spillage, the evaluation of feed consumption was difficult. There were no treatment-related haematological effects. Occasionally observed statistically significant findings were not dose dependent and/or not consistently found during the course of the treatment period.

At 24 months, spleen weight relative to brain weight was higher in high-dose males (+24%) and females (+23%). Liver weight relative to brain weight was higher in high-dose males (+46%) and females (+53%) and in males at 5000 ppm (+11%). At 12 months in the high-dose females, increased weights of spleen (+46%) and liver (+18%) relative to brain weight were observed. Macroscopic examination revealed no treatment-related findings at the interim kill. At termination, examination of the livers at 50 000 ppm revealed increased incidences of enlargement, exaggerated lobular pattern, pallor, pale areas or foci (all in both sexes) or dark areas or foci (in males only) compared with controls (see Table 13). A higher incidence of ulcer of the forestomach was noted in high-dose males (9 versus 0 in controls). The incidence of seminal vesicle enlargement in high-dose males (2) was lower than in controls (15).

Table 13. Macroscopic changes in the liver of mice at 24 months

	Incidence of liver finding							
	Males				**Females**			
Liver finding	**0 ppm**	**500 ppm**	**5 000 ppm**	**50 000 ppm**	**0 ppm**	**500 ppm**	**5 000 ppm**	**50 000 ppm**
Enlargement	1	2	3	11	4	6	4	18
Exaggerated lobular pattern	1	0	1	33	0	1	0	27
Pallor	11	7	7	33	13	7	13	44
Pale areas/foci	10	17	24	19	7	10	4	13
Dark areas/foci	4	2	5	14	11	10	5	13

ppm: parts per million

Source: Esdaile (1990a)

At the interim kill at 12 months, Kupffer cell aggregates were observed in 9/10 animals in both sexes at 50 000 ppm, versus 0/10 in male and female control animals. Individual cell necrosis and hypertrophy of hepatocytes were noted in single animals at this dose. At 24 months, non-neoplastic changes were predominantly observed in liver and spleen of both sexes at 50 000 ppm and in liver of females at 5000 ppm (Table 14). The inflammation and cell necrosis in the liver are indicative of chronic liver damage. The reduced incidences of splenic lymphoid hyperplasia and seminal vesicle enlargement in high-dose males are not considered adverse.

In terminal male animals, the total incidences of primary tumours (high dose), the number of animals with multiple tumours and malignant metastatic tumours (middle and high doses) and the number of malignant tumours (all doses) tended to be higher than in controls, although no statistical significance was found (see Table 15). This apparent increase in tumour incidence was due to increases in tumour frequency in the liver of treated males. The frequency of hepatocellular carcinoma was considerably higher in all treated male groups (at least 5-fold difference), but not in females. Also, the total incidence of hepatocellular tumours (adenoma and carcinoma combined) in these groups of male mice was above the control value, although not statistically significantly. However, the incidences of carcinomas in the livers of treated mice were not dose dependent and are within the historical control range for B6C3F1 mice (21.1% with a range of 8–32% based on 36 dietary

Table 14. Selected non-neoplastic findings of mice fed flufenoxuron for 24 months

	Incidence of non-neoplastic finding							
	Males				Females			
	0 ppm	500 ppm	5 000 ppm	50 000 ppm	0 ppm	500 ppm	5 000 ppm	50 000 ppm
Liver (no. examined)	(50)	(50)	(50)	(50)	(50)	(50)	(50)	(50)
Inflammation	2	3	4	12**	6	5	9	10
Cell necrosis, individual	2	1	1	42***	0	0	1	38***
Kupffer cell aggregates	0	0	2	47***	0	1	11***	44***
Hypertrophy, hepatocellular	0	0	1	31***	0	0	0	35***
Spleen (no. examined)	(50)	(50)	(50)	(49)	(50)	(50)	(50)	(50)
Macrophages, syncytial	0	0	0	18***	0	0	0	10***
Hyperplasia, lymphoid	10	6	8	2*	4	7	9	7
Stomach, glandular (no. examined)	(50)	(43)	(38)	(47)	(49)	(48)	(49)	(48)
Inflammation	0	1	0	5*	0	0	0	1
Seminal vesicle (no. examined)	(50)	(50)	(49)	(49)	NA	NA	NA	NA
Enlargement	23	13	19	3***	–	–	–	–

NA: not applicable; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$

Source: Esdaile (1990a)

administration studies: Haseman et al., 1985; 20.4% with a range of 10–40% based on 19 studies: NTP, 1999). It is noted that in the present study, the controls showed an unusually low incidence (6%). This indicates that the apparent increased incidence in hepatocellular tumours in treated male mice in this study is due to the low incidence in control males. Splenic haemangiosarcomas occurred more frequently in high-dose females, but not in males. However, as this finding was not accompanied by an increase in angiomas, it was not considered treatment related. The incidence of all other tumours was comparable between control and treated groups.

The NOAEL was 500 ppm (equal to 56 mg/kg bw per day), based on an increased incidence of lordotic episodes, decreased body weight gain, increased Kupffer cell aggregates in females and increased liver weight relative to brain weight in males at 5000 ppm (equal to 559 mg/kg bw per day). Flufenoxuron was not carcinogenic in B6C3F1 mice under the conditions of the study (Esdaile, 1990a, 1991d; Berry, 1992c; Finn, 1993).

Rats

In a 2-year dietary toxicity study, flufenoxuron (purity 97.6–98.1%; batch no. 7003 = ST87/002) was administered to groups of 30 male and 30 female Fischer F344/DuCrj (SPF) rats at 1, 5, 50, 500, 5000 or 50 000 ppm (equal to 0.044, 0.23, 2.2, 22, 230 and 2470 mg/kg bw per day for males and 0.055, 0.28, 2.8, 28, 300 and 3210 mg/kg bw per day for females, respectively). A control group of 60 males and 60 females was fed basal diet. Ten rats of each sex per treatment group and 20 rats of each sex in the control group were designated for interim kill after 52 weeks of treatment. The rats were checked daily for mortality and clinical signs. Group feed consumption and individual body weights were recorded weekly for the first 13 weeks and once every 4 weeks thereafter. At 3, 6, 12, 18 and 24 months, haematology, measurement of non-cyanide binding haemoglobin and more specifically methaemoglobin levels (using Evelyn and Malloy's specific method), blood smear examination and clinical chemistry (not at 3 months) were performed in the rats designated for the 2-

year treatment. Urine analysis was performed on 10 animals of each sex per dose level (including controls) 1–3 weeks before each blood sampling, except for the 3-month sampling. All animals found dead, killed prematurely or killed at termination at 12 or 24 months were necropsied. Weights of adrenals, brain, liver, kidneys, spleen, heart and ovaries/testes of animals after scheduled death were recorded. Histological examinations were performed on a wide range of organs and tissues of all rats in the control and 50 000 ppm groups and of dead or moribund animals. Kidneys, liver, lungs and spleen were also examined microscopically in all other animals. Bone marrow smears prepared from high-dose and control interim kill rats were examined.

Table 15. Incidence of neoplastic findings in mice fed flufenoxuron for 24 months

	Incidence of neoplastic findings							
	Males				**Females**			
	0 ppm	**500 ppm**	**5 000 ppm**	**50 000 ppm**	**0 ppm**	**500 ppm**	**5 000 ppm**	**50 000 ppm**
Total no. of animals per group	50	50	50	50	50	50	50	50
Overview								
Total no. of primary tumours	50	52	70	64	70	60	42	74
Total no. of animals with tumours	33	37	45	38	44	37	31	42
Total no. of animals with multiple tumours	14	13	22	21	19	16	11	24
Total no. of benign tumours	30	16	33	25	33	30	16	31
Total no. of malignant tumours	20	36	37	39	37	30	26	43
Total no. of malignant tumours with metastasis	12	10	16	17	32	16	16	32
Individual tumours								
Liver	(50)	(50)	(50)	(50)	(50)	(50)	(50)	(50)
Hepatocellular adenoma	15	3	11	10	10	6	2	13
Hepatocellular carcinoma	3	19***	15**	15**,$	3	9*	7	5
Hepatocellular tumours, total incidence	18	22	26	25	13	15	9	18
Cholangioma	0	0	0	0	0	0	1	0
Haemangiosarcoma	2	1	0	5	0	0	0	1
Haemangioma	0	0	0	2	0	0	0	0
Spleen	(50)	(50)	(50)	(49)	(50)	(50)	(50)	(50)
Haemangioma	0	0	0	0	0	0	1	0
Haemangiosarcoma	4	3	0	3	0	1	1	7**,$$
Total vascular tumours (haemangioma + haemangiosarcoma)	8	6	1	11	1	2	3	11

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ (Fisher's exact test); $: $P < 0.05$: $$: $P < 0.01$ (trend analysis)

Source: Esdaile (1990a); Finn (1993)

There were no effects of treatment on mortality or clinical signs. Body weight gain was statistically significantly reduced at 5000 and 50 000 ppm in males (up to 10% and 13%, respectively) and females (up to 12% and 14%, respectively) from about week 28 to the end of the study. In these treatment groups, feed consumption was slightly increased compared with controls. Haematological investigations revealed toxicologically relevant changes in high-dose females, which showed lower red blood cell counts and haemoglobin levels (both up to −8%) at 3 and 12 months and lower haematocrit at 3 months (−7%). In males treated at 5000 and 50 000 ppm, higher red blood cells, haemoglobin and haematocrit at 24 months were considered to reflect a healthier condition of old high-dose males. Methaemoglobin levels were not affected by treatment. A small increase in non-cyanide binding haemoglobin in both sexes treated at 5000 and 50 000 ppm (up to +29% at 24 months) was considered to reflect sulfhaemoglobin. No toxicologically relevant changes in white blood cell parameters were observed. At 12 months, bone marrow smears showed no toxicologically relevant findings. Small increases in erythropoietic activity observed in bone marrow smears of high-dose males and females were consistent with the mild anaemia observed in these animals. As these effects were small, they were considered not toxicologically relevant. Triglyceride levels were lower in 5000 or 50 000 ppm treated animals, which is consistent with the lower body weight. Other changes in clinical chemistry parameters were small or not dose dependent or not consistent over time or between sexes and were not considered to be toxicologically relevant. Urine analysis showed an increase in glucose levels at 24 months in males treated at 5000 or 50 000 ppm (0.6 and 0.8 mmol/L, respectively, compared with 0.2 mmol/L in controls), but not in females. As no increase was observed at other time points and as other urinary parameters and blood glucose levels were not affected, the increased urinary glucose levels were not considered toxicologically relevant.

At the interim kill at 5000 and 50 000 ppm, slight increases were observed in relative heart weight in males (+5% and +10%, respectively) and females (+8% and +9%, respectively). In addition, in females at 50 000 ppm, increases in relative weights of spleen (+22%), liver (+12%) and adrenals (+11%) were observed. Increases in relative weights of these organs in females at 5000 ppm were small (≤ 8%). At the 24-month kill, only lower relative spleen weights in males at 5000 and 50 000 ppm (−34% and −35%, respectively) and higher relative adrenal weights in high-dose females (+11%) were observed. The reduced relative spleen weights were due to a lower incidence and severity of enlarged spleens compared with controls. The small increase in relative adrenal weight in females was not accompanied by histological changes. None of the organ weight differences observed at 24 months were considered to be of toxicological relevance. Necropsy revealed no treatment-related adverse effects. At the interim kill, rats at 500 ppm and higher had increased incidences of basophilic parenchymal foci in the liver. However, as these incidences were comparable between all groups at 24 months and as no increased frequency of liver neoplasms was observed, this finding is not considered toxicologically relevant. At 24 months in males, a 2-fold increased incidence of zonal (periportal) fatty vacuolation was observed at 50–50 000 ppm (not dose dependent, statistically significant at 50, 500 and 5000 ppm, but not at 50 000 ppm). This increase was not observed in females. The incidence of lymphocyte infiltration of the liver was 4 times higher in high-dose females (6/20) than in controls (3/40). There were no treatment-related increases in the incidence of neoplastic lesions.

The NOAEL was 500 ppm (equal to 22 mg/kg bw per day), based on decreased body weight gain, a small increase in non-cyanide binding haemoglobin, considered to reflect sulfhaemoglobin, and decreased triglyceride levels in both sexes at 5000 ppm (equal to 230 mg/kg bw per day). Flufenoxuron was not carcinogenic in Fischer F344/DuCrj (SPF) rats under the conditions of the study (Esdaile, 1990b,c; Berry, 1992d).

In a 2-year dietary toxicity study, flufenoxuron (purity 97.6–98.1%; batch no. WB16867 = 7003 = ST87/002) was administered to groups of 50 male and 50 female Fischer F344/DuCrj (SPF) rats at 0, 500, 5000 or 50 000 ppm (equal to 0, 21.6, 218 and 2290 mg/kg bw per day for males and 0, 25.9, 276 and 2901 mg/kg bw per day for females, respectively). The rats were checked daily for mortality and clinical signs. Group feed consumption and individual body weights were recorded

weekly for the first 13 weeks and once every 4 weeks thereafter. At the same time points, the rats were palpated for masses. Ophthalmological examinations were performed on 20 rats of each sex in each group prior to the start of the study and in control and high-dose groups at the end of the treatment. At 12, 18 and 24 months, blood was sampled for blood smear examination. All animals found dead, killed prematurely or killed at termination were necropsied. Weights of adrenals, brain, liver, kidneys, spleen, heart and ovaries/testes of animals after scheduled death were recorded. Histological examinations were performed on a wide range of organs and tissues of all rats in the control and 50 000 ppm groups and on dead or moribund animals. Kidneys, liver, lungs and gross lesions in all other animals were also examined microscopically.

No adverse effects of treatment on mortality or clinical signs were observed. Body weight gain was statistically significantly reduced throughout most of the study in females at 50 000 ppm (up to 14%). In females at 5000 ppm, body weight gain reduction during the study was small (up to 9%), but statistically significant. In males at 5000 and 50 000 ppm, feed consumption was slightly increased compared with controls, which is probably related to the lower caloric value of the diet. A dose-related lower spleen weight in males was actually correlated with a decreased incidence of spleen enlargement corresponding to splenic mononuclear cell leukaemia. Examination of blood smears revealed no toxicologically relevant changes. The incidence of leukaemia was inversely dose related (11, 3, 1 and 1 in males and 8, 3, 1 and 0 in females at 0, 500, 5000 and 50 000 ppm, respectively). Correspondingly, in males, a dose-relatedly lower spleen weight (−13%, −28% and −31% at 500, 5000 and 50 000 ppm, respectively) was correlated with a decreased incidence of spleen enlargement related to splenic mononuclear cell leukaemia. In high-dose females, an increase in adrenal weights (13%) was not accompanied by histopathological changes. At necropsy, an exaggerated lobular pattern of the liver was observed in all treated groups of males, which was attributed to the high frequency of spongiosis hepatitis in control males. Histopathology revealed an increased incidence of basophilic foci observed in the livers of high-dose males. However, the study pathologist suggested that it was possible that the higher incidence of mononuclear cell leukaemia, which infiltrates the liver in control males, could have obscured the diagnosis of this focal change in these controls. Accordingly, for the subset of male rats without mononuclear cell leukaemia, basophilic foci were not observed at significantly different frequencies between groups.

There were no treatment-related increases in the incidence of any neoplastic lesion for treated males or females. Actually, the incidences of malignant primary tumours in males, of multiple primary benign tumours in females and of lymphatic mononuclear cell leukaemia in both sexes were higher in controls than in treated animals. In high-dose females, the incidence of mammary fibroadenoma was also statistically significantly reduced.

The NOAEL was 500 ppm (equal to 25.9 mg/kg bw per day), based on reduced body weight gain in females at 5000 ppm (equal to 276 mg/kg bw per day). Flufenoxuron was not carcinogenic in Fischer F344/DuCrj (SPF) rats under the conditions of the study (Esdaile, 1990d; Basford, 1991; Berry, 1992e).

2.4 Genotoxicity

Flufenoxuron was tested for genotoxicity in a range of assays (summarized in Table 16). Several of these studies, although indicating no genotoxic effect of flufenoxuron, were not entirely compliant with the OECD test guidelines. This is indicated in Table 16, and the deviations from the guidelines are described in the footnotes. An in vitro chromosomal aberration study in human lymphocytes (McEnaney, 1992) and an in vivo micronucleus test in the rat (Allen, Proudlock & Brooker, 1986) have not been considered in this evaluation because of their unacceptable deviations from OECD test guidelines. In one in vitro chromosomal aberration study in Chinese hamster ovary cells, flufenoxuron induced increased numbers of chromosomal aberrations (excluding gaps) in the presence of S9 mix. In a repeat study, a positive effect was again observed when cells were exposed to flufenoxuron in the presence of S9 mix. However, in the presence of phosphate-buffered glutathione at a concentration of 5 mmol/L, the extent of the chromosomal damage was substantially

Table 16. Overview of genotoxicity tests with flufenoxuron[a]

	Test object	Concentration	Purity (%)	Results	Reference
In vitro					
Gene mutations	*Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537; *Escherichia coli* WP2 uvrA pKM101	31.25–4 000 µg/plate (±S9)	99	Negative Study not fully adherent to OECD guideline	Brooks & Wiggins (1986); Brooks (1991)[b]
Gene mutations	*Saccharomyces cerevisiae*	0.01–1 000 µg/mL (±S9)	99	Negative Study not fully adherent to OECD guideline	Brooks & Wiggins (1986)[c]
Gene mutations	*S. typhimurium* strains TA98, TA100, TA1535, TA1537; *E. coli* WP2 uvrA	3–5 000 µg/plate (±S9)	97.1	Negative	Sokolowski (2007)[d]
Gene mutations	V79 Chinese hamster cells, HPRT test	50–1 350 µg/mL (±S9)	99	Negative Study not fully adherent to OECD guideline	Clare & Wiggins (1986); Brooks (1991)[e]
Gene mutations	V79 Chinese hamster cells, HPRT test	6–1 600 µg/mL (±S9)	97.1	Negative	Wollny (2007)[f]
Chromosomal aberrations	CHO cells	15–150 µg/mL (±S9)	96.6	Positive in the presence of S9 Increases in chromosomal aberrations at concentrations of 15–150 µg/mL, statistically significant at 75 µg/mL	Meyer (1987, 1991a)[g]
Chromosomal aberrations	CHO cells	150 µg/mL (±S9, ± 5 mmol/L glutathione)	96.6	Positive in the presence of S9 Statistically significant increase in chromosomal aberrations at 150 µg/mL Negative in the presence of S9 and glutathione at a concentration of 5 mmol/L	Meyer (1988a, 1991b)[h]
Chromosomal aberrations	Rat epithelial RL4 cells	45–450 µg/mL (−S9) 16–160 µg/mL (+S9)	96.6	Negative Study not fully adherent to OECD guideline	Meyer (1988b, 1991c)[i]
In vivo					
Micronucleus formation	ICR mice bone marrow	Two intra-peritoneal doses of 500, 1 000 or 2 000 mg/kg bw (24 h interval)	98.2	Negative	Nishitomi (1993)[j]

Table 16 (continued)

	Test object	Concentration	Purity (%)	Results	Reference
Chromosomal aberrations	Wistar Hanlbm rat bone marrow	One gavage dose of 500, 1 000 or 2 000 mg/kg bw	97.1	Negative	Honarvar (2007)[k]
Unscheduled DNA synthesis	Fischer 344 rat liver cells	One gavage dose of 188, 375, 750 or 1 500 mg/kg bw	97.4	Negative	Cifone (1991)[l]
Replicative DNA synthesis	Fischer 344 rat liver cells	Two gavage dose of 2 000 or 4 000 mg/kg bw (24 h interval)	98.2	Negative	Miyagawa (1992)[m]

ADME: absorption, distribution, metabolism and excretion; bw: body weight; CHO: Chinese hamster ovary; DMSO: dimethyl sulfoxide; DNA: deoxyribonucleic acid; HPRT: hypoxanthine–guanine phosphoribosyltransferase; OECD: Organisation for Economic Co-operation and Development; RDS: replicative DNA synthesis; S9: 9000 × *g* supernatant fraction from rat liver homogenate

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

[b] Batch no. 9 = ST85/113. The dose levels were selected on the basis of cytotoxicity and solubility. No positive results were observed. However, it is noted that for four *Salmonella* strains (TA98, TA100, TA1537, TA1538), the positive control substances need metabolic activation, and therefore these substances are not suitable to use in the plates without S9. Normally, positive control substances that do not require metabolic activation are used.

[c] Batch no. 9 = ST85/113. Study design resembles OECD Test Guideline 481. Liquid suspension cultures of log phase yeasts received the test material in DMSO at a dose level of 0.01, 0.1, 0.25, 0.5 or 1.0 mg/mL with and without S9 metabolic activation. Flufenoxuron did not have any effect on cell viability, and the results obtained from both trials showed no increase in the frequency of mitotic gene conversion in the presence or absence of metabolic activation. The positive control materials induced mitotic gene conversion, indicating that the test organism and metabolic activation system were functioning properly.

[d] Batch no. V5-7211L28. Performed according to OECD Test Guideline 471. Flufenoxuron was dissolved in DMSO and tested at 3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate. Toxicity was observed without S9 in the TA1535 strain at 1000 and 5000 mg/plate, in strain TA1537 at 2500 µg/plate and in strain TA98 at 1000–5000 µg/plate. Precipitation was seen at concentrations of 333 µg/plate and above. No positive results were observed.

[e] Batch no. 10B = ST85/159. Study design resembles OECD Test Guideline 476. Concentrations were based on the results of preliminary cytotoxicity assays in which the highest tested concentration of 1000 µg/mL reduced the cloning efficiency to 19% with metabolic activation and to 48% without metabolic activation. The choice of the high dose of 1350 µg/mL for the definitive mutagenicity assay was therefore validated for the +S9 condition but not for the –S9 condition. In the main tests, the highest concentration did not induce any significant cytotoxicity in test cells. No statistically significant increases in gene mutations attributable to flufenoxuron were observed at concentrations where neither cytotoxicity nor flufenoxuron precipitation was observed.

[f] Batch no. V5-7211L28. Performed in accordance with OECD Test Guideline 476. Precipitation of test material was seen in all cultures above 25 µg/mL. There was no evidence of an increase in cell mutation up to 1600 µg/mL.

[g] Batch no. 6001 = ST86/176. Study design resembles OECD Test Guideline 473. Doses used in this assay were based on results from cytotoxicity assays with flufenoxuron conducted at concentrations of up to 250 µg/mL without S9 mix and 300 µg/mL with S9 mix. In the preliminary test, a 50% inhibition of cell growth was observed with and without metabolic activation, at a flufenoxuron concentration of 150 µg/mL. Therefore, this concentration was chosen as the highest concentration for the main test. However, in the main test, only 24% lower mitotic indices were observed at the highest concentration with S9 mix. In the absence of S9 mix, there was no evidence of induced chromosomal damage in any treatment group. In the presence of S9 mix, cultures treated with flufenoxuron at 75 µg/mL showed an increase in chromosomal aberrations (excluding gaps) at the 24-hour harvest. However, the increases in chromosomal aberrations at 15 and 150 µg/mL were not statistically significant. The study author concluded that the data indicate that flufenoxuron is clastogenic in the presence of S9 mix.

[h] Batch no. 6001 = ST86/176. Study design resembles OECD Test Guideline 473. Flufenoxuron was tested for its potential to induce chromosomal aberrations in CHO cells in the presence of 5 mmol/L glutathione. Glutathione plays a major role in the detoxification of xenobiotics, including carcinogens. A single concentration of 150 µg/mL was used in this study, based on a 50% inhibition in cell growth observed at this dose in the previous study, but which was not validated as a

toxic concentration due to the lack of a dose–response relationship even at a 2 times higher concentration. When glutathione was not supplemented with the culture medium, flufenoxuron induced statistically significant increases in the number of cells with chromosomal aberrations (gaps included or not) in the presence of S9 mix compared with the negative controls. In the presence of phosphate-buffered glutathione at a concentration of 5 mmol/L, the extent of the chromosomal damage was substantially reduced to levels comparable with those in the negative controls. The positive control (cyclophosphamide) showed significant increases in chromosomal aberrations, validating the test system. The results of this study suggest that flufenoxuron is clastogenic to CHO cells in the presence of S9 mix activation. Glutathione at a concentration of 5 mmol/L displays a scavenger effect on this clastogenic potential. Although it is stated that 5 mmol/L is a physiological glutathione concentration, no reference is given. The Meeting noted that physiological glutathione levels are about 0.5–1 mmol/L in human whole blood, about 1–2 mmol/L in red blood cells and about 0.005 mmol/L in plasma. Intracellular levels in other cells range from 0.5 to 10 mmol/L, whereas extracellular levels in animals are 1–3 orders of magnitude lower. Thus, the 5 mmol/L glutathione concentration in the medium appears to be some orders of magnitude higher than the concentration in extracellular fluid or plasma. Therefore, the Meeting concluded that the glutathione concentration used in the study with CHO cells is well above the physiological glutathione concentration, and consequently the role of the glutathione supplement in this assay has not been clarified.

[i] Batch no. 6001 = ST86/176. Study design resembles OECD Test Guideline 473. Cultured rat epithelial RL4 cells were exposed to flufenoxuron for 3 hours in the presence or absence of S9 metabolic activation and harvested for chromosome preparations at 8, 12 and 24 hours after the start of the treatment. The doses evaluated in the definitive chromosomal aberration assay were 45, 225 and 450 µg/mL in the absence of metabolic activation and 16, 80 and 160 µg/mL in the presence of metabolic activation. The total cell counts were reduced to 45.9% of solvent control values at a flufenoxuron concentration of 450 µg/mL in the absence of S9 mix and reduced to 61.7% of solvent control values at a test substance concentration of 160 µg/mL in the presence of S9 mix. It is noted that the reduction in cell count in the presence of S9 mix is not low enough according to OECD Test Guideline 473. Flufenoxuron did not induce any increases in structural chromosomal aberrations at any dose level, at any harvest time, when tested up to 450 µg/mL without activation and up to 160 µg/mL with S9 mix. As the maximal tested concentrations were not toxic enough (mitotic indices were decreased by only 21% without S9 mix and by 14% with S9 mix compared with solvent controls), induced no precipitation and were below 5 mg/mL, this study is of limited value.

[j] Batch no. 16/91-1. Study design resembles OECD Test Guideline 474. Vehicle was corn oil. Cyclophosphamide at 40 mg/kg bw was used as a positive control. No mortalities or notable clinical signs of toxicity were observed in any animal. No statistically significant increases in polychromatic to normochromatic erythrocyte ratios were observed, so there was no relevant cytotoxicity with flufenoxuron treatment. The number of micronucleated polychromatic erythrocytes was similar between any test item–treated group and the olive oil control group. Data from toxicokinetic studies indicate that flufenoxuron reaches the bone marrow (see section 1.1).

[k] Batch no. V5-7211L28. Performed in accordance with OECD Test Guideline 475. Six male and six female rats per dose and per collection time were treated by gavage with flufenoxuron at 500, 1000 or 2000 mg/kg bw. At all doses, reduction of spontaneous activity, hunched posture and ruffled fur were observed. Bone marrow cells were collected for chromosomal aberration analysis at 24 hours (all doses) and 48 hours (only the high-dose group) after the treatment. No increase in chromosomal aberrations in rat bone marrow cells following oral dosing at up to 2000 mg/kg bw was found. The test item was formulated in corn oil. The mitotic indices were not relevantly reduced after treatment with flufenoxuron. The author considered this to be an indication that the test item did not have cytotoxic effects in the bone marrow. Exposure of bone marrow was confirmed in the ADME studies (see section 1.1).

[l] Batch no. 7016 = ST87/129. Study design resembles OECD Test Guideline 486. Three rats per dose group were given flufenoxuron at 0 (corn oil vehicle control), 188, 375, 750 or 1500 mg/kg bw. Three rats received 10 mg/kg bw of aqueous *N*,*N'*-dimethylnitrosamine solution by intraperitoneal injection as a positive control. None of the treatments with flufenoxuron caused nuclear labelling that was significantly different from that of the vehicle control. Treatment of animals with the positive control material induced large increases in nuclear labelling.

[m] Batch no. 16/91-1. Flufenoxuron was tested in the in vivo RDS assay to assess its potential hepatocarcinogenic promoter activity. Two groups of 12 male F344 rats were orally dosed, by gavage, with flufenoxuron in corn oil at a dose level of 2000 or 4000 mg/kg bw. The rats were killed 24, 39 or 48 hours after dosing (four rats per time point). Hepatocytes were prepared using a liver collagenase perfusion technique. Viability of the isolated hepatocytes was checked with the trypan blue exclusion test. Hepatocytes prepared at the concentration of 5×10^5/mL were exposed to 370 KBq/mL (methyl-^{3}H)-labelled thymidine and then incubated for 4 hours. After incubation, the cells were fixed onto slides. Dried slides were coated with a photographic emulsion, exposed for 7 days and subsequently stained with haematoxylin solution. For each animal, 2000 cells were scored to determine the percentage of cell nuclei in RDS. Evaluation criteria: Positive RDS test: maximum RDS incidence of 2.0% or above; negative RDS test: RDS incidence 1.0% or less; equivocal RDS test: RDS incidence between 1.0% and 2.0%. No mortalities or signs of toxicity were noted at either dose level of flufenoxuron. Cell viability was not affected. Flufenoxuron at 2000 or 4000 mg/kg bw did not induce RDS.

reduced to levels comparable with those in the negative controls. The Meeting concluded that the glutathione concentration used in the study with Chinese hamster ovary cells is well above the physiological glutathione concentrations in plasma and extracellular fluids, and for this reason the protective effect can only be an indication of what might happen in vivo, but does not constitute

strong evidence. Flufenoxuron induced no chromosomal aberrations in in vivo studies in mice and rats.

Based on the overall database, it is concluded that flufenoxuron is unlikely to be genotoxic or mutagenic in vivo.

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

In a two-generation dietary reproduction study performed according to OECD Test Guideline 416, Sprague-Dawley rats (28 of each sex per group for the F_0 generation, 24 of each sex per group for the F_1 generation) were fed flufenoxuron (purity 97–97.7%; batch no. 7016 = WB16869/02 = ST87/129) at a dietary concentration of 0, 50, 190, 710 or 10 000 ppm. The premating dietary intakes were equal to 0, 3.8, 14.3, 53.6 and 771 mg/kg bw per day for males and 0, 4.3, 16.0, 61.0 and 907 mg/kg bw per day for females of the F_0 generation and 0, 4.2, 16.1, 62.5 and 865 mg/kg bw per day for males and 0, 4.8, 18.7, 69.2 and 956 mg/kg bw per day for females of the F_1 generation. F_0 adults were treated over a 10-week premating period and throughout the 3-week mating period, gestation and 21-day lactation of two litters (F_{1A} and F_{1B}). The second mating was made specifically with males and females that failed at the first mating (so-called alternative pairing). F_0 parents were killed after weaning of the F_{1B} pups. F_{1B} adults (next generation) followed exactly the same protocol. Each litter (F_{1A}, F_{1B}, F_{2A}, F_{2B}) was randomly culled to eight pups on postnatal day 4 (PND 4). At weaning on PND 21, 24 rats of each sex per dose were selected for producing the F_2 generation. Parental (F_0) rats were exposed from 10 weeks before mating until termination, and F_1 rats were exposed from postnatal week 3 until termination. Clinical examination was performed daily. Feed consumption was recorded weekly during the premating period. Body weights of parental rats were recorded weekly; in addition, females were weighed on gestation days (GDs) 0, 7, 14, 17 and 20 and PNDs 0, 4, 7, 14 and 21. Pups were weighed on PNDs 0, 4, 8, 12 and 21. All litters were examined for number of pups, sex of pups, number of stillbirths, number of live births and gross anomalies. All F_1 animals were examined for presence of the auditory reflex and pupillary reflex. In 24 male and 24 female F_{1A} and F_{2A} pups, the day of cleavage of the balanopreputial gland (males) and the day of vaginal opening (females) were recorded. At termination, necropsy was performed on all F_0 and F_{1B} parental rats, and weights of adrenals, brain, kidneys, heart, lungs, liver, ovaries, prostate with seminal vesicles and coagulating glands, spleen, testes with epididymides, and thymus were recorded. Histological examination was performed on gross lesions and a wide range of organs and tissues of all F_0 and F_{1B} parental rats. In addition, in F_0 and F_{1B} control and high-dose adults, histopathology was extended to the reproductive tract (ovaries, uterus, vagina/prostate with seminal vesicles and coagulating glands, testes with epididymides), pituitary, spleen, liver and heart. The reproductive tract was also examined in males and females (F_0 and F_{1B}) that failed to reproduce at the second (B) mating. Pups culled at PND 8 and pups not selected for breeding were necropsied. Additionally, one male and one female weanling per litter of the F_{1A}, F_{1B}, F_{2A} and F_{2B} generations were also subjected to organ weighing and histopathological investigations (both examinations: same organs/tissues as for F_0 adults).

No treatment-related mortality or clinical signs were observed. Body weight gain was decreased (10–13%, not dose dependent) in F_{1B} males at doses of 190 ppm and above. Feed conversion efficiency was slightly higher in both sexes of the F_0 and F_{1B} generations at doses of 190 ppm and above. There were no treatment-related effects on mating performance, pregnancy rates, gestation duration, litter size or pup sex ratio. Macroscopic examination revealed a higher (but still moderate) incidence of minimal luminal dilatation of the uterus in high-dose F_0 and F_{1B} females, which may have been related to differences in estrous cycle stages but was considered as potentially treatment related. No other macroscopic findings were considered to be treatment related. Absolute adrenal weights were increased in high-dose F_0 and F_{1B} females (11–15%).

Combined data from both litters of all pregnant F_0 and F_{1B} females showed statistically significantly increased incidences of total litter loss (1, 1, 2, 8 and 13 litter losses during lactation at 0,

50, 190, 710 and 10 000 ppm, respectively). At PND 21 at 10 000 ppm in the F_{1A}, F_{2A} and F_{2B} generations, statistically significantly smaller litter sizes (5.5–6.6 versus 7.4–7.8 in controls) and higher cumulative pup loss (11–26% versus 1.3–3.0%) were observed. Pup mortality at 10 000 ppm and to a lesser extent at 710 ppm, both in litters totally lost and in litters where dams reared some young to weaning, was associated in many instances with failure to gain weight or actual weight loss in the period prior to death. At birth, mean body weights from pups in treated groups were comparable to, or even slightly higher than, those of control pups. At PND 21, significantly lower pup weights were observed at 10 000 ppm in three out of four generations (F_{1A}, F_{2A} and F_{2B}). Significantly lower pup weights were also observed at 190 or 710 ppm for some litters. Average body weight gains (birth to PND 21) for all generations were 43.9, 42.9, 38.8, 39.8 and 38.8 g at 0, 50, 190, 710 and 10 000 ppm, respectively (i.e. 98%, 88%, 91% and 88% of controls in the treatment groups). Preweaning development (i.e. startle and pupillary reflexes) was not affected by treatment. At 10 000 ppm, increased relative heart weights (12–14%) were observed in F_{1A}, F_{2A} and F_{2B} male pups and in F_{1A} female pups, and increased relative liver weight (10–12%) was observed in F_{2A} and F_{2B} male pups. Macroscopic and histopathological examination of weanlings revealed no treatment-related effects.

The NOAEL for parental toxicity was 50 ppm (equal to 4.2 mg/kg bw per day), based on decreased body weight gain in males of the F_{1B} generation at 190 ppm (equal to 16.1 mg/kg bw per day).

The NOAEL for offspring toxicity was 50 ppm (equal to 3.8 mg/kg bw per day), based on a reduction in average body weight gain during lactation of male and female pups of all generations at 190 ppm (equal to 14.3 mg/kg bw per day).

The NOAEL for reproductive toxicity was 10 000 ppm (equal to 771 mg/kg bw per day), the highest dose tested.

It is noted that substance intake data were available only during the premating period. It is expected that substance intake in females will have been higher during gestation and lactation than during the premating period (James et al., 1990; James, 1991, 1992).

A limited study was performed to investigate whether the adverse effects on pup survival and growth, as observed in the study of James et al. (1990), could be reproduced when flufenoxuron was administered during gestation and lactation only. Thus, a group of 15 female Sprague-Dawley rats was administered flufenoxuron (purity 97.4%; batch no. 7016) at a dietary concentration of 20 000 ppm from day 3 of pregnancy through to weaning. No control animals were employed in this study. Maternal body weight and feed consumption were measured on GDs 2, 3, 7, 10, 14, 17 and 20 and on PNDs 0, 7, 14 and 21. Offspring were examined daily and weighed regularly throughout the preweaning period. On postpartum day 21 or 22, mothers and pups were killed and examined externally and internally for abnormalities. The uterus of each female that gave birth was visually inspected for implantation sites, and the numbers of sites were recorded.

No mortality or treatment-related clinical signs were observed in mothers. Body weight development and feed consumption were within the range expected for pregnant and lactating rats of this strain and age. The mean gestation length of 21.9 days was within the expected range. The live birth index ([no. of live pups/no. of pups born] × 100), viability index ([no. of pups alive on day 4/no. of pups alive at birth] × 100) and lactation index ([no. of pups alive at PND 21/no. of pups alive at PND 4] × 100) were 98.2%, 98.2% and 98.8%, respectively, and thus not considered to be affected by the treatment. Pup weights were comparable to historical control values throughout lactation. Neither maternal nor offspring necropsy revealed any treatment-related findings.

The data suggest that dietary administration of flufenoxuron at 20 000 ppm to female rats from day 3 of pregnancy through to weaning of their offspring did not result in any adverse effects on the offspring (James & Jones, 1992).

In order to investigate the reasons for the increased postpartum mortality of pups at dose levels of 190 ppm and above in the two-generation study of James et al. (1990), a cross-fostering study was conducted using 50 male and 100 female Sprague-Dawley rats. The study design is depicted in Table 17. Flufenoxuron (purity 97.4; batch no. 7016 = ST 87/129) was administered to the treatment group (50 females) at a dietary dose level of 20 000 ppm (equal to 1633 mg/kg bw per day) for a 10-week premating period, after which the rats were mated for up to 2 weeks on a "one male to two females" ratio. The treated females continued to receive formulated diet until parturition. After parturition, treated females were fed control diet throughout the lactation period until study termination. Control females (50) and two groups each of 25 males were administered control diet throughout the study, with the exception that the males that were intended for mating with the treated females were also fed medicated diet 1 week before mating in order to become accustomed to the treated diet to which they were exposed during mating.

Table 17. Study design of the cross-fostering study

10-week premating 2-week mating 3-week gestation		**3-week lactation with control diet**	
Formulated diet	TD	TP (5 litters)	
		TP (12 litters)	→ milk and fat samples
		CP (26 litters)	→ cross-fostered
Control diet	CD	CP (15 litters)	
		CP (5 litters)	→ milk and fat samples
		TP (26 litters)	→ cross-fostered

C: control; D: dams; P: pups; T: treated

Source: Masters (1996)

At birth, the pups were counted, sexed, weighed and examined for external abnormalities, and subsequently the litters were culled to a litter size of eight pups. Culled pups were subjected to a macroscopic postmortem examination. A reciprocal cross-fostering of 26 litters was performed between control and treated dams; that is, control dams (CD) reared pups from treated dams (TP pups), and, reciprocally, pups from control dams (CP pups) were reared by previously treated dams (TD). Fifteen control and five treated dams reared their own offspring until weaning (CD/CP and TD/TP). Additionally, five control and 12 treated non-cross-fostered dams were used to obtain milk and fat samples on days 1, 7, 14 and 21 postpartum (one control and three treated dams per occasion) for analysis of residual flufenoxuron levels in milk and fat.

All adult rats and pups were observed daily for mortality and clinical signs. Body weights of adult animals were determined weekly. Body weights of females were also recorded on GDs 0, 7, 14, 17 and 20 and PNDs 0, 7, 14 and 21. Pup weights were measured on PNDs 0, 2, 4, 8, 12, 16 and 21. Feed consumption was measured weekly during the premating and lactation periods. Water consumption was determined on a daily basis for females during the initial and final 2 weeks of the gestation period. The dams selected for milk and fat sampling were administered oxytocin by intraperitoneal injection approximately 30 minutes before milking. After milking, the dams were killed, and abdominal fat was collected. Milk and fat samples were analysed for flufenoxuron content by HPLC. At PND 21, all pups and parental animals were killed and examined for external and internal abnormalities. The uterus of each female that gave birth was visually inspected for implantation sites, and the number of sites was recorded. The uteri of apparently non-pregnant females were examined for evidence of implantation.

No treatment-related clinical signs or changes in body weight and feed consumption were observed. Changes in pregnancy rate, precoital time and gestation length were not treatment related. There were no treatment-related effects on the number of implantations, prenatal loss, number of dead or live pups per dam, mean pup weight or sex ratio in any of these groups. After cross-fostering, survival of pups (in terms of viability and lactation indices and body weight gains) was similar between all subgroups. At necropsy, no treatment-related findings were observed in dams or pups.

Flufenoxuron levels in both milk and fat declined rapidly (Table 18), with half-lives of 2.3 days in milk and 7.6 days in fat. The fat half-life in the present study was much shorter than the half-life of 28 days observed in the study of Morrison & Huckle (1988). This is likely caused by the fact that in the present study, the females excreted flufenoxuron during lactation.

Table 18. Concentrations of flufenoxuron in milk and fat

No. of rats	PND	Mean (± SD) concentration in fat (µg/g)	Mean (± SD) concentration in milk (µg/g)
3	1	973 ± 82	450 ± 377
3	7	781 ± 240	91.3 ± 20.2
3	14	270 ± 77	9.4 ± 6.1
3	21	48.5 ± 29.6	1 sample: 9.54 2 other samples: fate unknown

PND: postnatal day; SD: standard deviation

Source: Masters (1996)

In contrast to the two-generation study, increased pup mortality and reduction of pup weight were not observed in pups of treated females (reared either by their mother or by an untreated dam after cross-fostering) or in pups of untreated dams reared by treated dams (after cross-fostering). This suggests that long-term exposure to flufenoxuron is required to cause the observed adverse effects of flufenoxuron in the multigeneration study.

Under the experimental conditions, the NOAEL was 20 000 ppm (equal to 1633 mg/kg bw per day), the only dose tested (Masters, 1996).

(b) Developmental toxicity

Rats

In a developmental toxicity study, groups of 26 pregnant female Sprague-Dawley rats were treated orally, by gavage, with flufenoxuron (purity 94.7%; batch no. 16 = ST86/165) in 0.5% aqueous carboxymethyl cellulose at a dose level of 0, 7.9, 81 or 967 mg/kg bw per day from days 6 through 16 of gestation (day 0 = day on which sperm were detected in the vaginal smear). Clinical signs and mortality were recorded daily. Body weight was measured on GDs 2, 6, 9, 13, 17 and 20. Feed consumption was measured daily from GD 3 onward. All females were killed on day 20 of gestation and subjected to gross examination. The uterus was examined, and the numbers of live and dead fetuses, corpora lutea, implantations and early and late resorptions were counted. Body weight and sex of the fetuses were recorded. About half of the fetuses from each litter were selected for skeletal examinations, and the other half for cross-sectional visceral examinations.

No treatment-related mortality, clinical signs or abortions were observed. The body weight gain was slightly and dose-dependently increased (85.6, 89.1, 92.9 and 94.2 g at 0, 7.9, 81 and 967 mg/kg bw per day, respectively). Feed consumption was not affected by treatment. Necropsy of the dams revealed no effect of flufenoxuron on the number of implantations, early and late resorptions,

number of live fetuses, fetal weight, sex ratio or uterine weight. Visceral and skeletal examination revealed no treatment-related findings. A slightly increased incidence of heart vessel branching abnormalities at the high dose (incidence 5/23 litters and 7/143 fetuses, versus incidence in controls of 1/25 litters and 1/156 fetuses) was observed. However, historical control data compiled by the Middle Atlantic Reproduction and Teratology Association (MARTA) in 1993 show that this is a common finding in Sprague-Dawley rats, with maximum incidences above those observed in the present study. Therefore, this finding was not considered toxicologically relevant.

The NOAEL for maternal toxicity was 967 mg/kg bw per day, the highest dose tested.

The NOAEL for embryo and fetal toxicity was 967 mg/kg bw per day, the highest dose tested (Hazelden, 1991a; Hazelden & Wilson, 1991a; MARTA, 1993; Christian, 1996).

In a special developmental toxicity study, groups of at least 15 presumed pregnant Fischer 344 rats were administered flufenoxuron (purity 99%; batch no. 9 = Ref 2014/092 = STL Ref. No. ST85/113) at 0, 10 or 1000 mg/kg bw per day by oral gavage during GDs 8–17. The rats were maintained until PND 5. The day on which sperm were observed in the vaginal lavage fluid was considered to be day 1 of gestation. Corn oil served as a vehicle. Dams were weighed daily during the treatment period, on day 21 of gestation and at termination on day 5 of lactation. At delivery, the day of gestation, number of live pups, total weight of the live litter and number of stillborn pups were recorded, and each pup was examined for abnormalities. On PND 5, the number of surviving pups, the total litter weight and the body weight of the dams were recorded again. The dams and pups were not further examined.

All dams remained healthy throughout the study. At 0, 10 and 1000 mg/kg bw per day, 17, 13 and 14 females, respectively, were pregnant and delivered offspring. No effects were observed on maternal body weight, on the number and weight of pups born or on pup body weight development. There was no indication of embryotoxicity. The only significant finding was that four of the high-dose dams failed to lactate properly. Two of these dams showed no signs of milk production, and their litters died. The other two had reduced milk production; some of the pups from their litters died, and the others failed to gain weight properly. In these animals, the mammary development was visibly reduced, and there was less than the average amount of milk or no milk visible in the stomachs of the neonates. The study author reported that reduced lactation is a rare observation in the historical control data of the laboratory (no data provided) and suggested that this finding is treatment related (Esdaile, 1986b).

Rabbits

In a developmental toxicity study, groups of 13–15 pregnant New Zealand White rabbits were treated orally, by gavage, with flufenoxuron (purity 94.7%; batch no. 16 = ST86/165) in aqueous carboxymethyl cellulose (0.5%) at a dose level of 0, 7.7, 100 or 1000 mg/kg bw per day from days 6 through 18 of gestation (the day after artificial insemination was designated as GD 0). Clinical signs were recorded daily. Body weight was measured on GDs 0, 6, 9, 12, 15, 19, 22, 26 and 29. Feed consumption was measured daily from GD 3. All females were killed on day 29 of gestation. All does were examined macroscopically for abnormalities. The reproductive tract was examined, and the numbers of live and dead fetuses, corpora lutea and implantations were counted. Body weight and sex of the fetuses were recorded. All fetuses were subjected to skeletal and visceral examinations.

No treatment-related mortality, clinical signs, abortions or effects on body weight gain or feed consumption were observed. Necropsy of the dams at day 29 revealed no treatment-related abnormalities. A reduced fetus weight (7%) at the high dose was attributed to the increased litter size in this group. No treatment-related effects were observed on the uterine weights, the numbers of corpora lutea, implantations, live fetuses and early or late deaths, or sex ratios. Occasional findings of minor or major abnormalities were not dose related, not statistically significant and/or within the control range of concurrent studies in the same laboratory and were therefore considered fortuitous.

At the high dose, a slight delay in skeletal ossification was observed. This was considered to be related to the slight, not treatment-related decrease in fetal weights and was considered fully reversible and non-adverse.

The NOAEL for maternal toxicity was 1000 mg/kg bw per day, the highest dose tested.

The NOAEL for embryo and fetal toxicity was 1000 mg/kg bw per, the highest dose tested (Hazelden, 1991b; Hazelden & Wilson, 1991b).

2.6 Special studies

(a) Neurotoxicity

Rats

In a 28-day neurotoxicity study, groups of 10 male and 10 female Wistar rats were given flufenoxuron (purity 99.2%; batch no. SNPE 38/00) at a dietary level of 0, 1000, 5000 or 20 000 ppm (equal to 0, 88, 435 and 1745 mg/kg bw per day for males and 0, 95, 475 and 1934 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 at day 27. At termination, rats were killed and examined macroscopically. The brains were weighed. Five rats of each sex from the control and high-dose groups were selected for neurohistological examination of the brain, ganglions, fibres, nerves, eyes and gastrocnemius muscle.

No treatment-related mortality or clinical signs were observed. Males at 5000 and 20 000 ppm showed a reduction in body weight gain (19% and 16%, respectively). There were no treatment-related effects observed in functional observational battery parameters, locomotor activity testing, brain weights, or macroscopic and histological examination.

The NOAEL was 1000 ppm (equal to 88 mg/kg bw per day), based on reductions in body weight gain in males at 5000 ppm (equal to 435 mg/kg bw per day). No evidence of neurotoxicity was observed in rats (Kaspers, Kaufmann & van Ravenzwaay, 2003).

(b) Studies with metabolites

Acute toxicity and genotoxicity studies with metabolites of flufenoxuron were available.

Reg. No. 241208 is a minor faecal (rats and dogs) and urinary (rats) metabolite.

Reg. No. 4064702 is a relevant soil metabolite, a minor metabolite found in the urine of rats (at most 1% of the administered flufenoxuron dose), a relevant water–sediment system product and a hen residue.

Acute toxicity

The results of studies of acute toxicity with metabolites of flufenoxuron are summarized in Table 19.

Genotoxicity

The results of genotoxicity studies with metabolites of flufenoxuron are summarized in Table 20.

3. Observations in humans

During the manufacturing of flufenoxuron at SNPE Chimie, Toulouse, France, over a period of 6 years, no unusual or abnormal health effects were observed among the operators and other employees involved with flufenoxuron production. Great Lakes (UK) Ltd, Widnes, did not observe unusual or abnormal health effects among 15 operators or other employees involved with

Table 19. Results of studies of acute toxicity with metabolites of flufenoxuron

Species	Strain	Sex	Route	Metabolite	Purity (%)	LD_{50} (mg/kg bw)	Reference[a]
Mouse	CD-1	M/F	Oral	Reg. No. 241208[b]	97	1 937 (M) 2 898 (F)	Gardner (1990a)
Mouse	CD-1	M/F	Oral	Reg. No. 4064702[c]	97	433 (M) 302 (F)	Gardner (1990b)

bw: body weight; DMSO: dimethyl sulfoxide; F: female; GLP: good laboratory practice; M: male; OECD: Organisation for Economic Co-operation and Development

[a] Statements of adherence to quality assurance and GLP were included in all studies.

[b] Reg. No. 241208 = WL115096. Batch no. 2 ST90/203. Study design resembles OECD Test Guideline 401. Doses of 625, 1000, 1600, 2560 and 4096 mg/kg bw were administered by gavage. Vehicle was DMSO. Mortality occurred at all doses in a dose-related manner (1, 2, 3, 7 and 6 mortalities at 625, 1000, 1600, 2560 and 4096 mg/kg bw, respectively). Clinical signs were lethargy, hunched posture, piloerection, pallor of the skin and eyes, abasia/ataxia, tachypnoea, cyanosis, hypothermia, prostration and unkempt appearance. Decedents showed darkening or congestion of the lungs, liver, spleen or kidneys, exaggerated hepatic lobular pattern and renal pallor. In mice killed at termination, no macroscopic changes were observed. Histological examination of the liver and kidneys of four mice that died after treatment at 4096 mg/kg bw revealed a minor adaptive change (centrilobular parenchymal hypertrophy) of the liver in two animals. There were no other microscopic indications of renal or hepatic toxicity.

[c] Reg. No. 4064702 = WL129183 = CL 932338. Batch no. 2 ST90/205. Study design resembles OECD Test Guideline 401. Doses of 0, 111, 200, 360 and 648 mg/kg bw were administered by gavage. Vehicle was DMSO. Mortalities numbered 0, 0, 1, 5 and 9 at 0, 111, 200, 360 and 648 mg/kg bw, respectively. Clinical signs at 111 mg/kg bw were lethargy, piloerection, hunched posture and pale eyes. In addition, at higher doses, unkempt appearance, rales, vocalization, lacrimation, corneal opacity, stereotyped behaviour involving circling movement and/or lateral leaning posture, abnormal gait/ataxia, hypothermia, tachypnoea, cyanosis and prostration were observed. Body weights were decreased at 200 mg/kg bw and above. Necropsy of animals that died before the end of the study included pallor or darkening of the liver and kidneys and distension and/or gaseous contents of the gastrointestinal tract. There were also isolated cases of softening of the brain and pituitary, inflammation of the stomach and lung congestion. There were no gross pathological abnormalities in the animals that were killed on day 15. Examination of histological samples of the liver and/or kidneys of three mice that died after treatment at 648 mg/kg bw and that presented pallor or darkening of the liver and kidneys revealed a minor adaptive change (centrilobular parenchymal hypertrophy) of the liver in two animals, but no abnormality in kidneys.

Table 20. Results of studies on the genotoxicity of metabolites of flufenoxuron

Metabolite	End-point	Test object	Concentration	Purity (%)	Results	Reference[a]
In vitro						
Reg. No. 241208	Gene mutation	*Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537, TA1538; *Escherichia coli* WP2 uvrA pKM101	7.8–5 000 µg/plate (±S9) 2.44–5 000 µg/plate (+S9)	97	Positive	Brooks & Wiggins (1990a)[b]
Reg. No. 4064702	Gene mutation	*S. typhimurium* strains TA98, TA100, TA1535, TA1537; *E. coli* WP2 uvrA pKM101	31.25–5 000 µg/plate (±S9)	97	Negative	Brooks & Wiggins (1990b)[c]
Reg. No. 241208	Gene mutation	Chinese hamster ovary cells	1–10 µg/mL (–S9), 2.5–40 µg/mL (+S9), experiment 1 3–8 µg/mL (–S9), 10–40 µg/mL (+S9),	99.4	Negative	Engelhardt (2005a,b)[d]

Metabolite	End-point	Test object	Concentration	Purity (%)	Results	Reference[a]
			experiment 2 20–90 µg/mL (+S9), experiment 3			
Reg. No. 241208	Chromosomal aberrations	Chinese hamster ovary cells	6.25–50 µg/mL (−S9) 5–640 µg/mL (+S9)	97	Negative	Brooks & Wiggins (1992)[e]
Reg. No. 241208	Cell transformation	Syrian hamster embryo cells	1 and 7 days: 0, 15, 20, 25, 30 and 35 µg/mL (±S9)	99.4	Negative	Engelhardt (2005c,d)[f]
In vivo						
Reg. No. 241208	Unscheduled DNA synthesis	Wistar rat, strain Crl:WI (Han)	0, 300 and 600 mg/kg bw	99.4	Negative	Engelhardt (2005e,f)[g]

bw: body weight; DMSO: dimethyl sulfoxide; DNA: deoxyribonucleic acid; GLP: good laboratory practice; OECD: Organisation for Economic Co-operation and Development; S9: 9000 × *g* supernatant fraction from rat liver homogenate

[a] Positive and negative (solvent) controls were included in all studies. In all studies, statements of adherence to GLP and quality assurance were included.

[b] Reg. No. 241208 = WL115096. Batch no. 2 ST90/203. Study design resembles OECD Test Guideline 471. The test compound formed oily smears on the surface of the top agar at 250–5000 µg/plate, showing that it was not totally miscible at these treatment levels, but no precipitate was reported at the highest concentrations. Reg. No. 241208 was cytotoxic to the bacterial strains *S. typhimurium* TA98, TA1537 and TA1538 and *E. coli* WP2 uvrA pKM101, especially in the absence of S9 fraction, so the test was not interpretable for these strains, as a too high cytotoxicity (> 20%) triggers some nonspecific mutations in the bacteria and because the cytotoxicity rates were not reported. The method of cytotoxicity assessment was not very sensitive, as some test concentrations at which the revertant count was dose-relatedly lower were not stated to induce cytotoxicity. Four tests yielded positive results in TA98 and TA100, all in the presence of S9 mix at a test item concentration of 125 or 250 µg/plate. However, the reproducibility of the positive results was questionable. Based on these weak increases in mutation rates, the test item was considered to have a weak mutagenic potential.

[c] Reg. No. 4064702 = WL129183. Batch no. 2 ST90/205. Study design resembles OECD Test Guideline 471. The test compound formed smears on the surface of the top agar at 125 µg/plate and above, and a white precipitate was evident at 5000 µg/plate. Cytotoxicity was observed only in strains TA1535 and TA1537 in the absence of S9 mix at doses of 2000 and 5000 µg/plate. No indication of mutagenic effects of Reg. No. 4064702 (WL129183) was observed at doses of up to 5000 µg/plate.

[d] Reg. No. 241208 = WL115096. Batch no. AC12642-12. Performed in accordance with OECD Test Guideline 476. Concentrations were chosen on the basis of cytotoxicity in a preliminary assay. Three experiments were performed. Test compound was dissolved in DMSO.

[e] Reg. No. 241208 = WL115096. Batch no. 3 ST92/179. Performed in accordance with OECD Test Guideline 473. Vehicle was DMSO. Doses used in this assay were based on results from preliminary cytotoxicity assays. There was no difference in the number of aberrant cells including and excluding gaps in Reg. No. 241208–treated cultures without or with S9 metabolic activation when compared with controls.

[f] Reg. No. 241208 = WL115096. Batch no. AC12642-12. Performed in accordance with a draft OECD test guideline (In vitro carcinogenicity: Syrian hamster embyo cell transformation assay). Reg. No. 241208 was assessed for its potential to induce morphological transformation in early passage Syrian hamster embryo cells after 24 hours or 7 days of exposure. The test substance did not cause a relevant increase in morphologically transformed colonies in either experiment. Thus, under the experimental conditions of this assay, Reg. No. 241208 is considered not to cause an increase in transformed colonies in the low pH 6.7 Syrian hamster embryo cell transformation assay.

[g] Reg. No. 241208 = WL115096. Batch no. AC12642-12. Performed in accordance with OECD Test Guideline 486. Tested in the unscheduled DNA synthesis test with mammalian liver cells in vivo. The test substance, dissolved in com oil, was administered once orally to groups of three male Wistar rats at a dose of 300 or 600 mg/kg bw in a volume of 10 mL/kg bw in each case. Hepatocytes were harvested 3 and 14 hours after administration of the test substance. At 300 and 600 mg/kg bw, the animals displayed piloerection and squatting posture. In addition, at 600 mg/kg bw, hypothermia was observed. Cell viability was not influenced by test substance treatment, morphological changes of the cells were not observed and cell material was not reduced. The single oral treatment with the test substance did not lead to an increase in the mean number of net nuclear grain counts at any dose level or exposure time in rat hepatocytes.

flufenoxuron production. Isochem, Toulouse, reported only one case of skin allergy in a worker potentially exposed to flufenoxuron. Symptoms did not recur after reinforcing individual protection

measures. No epicutaneous tests were performed in order to confirm flufenoxuron as causative agent because of the potential risk to the subject (Deweerdt & Mommee, 1997; Flynn, 2003; Evrard, 2004).

Comments

Biochemical aspects

After oral administration of flufenoxuron to rats, absorption was rapid, with a T_{max} of 3–6 hours; there was evidence of saturation at higher dose levels (> 80% absorption at 3.5 mg/kg bw compared with < 15% at 350 mg/kg bw in rats). Absorption was lower in dogs than in rats (< 30% in dogs at 3.5 mg/kg bw). Absorbed flufenoxuron was widely distributed throughout the body, with highest levels in fat and bone marrow. Unchanged flufenoxuron was the major residue in all tissues, faeces and urine. Flufenoxuron is metabolized by cleavage of the bond adjacent to the 2,6-difluorobenzoyl moiety, followed by oxidation or hydroxylation. The major metabolite identified in rats was 2,6-difluorobenzoic acid, together with *N*-[4-[2-chloro-4-(trifluoromethyl)phenoxy]-2-fluorophenyl] urea and 2-amino-5-(2-chloro-4-(trifluoromethyl)phenoxy)-3-fluorophenol; 2,6-difluorobenzamide and 4-[2-chloro-4-(trifluoromethyl)phenoxy]-2-fluorobenzenamine were minor metabolites in rats. After repeated dosing, tissue radioactivity levels decreased slowly in rats (half-life 34 days, range 28–48 days) and in dogs (half-life 33 days). The routes of excretion were faecal and, to a lesser extent, urinary. Flufenoxuron was excreted in the milk of lactating rats. Flufenoxuron was shown to accumulate in body fat.

Toxicological data

The acute toxicity of flufenoxuron is low (oral LD_{50} > 3000 mg/kg bw; dermal LD_{50} > 2000 mg/kg bw; inhalation LC_{50} > 5.1 mg/L). Flufenoxuron was not irritating to the skin or the eyes of rabbits. Flufenoxuron was not a skin sensitizer in a Magnusson and Kligman test in guinea-pigs.

In repeated-dose toxicity studies with flufenoxuron in mice, rats and dogs, multiple adverse effects were observed, in particular body weight changes and toxicity to the haematological system indicative of haemolytic anaemia. Studies in dogs showed that these animals are particularly sensitive to the haematological effects of flufenoxuron. Owing to the saturation of absorption at higher doses, dose–response curves are often flat.

In a 28-day study in mice using dietary flufenoxuron concentrations of 0, 50, 500, 5000, 10 000 and 50 000 ppm (equivalent to 0, 7.1, 71, 710, 1400 and 7100 mg/kg bw per day, respectively), the NOAEL was 10 000 ppm (equivalent to 1400 mg/kg bw per day), based on reduced feed consumption observed at 50 000 ppm (equivalent to 7100 mg/kg bw per day).

In a 13-week study in mice using dietary flufenoxuron concentrations of 0, 50, 500, 5000, 10 000 and 50 000 ppm (equal to 0, 10, 103, 1069, 2139 and 11 071 mg/kg bw per day for males and 0, 12, 124, 1247, 2482 and 12 619 mg/kg bw per day for females, respectively), the NOAEL was 50 ppm (equal to 10 mg/kg bw per day), based on increased serum bilirubin concentrations in males and females at 500 ppm (equal to 103 mg/kg bw per day).

In a 28-day study in rats using dietary flufenoxuron concentrations of 0, 50, 500, 5000, 10 000 and 50 000 ppm (equal to 0, 4.8, 49, 475, 997 and 5147 mg/kg bw per day for males and 0, 5.3, 53, 534, 1067 and 5432 mg/kg bw per day for females, respectively), the NOAEL was 50 000 ppm (equal to 5147 mg/kg bw per day), the highest dose tested.

In a 13-week study in rats using dietary flufenoxuron concentrations of 0, 50, 500, 5000, 10 000 and 50 000 ppm (equal to 0, 3.5, 35, 351, 689 and 3637 mg/kg bw per day for males and 0, 4.1, 41, 399, 820 and 4151 mg/kg bw per day for females, respectively), the NOAEL was 500 ppm (equal to 41 mg/kg bw per day), based on slightly higher spleen weights and haematological changes indicative of mild anaemia observed in females at 5000 ppm (equal to 399 mg/kg bw per day).

In a 13-week study in dogs using dietary flufenoxuron concentrations of 0, 500, 5000 and 50 000 ppm (equal to 0, 18, 163 and 1961 mg/kg bw per day for males and 0, 21, 182 and 2039 mg/kg bw per day for females, respectively), the LOAEL was 500 ppm (equal to 18 mg/kg bw per day), based on haemolytic anaemia and associated changes. No NOAEL could be identified.

In a 1-year study in dogs using dietary flufenoxuron concentrations of 0, 10, 100, 500 and 50 000 ppm (equal to 0, 0.36, 3.5, 19 and 1898 mg/kg bw per day for males and 0, 0.36, 3.8, 19 and 1879 mg/kg bw per day for females, respectively), the NOAEL was 100 ppm (equal to 3.5 mg/kg bw per day), based on evidence of haemolytic anaemia and related changes at 500 ppm (equal to 19 mg/kg bw per day).

The overall NOAEL for the 13-week and 1-year dog studies was 100 ppm (equal to 3.5 mg/kg bw per day). The overall LOAEL was 500 ppm (equal to 18 mg/kg bw per day).

In a 2-year carcinogenicity study in mice using dietary flufenoxuron concentrations of 0, 100, 1000 and 10 000 ppm (equal to 0, 15.3, 152 and 1592 mg/kg bw per day for males and 0, 17.4, 187 and 1890 mg/kg bw per day for females, respectively), the NOAEL was 1000 ppm (equal to 187 mg/kg bw per day), based on decreased body weight gain in females at 10 000 ppm (equal to 1890 mg/kg bw per day). There was no evidence of carcinogenicity.

In a second 2-year carcinogenicity study in mice, using dietary flufenoxuron concentrations of 0, 500, 5000 and 50 000 ppm (equal to 0, 56, 559 and 7356 mg/kg bw per day for males and 0, 73, 739 and 7780 mg/kg bw per day for females, respectively), the NOAEL was 500 ppm (equal to 56 mg/kg bw per day), based on an increased incidence of lordotic episodes, decreased body weight gain, increased Kupffer cell aggregates in females and increased liver weight relative to brain weight in males at 5000 ppm (equal to 559 mg/kg bw per day). An increased incidence of splenic haemangiosarcomas was observed in females dosed at 50 000 ppm. However, as this finding was not accompanied by an increase in angiomas, it was not considered to be treatment related. An apparent increased incidence of hepatocellular carcinomas in treated male mice in this study was considered not treatment related because the incidence in control males was low.

The overall NOAEL for systemic toxicity in the 2-year studies in mice was 1000 ppm (equal to 187 mg/kg bw per day). The overall LOAEL was 5000 ppm (equal to 559 mg/kg bw per day).

In a 2-year toxicity and carcinogenicity study in rats using dietary flufenoxuron concentrations of 0, 1, 5, 50, 500, 5000 and 50 000 ppm (equal to 0, 0.044, 0.23, 2.2, 22, 230 and 2470 mg/kg bw per day for males and 0, 0.055, 0.28, 2.8, 28, 300 and 3210 mg/kg bw per day for females, respectively), the NOAEL was 500 ppm (equal to 22 mg/kg bw per day), based on decreased body weight gain, a small increase in non-cyanide-binding haemoglobin, considered to reflect sulfhaemoglobin, and decreased triglyceride levels in both sexes at 5000 ppm (equal to 230 mg/kg bw per day). Flufenoxuron was not carcinogenic under the conditions of the study.

In a second 2-year toxicity and carcinogenicity study in rats, using dietary flufenoxuron concentrations of 0, 500, 5000 and 50 000 ppm (equal to 0, 21.6, 218 and 2290 mg/kg bw per day for males and 0, 25.9, 276 and 2901 mg/kg bw per day for females, respectively), the NOAEL was 500 ppm (equal to 25.9 mg/kg bw per day), based on reduced body weight gain in females at 5000 ppm (equal to 276 mg/kg bw per day). No carcinogenic potential of flufenoxuron was observed.

The overall NOAEL for systemic toxicity in the 2-year studies in rats was 500 ppm (equal to 25.9 mg/kg bw per day). The overall LOAEL was 5000 ppm (equal to 230 mg/kg bw per day).

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

Flufenoxuron was tested for genotoxicity in an adequate range of assays, both in vitro and in vivo. There was no evidence of genotoxicity in vitro, with the exception of two assays of chromosomal aberrations in Chinese hamster ovary cells, and there was no evidence of genotoxicity in vivo. The Meeting concluded that flufenoxuron is unlikely to be genotoxic in vivo.

In view of the lack of genotoxicity in vivo and the absence of carcinogenicity in mice and rats at exposure levels that are relevant for human dietary risk assessment, the Meeting concluded that flufenoxuron is unlikely to pose a carcinogenic risk to humans from the diet.

In a two-generation reproductive toxicity study in rats using dietary flufenoxuron concentrations of 0, 50, 190, 710 and 10 000 ppm (premating dietary intakes were equal to 0, 3.8, 14.3, 53.6 and 771 mg/kg bw per day for males and 0, 4.3, 16.0, 61.0 and 907 mg/kg bw per day for females of the F_0 generation and 0, 4.2, 16.1, 62.5 and 865 mg/kg bw per day for males and 0, 4.8, 18.7, 69.2 and 956 mg/kg bw per day for females of the F_1 generation, respectively), the NOAEL for parental toxicity was 50 ppm (equal to 4.2 mg/kg bw per day), based on decreased body weight gain in males of the F_{1B} generation at 190 ppm (equal to 16.1 mg/kg bw per day). The NOAEL for offspring toxicity was 50 ppm (equal to 3.8 mg/kg bw per day), based on a reduction in average body weight gain during lactation of male and female pups of all generations at 190 ppm (equal to 14.3 mg/kg bw per day). The NOAEL for reproductive toxicity was 10 000 ppm (equal to 771 mg/kg bw per day), the highest dose tested. In this study, adverse effects on pup survival and growth were observed at 710 ppm (equal to 53.6 mg/kg bw per day). Subsequent studies, including a cross-fostering study in rats, failed to further elucidate the mechanism for the adverse effects on pup survival.

In a developmental toxicity study in rats using gavage flufenoxuron doses of 0, 7.9, 81 and 967 mg/kg bw per day, the NOAEL for maternal toxicity and for embryo and fetal toxicity was 967 mg/kg bw per day, the highest dose tested.

In a developmental toxicity study in rabbits using gavage flufenoxuron doses of 0, 7.7, 100 and 1000 mg/kg bw per day, the NOAEL for maternal toxicity and for embryo and fetal toxicity was 1000 mg/kg bw per day, the highest dose tested.

The Meeting concluded that flufenoxuron is not teratogenic.

In a 28-day neurotoxicity study in rats using dietary flufenoxuron concentrations of 0, 1000, 5000 and 20 000 ppm (equal to 0, 88, 435 and 1745 mg/kg bw per day for males and 0, 95, 475 and 1934 mg/kg bw per day for females, respectively), the NOAEL was 1000 ppm (equal to 88 mg/kg bw per day), based on reductions in body weight gain in males at 5000 ppm (equal to 435 mg/kg bw per day). No evidence of neurotoxicity was observed in this or other studies.

The Meeting concluded that flufenoxuron is not neurotoxic.

Toxicological data on metabolites and/or degradates

Acute toxicity and genotoxicity studies were performed with Reg. No. 241208 (4-[2-chloro-4-(trifluoromethyl)phenoxy]-2-fluorobenzenamine), a minor faecal (rats and dogs) and urinary (rats) metabolite and a minor residue in hens; and with Reg. No. 4064702 (*N*-[4-[2-chloro-4-(trifluoromethyl)phenoxy]-2-fluorophenyl] urea), which is a soil metabolite, a minor metabolite found in the urine of rats (at most 1% of the administered flufenoxuron dose), a water–sediment system product and a minor residue in hens.

In studies of acute oral toxicity in mice with Reg. No. 241208, the oral LD_{50} was 1937 mg/kg bw. There was no evidence of genotoxicity in a number of in vitro and in vivo studies, except for one Ames test that indicated a weak positive response.

For Reg. No. 4064702, the oral LD_{50} was 302 mg/kg bw. An Ames test was negative.

The Meeting concluded that these metabolites are not toxicologically relevant for a dietary risk assessment.

Human data

Medical surveillance of personnel at flufenoxuron manufacturing plants revealed no unusual or abnormal health effects, except for one case of skin allergy in a worker potentially exposed to flufenoxuron.

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

Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) of 0–0.04 mg/kg bw for flufenoxuron, on the basis of the overall NOAEL of 3.5 mg/kg bw per day for a range of effects indicative of haemolytic anaemia in 13-week and 1-year dietary studies in dogs, using a safety factor of 100. This ADI was supported by a two-generation dietary reproductive toxicity study in rats, with a NOAEL for parental toxicity of 4.2 mg/kg bw per day, based on decreased body weight gain in males, and a NOAEL for offspring toxicity of 3.8 mg/kg bw per day, based on a reduction in average body weight gain during lactation of male and female pups of all generations. An additional safety factor to extrapolate to lifetime exposure was considered unnecessary, as the LOAELs in the 13-week and 1-year studies in dogs were both 500 ppm (equal to 18–19 mg/kg bw per day) and as the concentrations of flufenoxuron in blood and fat in dogs appear to reach steady state after 3 months of treatment, indicating that effects at lower doses with more prolonged exposure are not expected.

The Meeting concluded that it was not necessary to establish an acute reference dose (ARfD) for flufenoxuron in view of its low acute toxicity, the absence of developmental toxicity and the absence of any other toxicological effects that would be likely to be elicited by a single dose.

Levels relevant to risk assessment of flufenoxuron

Species	Study	Effect	NOAEL	LOAEL
Mouse	Ninety-day study of toxicity[a]	Toxicity	50 ppm, equal to 10 mg/kg bw per day	500 ppm, equal to 103 mg/kg bw per day
	Two-year studies of carcinogenicity[a,b]	Toxicity	1 000 ppm, equal to 187 mg/kg bw per day	5 000 ppm, equal to 559 mg/kg bw per day
		Carcinogenicity	50 000 ppm, equal to 7 356 mg/kg bw per day[c]	–
Rat	Two-year studies of toxicity and carcinogenicity[a,b]	Toxicity	500 ppm, equal to 25.9 mg/kg bw per day	5 000 ppm, equal to 230 mg/kg bw per day
		Carcinogenicity	50 000 ppm, equal to 2 470 mg/kg bw per day[c]	–
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	10 000 ppm, equal to 771 mg/kg bw per day[c]	–
		Parental toxicity	50 ppm, equal to 4.2 mg/kg bw per day	190 ppm, equal to 16.1 mg/kg bw per day

Species	Study	Effect	NOAEL	LOAEL
		Offspring toxicity	50 ppm, equal to 3.8 mg/kg bw per day	190 ppm, equal to 14.3 mg/kg bw per day
	Developmental toxicity study[d]	Maternal toxicity	967 mg/kg bw per day[c]	–
		Embryo and fetal toxicity	967 mg/kg bw per day[c]	–
Rabbit	Developmental toxicity study[d]	Maternal toxicity	1 000 mg/kg bw per day[c]	–
		Embryo and fetal toxicity	1 000 mg/kg bw per day[c]	–
Dog	Ninety-day and 1-year studies of toxicity[a,b]	Toxicity	100 ppm, equal to 3.5 mg/kg bw per day	500 ppm, equal to 18 mg/kg bw per day

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

Estimate of acceptable daily intake (ADI)

0–0.04 mg/kg bw

Estimate of acute reference dose (ARfD)

Unnecessary

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

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

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

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rats: Rapid; > 80% in both sexes at 3.5 mg/kg bw; > 3% in males and 12% in females at 350 mg/kg bw Dogs: Rapid; < 30% in both sexes at 3.5 mg/kg bw
Dermal absorption	No data
Distribution	Rats and dogs: Widespread distribution, highest concentrations found in fat and, to a lesser extent, bone marrow Present in milk in lactating rats

Potential for accumulation	Fat/blood residue concentration ratios were about 53 (rats) and 12 (dogs) 7 days post-dosing Rats: Half-life in various organs after repeated administration is 28–48 days Dogs: Half-life in blood is 33 days Potential for accumulation over repeated dosing
Rate and extent of excretion	Slow, mainly via faeces (biliary excretion) and urine
Metabolism in animals	Limited metabolism; major metabolite was 2,6-difluorobenzoic acid, accounting for 10–12% of the administered dose in 0- to 48-hour urine
Toxicologically significant compounds in animals and plants	Parent
Acute toxicity	
Rat, LD_{50}, oral	> 3 000 mg/kg bw
Rat, LD_{50}, dermal	> 2 000 mg/kg bw
Rat, LC_{50}, inhalation	> 5.1 mg/L
Rabbit, dermal irritation	Not irritating
Rabbit, ocular irritation	Not irritating
Guinea-pig, dermal sensitization	Not sensitizing (maximization test)
Short-term studies of toxicity	
Target/critical effect	Haemolytic anaemia
Lowest relevant oral NOAEL	3.5 mg/kg bw per day (dogs)
Lowest relevant dermal NOAEL	No data
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Reduced body weight gain, haemolytic anaemia
Lowest relevant NOAEL	25.9 mg/kg bw per day (rats)
Carcinogenicity	Unlikely to pose a carcinogenic risk to humans from the diet
Genotoxicity	
	Unlikely to be genotoxic in vivo
Reproductive toxicity	
Target/critical effect	None
Lowest relevant parental NOAEL	4.2 mg/kg bw per day
Lowest relevant offspring NOAEL	3.8 mg/kg bw per day
Lowest relevant reproductive NOAEL	771 mg/kg bw per day, highest dose tested
Developmental toxicity	
Target/critical effect	None
Lowest relevant maternal NOAEL	967 mg/kg bw per day, highest dose tested (rat)
Lowest relevant embryo/fetal NOAEL	967 mg/kg bw per day, highest dose tested (rat)
Neurotoxicity	
Acute neurotoxicity NOAEL	No data

Subchronic neurotoxicity NOAEL	1 745 mg/kg bw per day, highest dose tested
Developmental neurotoxicity NOAEL	No data
Other toxicological studies	
Studies on toxicologically relevant metabolites	*Reg. No. 241208:* Mouse LD_{50} oral = 1 937 mg/kg bw Genotoxicity: unlikely to be genotoxic *Reg. No. 406 4702:* Mouse LD_{50} oral = 302 mg/kg bw Not mutagenic in Ames test
Medical data	
	One case of skin allergy possibly related to exposure to flufenoxuron

Summary

	Value	Study	Safety factor
ADI	0–0.04 mg/kg bw	Thirteen-week and 1-year studies of toxicity (dog)	100
ARfD	Unnecessary	–	–

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IMAZAMOX

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Explanation

Imazamox is the International Organization for Standardization (ISO)–approved common name for (+)-2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)-5-(methoxymethyl) nicotinic acid (International Union of Pure and Applied Chemistry) (Chemical Abstracts Service No. 114311-32-9). Imazamox is an imidazolinone herbicide used pre- or post-emergence of weeds.

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

This evaluation is based mainly on the study reports submitted by the sponsor. All critical studies contained statements of compliance with good laboratory practice (GLP), unless otherwise specified. However, GLP status was not specifically checked. In general, studies were conducted with technical material.

A literature search was conducted by the authors on 20 June 2014 in the databases PubMed and PubMedCentral (keyword: imazamox, no restriction for publication date). Forty-three and 16 references were retrieved, respectively. Articles appearing to be obviously non-relevant for a toxicological or human health evaluation were excluded from the results list based on their titles

and/or abstracts. For the remaining five references, the full articles were retrieved. One article (Peterson & Shama, 2005) did not include toxicological data and was not further considered. Another article (Fragiorge et al., 2008) investigated the effects of an imazamox-containing formulation on *Drosophila melanogaster* larvae and is therefore less relevant for the evaluation of the active ingredient. The remaining three articles were included in the evaluation and are described in the appropriate sections.

Evaluation for acceptable daily intake

1. Biochemical aspects

1.1 Absorption, distribution and excretion

The absorption, tissue distribution, metabolism (see section 1.2) and excretion (ADME) of [6-pyridine-^{14}C]imazamox or [6-pyridine-^{13}C]imazamox[1] were studied in male and female Sprague-Dawley rats administered either an intravenous or oral dose of these compounds (Chiu, 1995). Forty-eight rats were placed on test, including four preliminary-phase animals (two of each sex), four control animals and 40 test animals (five of each sex for each of the following four dose groups: low-dose intravenous 10 mg/kg body weight [bw]; low-dose oral 10 mg/kg bw; low-dose multiple oral 10 mg/kg bw; high-dose oral 1000 mg/kg bw). Urine and faeces were collected from all treated groups at prescribed intervals. Animals were terminated 7 days after administration of the radiolabelled dose. Selected tissue samples were collected, weighed and analysed for total radioactivity.

Imazamox was eliminated primarily via the urine and secondarily via the faeces (Table 1). Elimination occurred rapidly within 6 hours post-dosing. Radiolabelled imazamox equivalents in the tissues were generally below, or in a few cases only just slightly above, detection limits and accounted for at most about 0.007% of the actual administered dose for all treatment groups (Chiu, 1995).

In another ADME study, male and female Sprague-Dawley rats were administered an oral dose of [6-pyridine-^{14}C]imazamox or [6-pyridine-^{13}C]imazamox (see footnote 1 for chemical structure) (Chiu, 1996). Seventy-six animals were placed on test, including four preliminary-phase animals (two of each sex), four control animals and 68 test animals (four of each sex for each of low-dose oral 10 mg/kg bw and high-dose oral 1000 mg/kg bw for blood pharmacokinetics; nine of each sex for each of low-dose oral 10 mg/kg bw and high-dose oral 1000 mg/kg bw for tissue distribution/balance; four of each sex for each of low-dose oral 10 mg/kg bw and high-dose oral 1000 mg/kg bw for biliary excretion). The results of a further control group receiving sham treatment only are of little relevance for the present evaluation. Urine and faeces were collected from four of the five treated groups at prescribed intervals. Those rats were terminated 7 days after administration of the radiolabelled dose. Selected tissue and excreta samples were collected and analysed for total radioactivity.

Imazamox was eliminated primarily via the urine and secondarily via the faeces. Biliary excretion was not an important route of elimination (Table 2). Elimination was very efficient and occurred within 48 hours post-dosing. Maximum blood concentrations were reached within 0.5–1 hour, depending on the dose levels (Table 3); the terminal half-life was relatively fast (20 minutes to 1 hour) (Chiu, 1996).

[1] Chemical structure (* denotes the position of the ^{14}C or ^{13}C label):

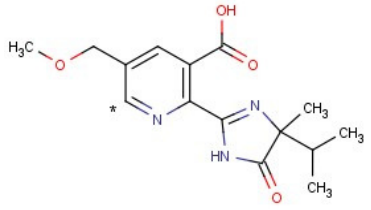


Table 1. Route of excretion and total recovery of imazamox in rat

Group	Target dose (mg/kg bw)	Route of administration	Sex of animal	% of radioactive dose ± standard deviation		
				Urine[a]	Faeces	Total
P (preliminary)	10	Single oral	M + F	68.6	19.9	88.5
A (low dose)	10	Intravenous	M	84.5 ± 10.9	2.7 ± 0.7	88.8[b] ± 10.3
			F	91.2 ± 7.0	1.9 ± 0.9	93.3[b] ± 6.4
B (low dose)	10	Single oral	M	74.5 ± 16.0	18.7 ± 10.7	93.2 ± 7.3
			F	74.4 ± 3.7	24.0 ± 2.8	98.3 ± 1.7
C (low dose)	10	Repeated oral	M	82.1 ± 11.3	17.2 ± 9.8	99.3 ± 1.8
			F	74.0 ± 10.6	24.2 ± 10.2	98.2 ± 1.0
D (high dose)	1 000	Single oral	M	80.2 ± 3.6	12.2 ± 3.7	92.4 ± 1.6
			F	79.3 ± 3.6	12.8 ± 3.7	92.1 ± 1.6

F: female; M: male

[a] Includes cage rinse, cage wash and cage wipe.

[b] Includes swab.

Source: Chiu (1995)

Table 2. Route of excretion and total recovery of imazamox in rat

Group	Target dose (mg/kg bw)	Route of administration	Sex of animal	% of radioactive dose			
				Urine	Faeces	Bile	Total
P (preliminary)	10	Single oral	M	74.9	21.8	NA	96.7
			F	77.6	16.6	NA	94.2
G (low dose)	10	Single oral	M	80.0	17.1	NA	97.1
			F	79.4	19.9	NA	99.3
H (high dose)	1 000	Single oral	M	88.1	11.5	NA	99.6
			F	79.6	17.2	NA	96.8
I (bile cannulation, low dose)	10	Single oral	M	67.6[a]	27.8	2.55	98.4
			F	68.5[a]	27.6	1.37	98.3
J (bile cannulation, high dose)	1 000	Single oral	M	67.1[a]	25.7	2.62	95.9
			F	75.5[a]	22.5	1.75	101.3

F: female; M: male; NA: not applicable

[a] Includes cage wash and cage wipe.

Source: Chiu (1996)

Table 3. Toxicokinetic parameters of imazamox in rat

Group	Target dose (mg/kg bw)	Route of administration	Sex of animal	T_{max} (h)	C_{max} (ppm)	$t_{½\ term}$ (h)	AUC_{0-t} (ppm·h)
E (low dose)	10	Single oral	M	0.53	4.59	0.36	6.40
			F	0.44	5.60	0.34	6.91
F (high dose)	1 000	Single oral	M	1.02	354	0.97	1 047
			F	1.03	342	1.00	1 099

AUC_{0-t}: area under the concentration–time curve from time 0 to time t; C_{max}: maximum concentration in blood; F: female; M: male; ppm: parts per million; $t_{½\ term}$: terminal half-life; T_{max}: time to reach C_{max}

Source: Chiu (1996)

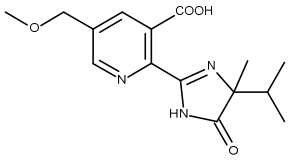
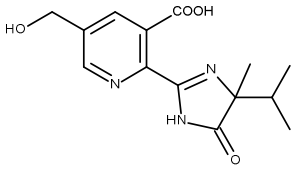
The toxicokinetic properties of ^{14}C-^{15}N-labelled imazamox[2] were investigated in male and female Wistar rats after application of a single oral dose of 500 mg/kg bw (Fabian & Landsiedel, 2012). The mean total recovery of radioactivity was found to be approximately 98% of the administered dose for male and female rats. The excretion via urine over the observation period of 168 hours amounted to 78% of the dose for males and 74% for females. The major part of the urinary excretion (73% of the administered dose for males and 65% for females) occurred within the first day after test substance administration. Smaller amounts of the test substance were excreted through faeces. These amounts accounted for 19% of the dose for males and 23% for females. After the observation period, the remaining radioactivity in the carcass amounted to 0.01% of the dose for both sexes, indicating fast excretion of the orally administered test substance. The absorbed dose (calculated as sum of percentage dose values in urine, cage wash, carcass and organs, with the exception of the gastrointestinal tract) showed similar values for both sexes and was estimated to be 78% and 75% of the administered dose for males and females, respectively.

In conclusion, orally dosed ^{14}C-^{15}N-labelled imazamox (500 mg/kg bw) was excreted mainly in the urine of male and female rats during the 1-week observation period. Under the test conditions, the estimated absorption of imazamox was comparable for both sexes and was determined to be about 75% of the administered dose (Fabian & Landsiedel, 2012).

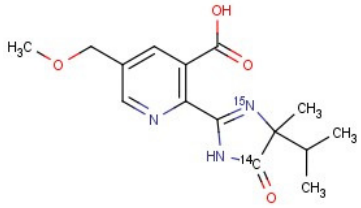
1.2 Biotransformation

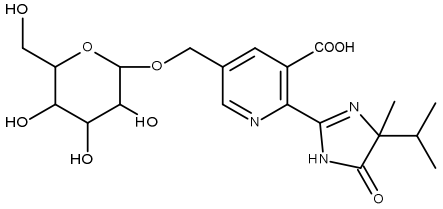
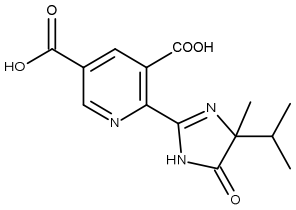
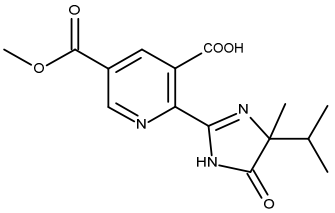
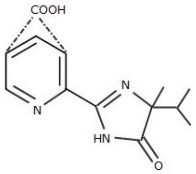
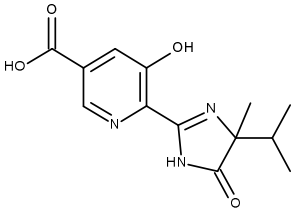
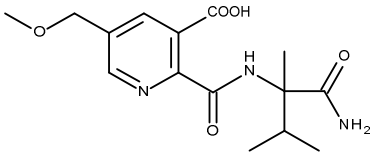
Selected excreta samples from the first ADME study (Chiu, 1995) were analysed for the presence and identity of parent compound and metabolites. Imazamox was primarily eliminated as unchanged test compound via the urine. The amount of total dose that was excreted via faeces consisted mostly of unchanged test compound plus minor amounts of the metabolites *O*-demethylated imazamox (CL 263284) and its subsequent carboxylic acid (CL 312622), together with likely trace amounts of the methyl ester of imazamox (CL 303190) and *N*-methyl-imazamox, in which the imidazole ring is *N*-methylated (Table 4, Fig. 1).

Table 4. Identity of selected substances

Codes	Structure
Imazamox BAS 720 H; CL 299263	
CL 263284; M715H001; 4110773	

[2] Chemical structure showing position of labels:

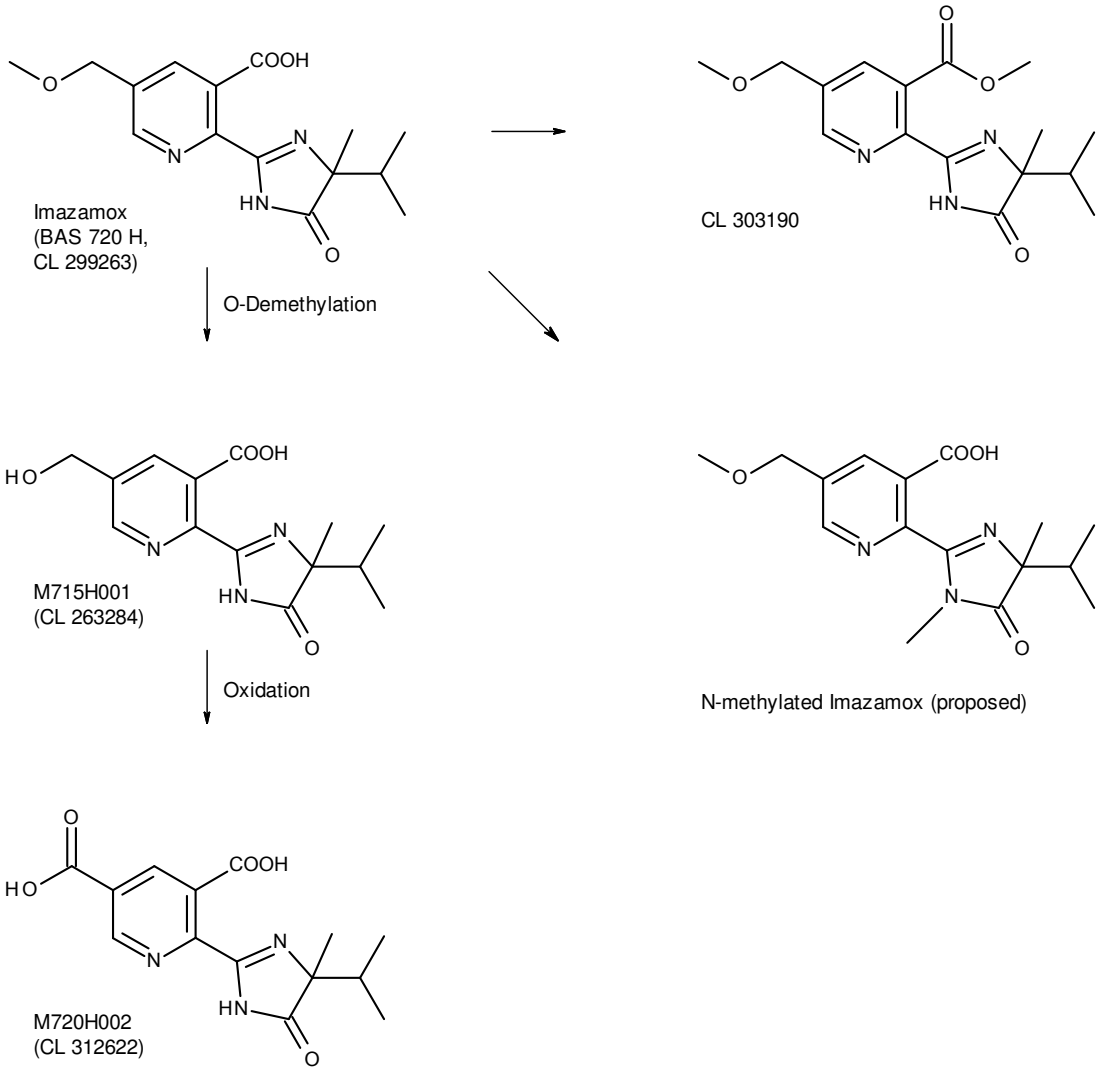


Codes	Structure
CL 189215; M715H002; 4110445	
CL 312622; M720H002; 4110542	
SES15698	
SES15996	
CL 354825	
CL 336554	

Source: Modified from Doi & Amouroux (2013)

Selected tissue and excreta samples from the second ADME study (Chiu, 1996) were analysed for the presence and identity of parent compound and metabolites. Imazamox was primarily eliminated as unchanged test compound via the urine and secondarily via the faeces. The amount of total dose that was excreted via urine, faeces and bile consisted mostly of unchanged test compound plus minor amounts of *O*-demethylated imazamox (CL 263284) and its subsequent carboxylic acid (CL 312622). The same metabolite profile was also found in liver, kidney and muscle extracts.

Fig. 1. Proposed metabolic pathway of imazamox in rats



Source: Doi & Amouroux (2013)

Urinary and faecal samples from the study by Fabian & Landsiedel (2012) were further analysed for the presence and identity of parent compound and metabolites (Thiaener & Lutz, 2012). In both sexes, imazamox was rapidly excreted via urine and faeces as the unchanged parent compound. About 73% (females) and 77% (males) of the applied dose were excreted within 96 hours as parent compound via urine. Additionally, about 21% (females, 0–72 hours) and 15% (males, 0–72 hours) of the dose were excreted as parent compound via faeces. Therefore, in total, 94% (females) and 92% (males) of the dose were excreted as imazamox via urine and faeces.

The *O*-demethylated imazamox (CL 263284), its subsequent carboxylic acid (CL 312622) and the respective ester (SES15698) (Table 4), which were identified in urine and faeces of both sexes, were already present as impurities in the dosing solution at a comparable order of magnitude. No significant increase in these components in rat excreta was observed, which could have been attributed to metabolism. Moreover, the isotope patterns of these components did not match those of the parent compound, which confirms the assumption that the amounts of CL 263284, CL 312622 and SES15698 detected in urinary and faecal samples were not formed by metabolism of imazamox in the rat. A further metabolite (SES15996) was detected in faeces, which is most probably a

decarboxylation product of CL 312622. As its isotope pattern also did not match that of the parent compound, it was likely not a rat metabolite of imazamox (Thiaener & Lutz, 2012).

2. Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

Submitted studies on the median lethal dose (LD_{50}) values determined in experimental animals are summarized in Table 5.

Table 5. LD_{50} values determined in experimental animals

Route	Species, study design	Results	Source
Oral	Rat Similar to OECD TG 401	LD_{50} > 5 000 mg/kg bw	Fischer (1995a)
Oral	Mouse OECD TG 401	LD_{50} > 5 000 mg/kg bw	Bradley (1995a)
Dermal	Rabbit Similar to OECD TG 402	LD_{50} > 4 000 mg/kg bw	Fischer (1995c)
Inhalation	Rat OECD TG 403	LC_{50} > 6.3 mg/L (> 1.6 mg/L of respirable particles, i.e. size < 3 µm)	Hoffman (1994)

bw: body weight; LC_{50}: median lethal concentration; LD_{50}: median lethal dose; OECD TG: Organisation for Economic Co-operation and Development Test Guideline

(b) Dermal irritation

In a skin irritation study in rabbits (Fischer, 1995d), conducted similarly to Organisation for Economic Co-operation and Development (OECD) Test Guideline 404, very slight erythema was reported for 2/6 treated animals at the 24-hour observation time, which had resolved at the 48-hour observation time. No erythema was noted in the other animals at the 24-hour observation time or in any animals at all other observation times. No signs of oedema or other observations were reported in any animals at any observation time. No mortalities or systemic reactions occurred during the study period.

(c) Ocular irritation

In an eye irritation study (Fischer, 1996b) conducted according to OECD Test Guideline 405, 0.1 g of the test substance, imazamox technical, was instilled into the conjunctival sac of each of six female New Zealand White rabbits. After a 24-hour exposure period, treated eyes were rinsed with tap water. One hour after application, slight redness of the conjunctivae, slight to moderate chemosis and moderate ocular discharge were noted in all six rabbits. At the 24-hour observation time, all animals exhibited slight to moderate redness of the conjunctivae; four of six rabbits exhibited scattered and diffuse areas of corneal opacity, slight chemosis and a mild to moderate ocular discharge; and one of six rabbits exhibited mild iritis. By 48 hours, corneal opacities and iritis had resolved in all animals; however, all six animals continued to exhibit slight to moderate conjunctival redness, whereas 2/6 exhibited slight chemosis and slight ocular discharge. At 72 hours, all signs of irritation had resolved in 2/6 animals, with the remaining four rabbits still exhibiting slight conjunctival redness. All symptoms ceased after 7 days. No mortalities or systemic reactions occurred during the study period.

(d) Dermal sensitization

The skin sensitizing potential of imazamox was assessed according to the Buehler method in a study in which 10 guinea-pigs were treated with 0.2 g imazamox once per week for 3 weeks (Glaza, 1992). Upon challenge, no animal showed skin reactions. No mortalities or systemic reactions occurred during the study period. All guinea-pigs treated with the positive control substance, 2,4-dinitrochlorobenzene, developed skin reactions during both the induction and the challenge phases. It should be noted that a group size of 10 treated animals is too small to allow a firm conclusion to be reached; OECD Test Guideline 406 recommends a minimum of 20 animals per treatment group.

In another study, the skin sensitizing potential of imazamox was assessed in guinea-pigs using the maximization method of Magnusson and Kligman, according to OECD Test Guideline 406 (Glaza, 1996). In the induction phase, the test group received 0.1 mL intradermal injections of Freund's Complete Adjuvant (FCA), the test substance (5% weight per volume [w/v] suspension in mineral oil) and a mixture of FCA and the test substance (5% w/v suspension in a 50% solution of FCA in water) at posterior dorsal injection sites. Controls received FCA, 1:1 vehicle:FCA or vehicle alone, respectively. To induce skin irritation, the animals were dermally treated with 10% sodium lauryl sulfate after 7 days for 24 hours and then treated with a 25% weight per weight [w/w] mixture of imazamox or vehicle alone under occlusive conditions for 48 hours. Two weeks later, following a 24-hour occlusive topical challenge with the test substance or vehicle, the test sites were assessed. Upon challenge, none of the 20 treated guinea-pigs showed skin reactions. No mortalities or systemic reactions occurred during the study period. There was no effect on body weight gain. Although no concurrent positive control was included, in a separate study (same laboratory, same method, similar time frame), 10/10 guinea-pigs treated with hexylcinnamaldehyde showed skin reactions during the challenge phase.

2.2 Short-term studies of toxicity

(a) Oral administration

Mice

No short-term toxicity studies conducted with mice were submitted.

Rats

In a 28-day range-finding study, groups of five male and five female Sprague-Dawley rats received diets containing imazamox at a concentration of 0, 5000, 10 000 or 20 000 parts per million (ppm) (equal to 0, 607, 1248 and 2434 mg/kg bw per day for males and 0, 616, 1217 and 2441 mg/kg bw per day for females, respectively) (Fischer, 1996a). Rats were observed daily for signs of overt toxicity, morbidity and mortality. Detailed clinical observations, individual body weights and individual feed consumption were recorded weekly. At termination, all surviving rats were subjected to a gross necropsy, and selected organs were weighed. Samples of selected tissues were submitted for histopathological evaluation from all rats in the 0 and 20 000 ppm groups only.

No mortalities or treatment-related clinical signs were observed during the study period. Feed consumption values for treated males and females were generally similar to those of control rats at all measurement times. Body weights and body weight gains of males at all treatment levels were similar to, or in excess of, those of control rats at all measurement times. Body weights were slightly decreased in females of all treated groups at most measurement times, decreases attaining statistical significance among females of the 10 000 and 20 000 ppm groups during study week 3. Body weight gains were statistically significantly reduced among females of all dose groups during study week 1, resulting in overall body weight gain depressions of 7%, 10% and 8% in the 5000, 10 000 and 20 000 ppm groups, respectively. Decreases in body weight and body weight gain observed for treated females were not considered toxicologically significant, as no dose–response relationship was evident, similar findings were not observed in males and similar findings were not observed in females in the 13-week rat dietary toxicity study (see below) or during the first 4 weeks of treatment in the 2-year rat study (see below), at concentrations up to and including 20 000 ppm.

No haematology, blood chemistry, eye or urinary parameters were evaluated in this study. Absolute and relative (to body weight) liver weights were statistically significantly increased for males in the 10 000 ppm group only. These increases were not considered treatment related, given the absence of an effect at 20 000 ppm. No gross pathological or histopathological changes were reported.

The no-observed-adverse-effect level (NOAEL) was 20 000 ppm (equal to 2434 or 2441 mg/kg bw per day for males and females, respectively), based on the absence of adverse effects up to the highest dose level tested (Fischer, 1996a).

In a 13-week dietary toxicity study, groups of 10 male and 10 female Sprague-Dawley rats received diets containing imazamox at a concentration of 0, 1000, 10 000 or 20 000 ppm (equal to 0, 76, 785 and 1550 mg/kg bw per day for males and 0, 86, 880 and 1772 mg/kg bw per day for females, respectively) (Fischer, 1995b). Rats were observed daily for signs of overt toxicity, morbidity and mortality. Ophthalmological examinations were conducted on study day 0 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 rats at termination of the study. At termination, all surviving animals were subjected to a gross necropsy, and selected organs were weighed. Samples of selected tissues from all test rats were submitted for histopathological evaluation.

No mortalities were observed during the 13-week study period. No clinical signs of toxicity were reported that were attributed to treatment with imazamox. Body weights were not adversely affected by treatment. Feed consumption values for treated males and females were comparable to or greater than those of control animals at all measurement intervals, attaining statistical significance in females at 1000 ppm during weeks 2, 3 and 13 of the study. No statistically significant changes were reported in haematological, clinical chemistry or urinary parameters for either sex at any treatment level. No indication of treatment-related ocular abnormalities were observed at the termination of the study. Changes in some absolute organ weights (liver, kidney, heart and spleen) observed in females of the low-dose group were considered not adverse due to the lack of a dose–response relationship and corroborating findings. No gross pathological or histopathological changes were attributed to treatment with imazamox.

The NOAEL was 20 000 ppm (equal to 1550 or 1772 mg/kg bw per day for males and females, respectively), based on the absence of adverse effects up to the highest dose level tested (Fischer, 1995b).

Dogs

In a 90-day dietary toxicity study, groups of four male and four female Beagle dogs received diets containing imazamox at a concentration of 0, 1000, 10 000 or 40 000 ppm (equal to 0, 34, 329 and 1333 mg/kg bw per day for males and 0, 36, 381 and 1403 mg/kg bw per day for females, respectively) (Kelly, 1994). Physical observations, ophthalmoscopic examinations, body weight and feed consumption measurements, haematology, clinical chemistry and urine analyses were performed on all animals pretest and at selected intervals during the treatment period. After at least 90 days of treatment, all animals were terminated, selected organs were weighed and organ/body weight and organ/brain weight ratios were calculated. Complete gross postmortem examinations and histopathological evaluation of selected tissues were conducted on all animals.

No mortalities were reported during the 13-week study period. No clinical signs of toxicity were attributed to treatment with imazamox. Feed consumption and feed efficiency values as well as body weights and body weight gains were similar between control and treated animals. A haematological evaluation performed during study week 6 revealed a statistically significant increase in absolute lymphocyte values for males fed 40 000 ppm imazamox. However, these values fell within historical control data ranges and were not statistically significantly different from those of controls at termination. Thus, this increase was not considered to be treatment related. Clinical chemistry, urine analysis and ophthalmological parameters did not indicate any adverse effects due to dietary

administration of imazamox. Absolute and relative organ weights were similar between control and treated animals, and there were no gross or microscopic findings attributed to treatment with this test material.

The NOAEL was 40 000 ppm (equal to 1333 and 1403 mg/kg bw per day for males and females, respectively), based on the absence of adverse effects up to the highest dose level tested (Kelly, 1994).

In a 12-month dietary toxicity study, groups of five male and five female Beagle dogs received diets containing imazamox at a concentration of 0, 1000, 10 000 or 40 000 ppm (equal to 0, 29, 283 and 1174 mg/kg bw per day for males or 0, 30, 282 and 1156 mg/kg bw per day for females, respectively) (Kelly, 1995b). Physical observations, ophthalmoscopic examinations, body weight and feed consumption measurements, haematology, clinical chemistry and urine analyses were performed on all animals pretest and at selected intervals during the treatment period. After at least 1 year of treatment, all animals were terminated, selected organs were weighed and organ/body weight and organ/brain weight ratios were calculated. Complete gross postmortem examinations and histopathological evaluation of selected tissues were conducted on all animals.

No mortalities occurred during the study period. No clinical signs of toxicity were attributed to treatment with imazamox. Feed consumption and feed efficiency values as well as body weights and body weight gains for treated and control animals were comparable. Haematological and urine analysis parameters evaluated at 3 and 6 months and at termination were also comparable between treated and control animals. Although sporadic instances of statistically significant differences in clinical chemistry parameters were observed for treated and control animals, values for treated animals were considered to be within normal biological limits, were not consistently observed at different time intervals and were not observed in both sexes. Thus, these differences were not attributed to administration of imazamox. At study termination, evaluation for ocular changes gave no indication of treatment-related effects. Absolute and relative organ weights were comparable for control and treated animals. Macroscopic and microscopic changes observed occurred sporadically among control and treated groups and were considered to be incidental findings and not treatment related.

The NOAEL was 40 000 ppm (equal to 1174 and 1156 mg/kg bw per day for males and females, respectively), based on the absence of adverse effects up to the highest dose level tested (Kelly, 1995b).

(b) Dermal application

Rats

In a dermal toxicity study, groups of five male and five female Sprague-Dawley rats were dermally administered imazamox at a dose level of 0, 250, 500 or 1000 mg/kg bw per day (6 hours/day, 5 days/week) for 28 days (Blaszcak, 1995). The test material was uniformly spread on a gauze dressing and moistened with 0.5 mL of 0.9% saline, and then the gauze was applied to the skin of the animals. Physical observations, ophthalmoscopic examinations and body weight and feed consumption measurements were performed on all animals pretest and at selected intervals during the treatment period. Haematology and clinical chemistry evaluations were performed on all animals at study termination. After 28 days of treatment, all animals were euthanized. Selected organs were weighed, and organ/body weight and organ/brain weight ratios were calculated. Complete macroscopic postmortem examinations were conducted on all animals. Histopathological evaluation of selected tissues was performed on all animals in the control and high-dose groups; macroscopic lesions were examined for animals in all dose groups at study termination.

No mortalities or clinical signs of toxicity could be attributed to treatment throughout the study period. In addition, no signs of dermal irritation were observed. Feed consumption and mean body weight and body weight gains for treated groups were comparable to those of the controls. Haematology and clinical chemistry values were also similar among treated and control animals. At

termination of the study, no test material–related ocular changes were reported. Absolute organ weights were unaffected by the administration of imazamox. Changes in relative brain weights observed in males of the low-dose group were considered not adverse due to the lack of a dose–response relationship and corroborating findings. There were no compound-related macroscopic or microscopic changes observed in any evaluated rat.

The NOAEL was 1000 mg/kg bw per day, based on the absence of adverse effects up to the highest dose level tested (Blaszcak, 1995).

(c) Exposure by inhalation

No short-term toxicity studies with inhalation exposure were submitted.

2.3 Long-term studies of toxicity and carcinogenicity

Mice

In a carcinogenicity study, groups of 55 male and 55 female CD-1 mice received diets containing imazamox at a concentration of 0, 500, 3500 or 7000 ppm (equal to 0, 73, 535 and 1053 mg/kg bw per day for males and 0, 96, 664 and 1348 mg/kg bw per day for females, respectively) for 18 months (Kelly, 1995a). Physical observations and body weight and feed consumption measurements were performed on all animals pretest and on all survivors at selected intervals during the treatment period. Haematology was performed on 10 mice of each sex per group at month 12 and at termination. After at least 18 months of treatment, all survivors were euthanized, selected organs were weighed and organ/body weight and organ/brain weight ratios were calculated. Complete macroscopic postmortem examinations were performed on all animals. Microscopic evaluation of selected tissues was conducted on all animals in the control and high-dose groups at termination as well as for all mice dying during the study. In addition, the kidneys, liver, lungs and gross lesions were examined for all low- and mid-dose mice euthanized at termination.

At study termination, survival was similar among the control and treated groups. Survival rates were 76%, 76%, 73% and 73% for males and 82%, 76%, 74% and 74% for females in the 0, 500, 3500 and 7000 ppm groups, respectively. Observed clinical signs of toxicity were not attributed to treatment with imazamox. Although statistically significant differences were occasionally observed for mean weekly, biweekly and monthly body weights, body weight gains and feed consumption, no consistent trend towards increases or decreases in these parameters for males or females in any treatment group was evident. No treatment-related haematological effects were noted either at the 12-month observation time or at study termination. Absolute and relative organ weights were comparable with those of controls at termination of the study. Macroscopic or microscopic findings of toxicological significance at any treatment level were considered not to be associated with dietary administration of imazamox because of the lack of a dose–response relationship.

No carcinogenic effects were reported for the test material up to the highest dose level tested.

The NOAEL was 7000 ppm (equal to 1053 and 1348 mg/kg bw per day for male and female mice, respectively), based on the absence of adverse effects up to the highest dose level tested (Kelly, 1995a).

Rats

In a long-term toxicity and carcinogenicity study, groups of 65 male and 65 female Sprague-Dawley rats received diets containing imazamox at a concentration of 0, 1000, 10 000 or 20 000 ppm (equal to 0, 52, 528 and 1068 mg/kg bw per day for males and 0, 63, 626 and 1284 mg/kg bw per day for females, respectively) for 2 years (Fischer & Hess, 1995). The rats were observed daily for signs of overt toxicity, morbidity and mortality. Detailed clinical observations were recorded weekly during the 24-month study period. Ophthalmological examinations were done on all test animals on study day 2 and on all surviving animals at termination (24 months). Individual body weights and feed consumption data were recorded weekly during the first 14 weeks of the study, at biweekly intervals

from weeks 14 to 26 and monthly for the remainder of the 24-month study period. Samples for haematological, clinical chemistry and urine analysis determinations were collected from 10 rats of each sex per dose level at 6-month intervals during the study period. At termination, all surviving animals were subjected to a gross necropsy, and selected organs were weighed (10 rats of each sex per dose level). Gross necropsies were also performed on unscheduled deaths (found dead, euthanized moribund, accidental deaths) that occurred during the study. Samples of selected tissues were submitted for histopathological evaluation from all surviving test animals and from any unscheduled deaths that occurred during the study.

Survival was unaffected by dietary administration of the test material. Survival rates (excluding accidental deaths) were 28%, 28%, 31% and 27% for males and 33%, 26%, 31% and 33% for females in the 0, 1000, 10 000 and 20 000 ppm groups, respectively. None of the clinical signs of toxicity observed were attributed to treatment with imazamox technical. Feed consumption for both male and female rats at all treatment levels was generally comparable to or greater than that of controls. Body weights for male rats administered imazamox in the diet were comparable with those of controls at most measurement intervals. Slight decreases in body weights noted for females fed 20 000 ppm were not considered treatment related, given that they occurred late in the 24-month study period (from week 74), were observed in only one sex and were not statistically significant. Although weekly weight gains for both sexes fed the test diet were generally comparable with those of controls, overall body weight gains over the 24-month study period for males fed treated diets were slightly increased, whereas overall body weight gains for females treated at 20 000 ppm were slightly decreased (8.7%, Table 6). This slight decrease in overall body weight gain observed for females in the high-concentration group was considered unrelated to treatment, as it likely reflected the slight decrease in body weight observed for that group beginning at week 74. Moreover, total body weight gain for females in the 20 000 ppm group for the majority of the study period (from weeks 1 to 66) was comparable with that of controls.

Table 6. Body weight gains in treated rats

	Body weight gain (g, mean ± SD)							
	Males				**Females**			
Weeks	**0 ppm**	**1 000 ppm**	**10 000 ppm**	**20 000 ppm**	**0 ppm**	**1 000 ppm**	**10 000 ppm**	**20 000 ppm**
0–14	468.2 ± 55.86	469.3 ± 55.28	470.4 ± 63.90	468.1 ± 53.41	233.3 ± 33.38	227.9 ± 40.50	230.4 ± 38.88	229.6 ± 34.16
0–104	613.3 ± 124.30	725.9 ± 154.99	678.4 ± 160.13	730.5 ± 130.05	440.5 ± 167.13	457.3 ± 144.90	409.6 ± 115.96	402.0 ± 139.81
		(+18.4%)	(+10.6%)	(+19.1%)		(+3.8%)	(−7.0%)	(−8.7%)

ppm: parts per million; SD: standard deviation

Source: Fischer & Hess (1995)

No treatment-related haematological, clinical chemistry or urine analysis effects were noted at the 6-, 12- or 18-month observation times or at study termination (24 months). Ophthalmoscopic examination at study termination revealed no treatment-related findings. Absolute kidney weights as well as kidney/brain weight and kidney/body weight ratios for male rats fed 10 000 ppm were significantly increased compared with controls. Given the absence of a dose–response relationship and the lack of a similar response in corresponding females, this increase was not considered toxicologically significant. No other significant changes were observed in either absolute or relative organ weights for males or females at any treatment level. No macroscopic or microscopic findings of toxicological significance at any treatment level were attributed to dietary administration of imazamox technical.

No carcinogenic effects were induced by the test material; however, owing to the low survival, the power of the study design regarding this end-point is limited. The study did not include groups designated for an interim termination to allow a toxicological evaluation after 1 year.

The NOAEL was 20 000 ppm (equal to 1068 and 1284 mg/kg bw per day for males and females, respectively), based on the absence of adverse effects up to the highest dose level tested (Fischer & Hess, 1995).

2.4 Genotoxicity

Results of the submitted genotoxicity/mutagenicity studies are summarized in Table 7.

Table 7. Results of submitted genotoxicity/mutagenicity studies

End-point	Test object	Concentration	Purity (%)	Result	Reference
In vitro					
Bacterial mutagenicity test (similar to OECD TG 471)	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA1538 and *Escherichia coli* WP2 uvrA	0, 100, 500, 1 000, 2 500 and 5 000 µg/plate	98.2	Negative	Mulligan (1995a)
Chromosome aberration test (similar to OECD TG 473)	Chinese hamster ovary cells	0, 417, 833, 1 667 and 3 333 µg/mL	97.1	Negative[a]	Kumaroo (1994)
Hgprt locus mutation assay (similar to OECD TG 476)	Chinese hamster ovary cells	0, 50, 100, 500, 1 000, 2 000 and 4 000 µg/mL	98.2	Negative[a]	Sharma (1993b)
In vivo					
Micronucleus assay (similar to OECD TG 474)	Mouse	0, 1 250, 2 500 and 5 000 mg/kg bw (single dose administration)	98.2	Negative[b]	Sharma (1993a)

Hgprt: hypoxanthine–guanine phosphoribosyltransferase; OECD TG: Organisation for Economic Co-operation and Development Test Guideline

[a] Top dose limited by solubility but not by toxicity.
[b] No signs of toxicity (including changes in the proportion of polychromatic erythrocytes) were reported.

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

Groups of 30 male and 30 female Sprague-Dawley rats were fed diets containing imazamox at a concentration of 0, 1000, 10 000 or 20 000 ppm (equal to 0, 76, 770 and 1554 mg/kg bw per day for males and 0, 88, 892 and 1826 mg/kg bw per day for females, respectively, during the premating period) for two generations (Schroeder, 1995). Both parental generations were treated during a premating period of 10–11 weeks, and treatment continued during both a 20-day mating period and a post-mating period. Mated females continued to be treated during the ensuing gestation, lactation and post-weaning periods until termination. Parental animals (P, F_1) were observed twice daily for

mortality and unusual findings, and each animal received a detailed physical examination weekly; vaginal smear samples were evaluated daily for parental females (P, F_1) to evaluate estrous cycling for a 2-week period prior to initiation of mating. Body weights and feed consumption for the parental animals were recorded weekly during the premating treatment periods, and these parameters continued to be recorded weekly for males during the post-mating period until termination. Body weights and feed consumption were recorded for females at regular intervals during the gestation and lactation periods. Each parental generation produced a single litter, and pups were weaned on lactation day 21. On lactation day 4, litters with more than eight pups were culled to that number so as to equalize sex distributions (four of each sex) when possible; litters with eight or fewer pups on lactation day 4 were not adjusted. During lactation, litter size, pup weights and pup sex distribution data were recorded, and several pup developmental landmarks (pinna detachment, eye opening, hair growth and incisor eruption) were scored as achieved. Randomly selected pups from the F_1 litters (at least one pup of each sex per litter) were chosen to become the F_1 parental generation. Preputial separation and vaginal opening were scored for F_1 pups retained after the day 28 selections to eventually become the F_1 parental animals.

At termination, parental animals were given a gross postmortem examination, and reproductive tissues and pituitary glands were taken and preserved in 10% neutral buffered formalin; gross lesions were also taken and preserved for all parental animals. Reproductive tissues were evaluated histomorphologically for P and F_1 control and high-dose animals, and gross lesions were evaluated for all animals. The unselected F_1 pups were terminated soon after the day 28 weighing interval and evaluated for external irregularities; pups with external findings were also evaluated internally, and abnormal tissues were saved (10% neutral buffered formalin). Additionally, at day 21 or soon thereafter, one pup of each sex per litter per group for each litter interval was selected at random and given a detailed macroscopic evaluation, and abnormal tissues were saved.

No treatment-related mortality occurred. During the premating treatment period, mean weekly feed consumption for males and females of both parental generations was either comparable with or slightly higher than that of controls (10 000 and 20 000 ppm groups only). Additionally, no adverse effects on feed consumption were reported from treatment of either P or F_1 males with imazamox technical during the post-mating periods. No adverse effect of treatment was indicated by maternal feed consumption during the gestation and lactation intervals for both parental generations as well.

Mean weekly body weights for treated males and females of both parental generations were comparable with those of controls during the premating treatment period; mean body weights for treated males of both parental generations were also comparable with those of controls during the mating and post-mating periods. Mean weight gains for P and F_1 males and females treated with 1000 and 10 000 ppm and for P and F_1 males treated with 20 000 ppm were comparable with those of controls during the premating period (Table 8). Although P females in the 20 000 ppm group exhibited mean weight gains that were similar to those of controls during the premating period, F_1 females exhibited a statistically significant decrease (−11.3%) in mean weight gain over this period. Given the absence of similar decreases for P females or for P or F_1 males at 20 000 ppm, this slight decrease in mean weight gain was considered unlikely to be of biological significance. No treatment-related effects on maternal body weight or weight gain during the gestation and lactation intervals were observed for either parental generation.

Reproductive performance (estrous cycle data, mating indices, pregnancy rates, male fertility indices, gestation indices and parturition indices) was unaffected by treatment with imazamox technical. F_1 litter size was statistically significantly lower in the top-dose group, but stayed within the laboratory's historical control range. Mean litter size data both pre-cull (prior to neonatal day 4) and throughout the remainder of lactation for the treated groups were comparable with those for controls for both litter intervals. There were no treatment-related effects during either litter interval concerning litter or pup survival indices; mean pup weights at birth, during lactation and on neonatal day 28; pup sex distribution; pup developmental landmarks; or the number of dead pups at birth or during the 21-day lactation period. No gross macroscopic findings were observed for either parental or pup generations. The mean number of uterine implantation scars in the treated groups was considered comparable with control data for each litter interval and was also similar to the mean total number of

respective pups born. No microscopic compound-related changes were observed. In summary, the types and frequencies of observations/lesions seen among the treated animals were similar to those seen commonly in the performing laboratory for the strain of rats used.

Table 8. Premating body weight gains in treated rats

	Body weight gain (g, mean ± SD)							
	Males				**Females**			
	0 ppm	**1 000 ppm**	**10 000 ppm**	**20 000 ppm**	**0 ppm**	**1 000 ppm**	**10 000 ppm**	**20 000 ppm**
P generation (weeks 0–10)	304.0 ± 36.5	300.9 ± 32.9	295.1 ± 33.8	291.4 ± 25.4	110.9 ± 15.1	113.4 ± 17.9	111.6 ± 13.1	105.0 ± 15.0
F_1 generation (weeks 20–31)	304.0 ± 49.6	300.9 ± 43.7	312.4 ± 58.0	289.1 ± 52.0	127.5 ± 16.6	118.2 ± 19.1	122.1 ± 23.3	113.1* ± 19.0

F_1: first filial; P: parental; ppm: parts per million; SD: standard deviation; *: $P \leq 0.05$

Source: Schroeder (1995)

In summary, the NOAEL for adverse effects on parental animals, offspring and reproduction was 20 000 ppm (equal to 1554 and 1826 mg/kg bw per day for males and females, respectively), based on the absence of adverse effects up to the highest dose level tested (Schroeder, 1995).

(b) Developmental toxicity

Rats

In a developmental toxicity study, groups of 25 pregnant Sprague-Dawley rats were treated with an imazamox dose of 0, 100, 500 or 1000 mg/kg bw per day via gavage (Foss, 1994). The treatment period was gestation days 6–15. Current test guidelines require daily administration from implantation to the day prior to scheduled caesarean section; hence, the study design may have been less sensitive than required according to current standards. All rats were euthanized by carbon dioxide asphyxiation on day 20 of presumed gestation. The uterus of each rat was excised, weighed and examined for pregnancy, number and distribution of implantations, live and dead fetuses, and early and late resorptions. The number of corpora lutea was recorded for each dam. Each fetus was weighed and examined for sex, gross external alterations and soft tissue or skeletal alterations.

No mortalities, abortions or premature deliveries occurred during the study; no clinical signs were observed that were attributed to treatment. Absolute and relative feed consumption values for the entire dosing and post-dosing periods tended to be reduced in the 1000 mg/kg bw per day dose group (Table 9); however, none of these reductions was statistically significant. Reflecting these findings, body weights tended to be reduced in the 1000 mg/kg bw per day dose group on days 8 through 20 of gestation (Table 9). A statistically significant reduction in body weight gain during days 6–12 of gestation was observed for animals in the 1000 mg/kg bw per day dose group compared with controls; however, body weight gains were comparable among the four dose groups for the remainder of the dosing period as well as for the post-dosing period. Gravid uterine weights were not affected by administration of the test compound at any dose level, and there were no gross lesions identified at necropsy.

Fetal/litter evaluations occurred on day 20 of gestation following caesarean sectioning of the dams. Litter averages for corpora lutea, implantations, litter sizes, live fetuses, early and late resorptions, fetal weights, per cent resorbed conceptuses and per cent male fetuses were comparable among the four dose groups. Similarly, no gross external, soft tissue or skeletal malformations or variations were observed in fetuses that were caused by treatment of the dams with imazamox at doses as high as 1000 mg/kg bw per day. One fetus from the high-dose group, but none of the fetuses

from the other groups, was reported to have short, broad, bent ribs. As a result of this low incidence, this finding was considered not to be an adverse treatment-induced finding.

Table 9. Selected findings in treated pregnant rats

	Finding (mean ± SD)			
	0 mg/kg bw per day	**100 mg/kg bw per day**	**500 mg/kg bw per day**	**1 000 mg/kg bw per day**
Number of rats tested	25	25	25	25
Number of pregnant rats	24	24	25	25
Feed intake (absolute: g/day; relative: g/kg bw per day)				
Absolute, GDs 6–16	28.2 ± 2.6	28.7 ± 3.3	28.0 ± 2.6	27.0 ± 3.3
Absolute, GDs 16–20	33.1 ± 4.0	32.8 ± 2.3	32.2 ± 2.9	31.7 ± 3.5
Relative, GDs 6–16	88.6 ± 4.6	90.8 ± 6.9	88.7 ± 5.3	86.6 ± 6.1
Relative, GDs 16–20	82.6 ± 5.6	82.4 ± 4.4	81.9 ± 4.4	81.5 ± 5.5
Body weight (g)				
Maternal body weight, GD 8	293.8 ± 14.3	290.8 ± 12.7	292.7 ± 18.0	288.4 ± 17.7
Maternal body weight, GD 12	325.9 ± 18.8	322.4 ± 15.4	321.9 ± 18.6	315.2 ± 22.0
Maternal body weight, GD 16	363.2 ± 20.6	362.0 ± 20.3	358.7 ± 19.2	353.8 ± 26.2
Maternal body weight, GD 20	439.0 ± 30.5	436.4 ± 20.8	430.1 ± 31.0	426.4 ± 34.2
Gravid uterine weight	87.8 ± 9.7	88.2 ± 6.4	81.8 ± 20.2	84.3 ± 20.7
Body weight gain, GDs 6–12	44.0 ± 9.0	43.0 ± 7.4	38.4 ± 12.8	33.8* ± 14.0
Body weight gain, GDs 6–16	81.3 ± 10.2	82.6 ± 11.1	75.3 ± 13.8	72.4 ± 19.0
Body weight gain, GDs 16–20	75.8 ± 15.0	74.4 ± 6.8	71.4 ± 15.0	72.6 ± 13.0
Body weight gain, GDs 6–20	157.1 ± 23.2	157.0 ± 11.7	146.7 ± 25.8	145.0 ± 28.1

bw: body weight; GD: gestation day; SD: standard deviation; *: $P \leq 0.05$

Source: Foss (1994)

The NOAEL for adverse effects on maternal animals was 500 mg/kg bw per day, based on reduction of body weight gain and feed intake at 1000 mg/kg bw per day. The NOAEL for adverse effects on offspring was 1000 mg/kg bw per day, based on the absence of adverse effects up to the highest dose level tested (Foss, 1994).

Rabbits

In a developmental toxicity study, groups of 20 pregnant New Zealand White rabbits were treated with imazamox at a dose of 0, 300, 600 or 900 mg/kg bw per day via gavage (Hoberman, 1995). The treatment period was gestation days 7–19. Current test guidelines require daily administration from implantation to the day prior to scheduled caesarean section; hence, the study design may have been less sensitive than required according to current standards. All rabbits were observed for viability at least twice each day of the study and for general appearance several times during acclimation and on day 0 of presumed gestation. The rabbits were also examined for clinical observations of effects of the test substance, abortions, premature deliveries and deaths immediately before and after intubation (days 7 through 19 of presumed gestation). These observations were also made once daily during the post-dosing period (days 19 through 29 of presumed gestation). Body weights were recorded on days 0 and 7 through 29 of presumed gestation. Feed consumption values

were recorded daily throughout the study. The rabbits were terminated on day 29 of presumed gestation and necropsied. The number of corpora lutea in each ovary was recorded. The uterus was excised, weighed and examined for pregnancy, number and distribution of implantations, early and late resorptions, and live and dead fetuses. Each fetus was weighed, sexed and examined for gross external, soft tissue and skeletal alterations.

No mortalities or abortions occurred during the study. One doe in the 900 mg/kg bw per day group prematurely delivered a litter on day 29 of gestation; eight of the 10 conceptuses were live pups, and two of the 10 conceptuses were presumed cannibalized. This premature delivery was considered a possible effect of the test substance, as this doe had exhibited reduced body weight and feed consumption after day 11 of gestation. No other doe prematurely delivered a litter. No clinical signs were observed that were considered related to test substance intake.

Although absolute and relative feed consumption values for the entire dosing period were reduced in all treatment groups compared with control values (Table 10; 3% for 300 mg/kg bw per day, 12–13% for 600 mg/kg bw per day and 15–16% for 900 mg/kg bw per day), these effects were considered biologically significant only in the 600 and 900 mg/kg bw per day dose groups. For the majority of the dosing period (900 mg/kg bw per day) or for the entire dosing period (600 mg/kg bw per day), the pattern of decreased feed consumption increased with continued dosing. In addition, statistically significant reductions in absolute and relative feed consumption values were observed for the intermediate- and high-dose groups on days 7–29 of gestation. The reductions in feed intake were observed after a few days of dosing only. A statistically significant reduction in relative feed intake was observed in low-dose rabbits between gestation days 7 and 29. However, the mean relative feed consumption value was well within 10% of the control group value; thus, the change in the low-dose group was not considered biologically significant.

Table 10. Selected findings in treated pregnant rabbits

	Finding (mean ± SD)[a]			
	0 mg/kg bw per day	**300 mg/kg bw per day**	**600 mg/kg bw per day**	**900 mg/kg bw per day**
Number of rabbits tested	20	20	20	20
Number of pregnant rabbits	20	18	15	20
Feed intake (absolute: g/day; relative: g/kg bw per day)				
Absolute, GDs 7–20	181.4 ± 2.5 (*n* = 17)	175.7 ± 13.6	158.0 ± 20.9	152.4** ± 23.4 (*n* = 19)
Relative, GDs 7–20	49.1 ± 3.2 (*n* = 17)	47.8 ± 3.7	43.4** ± 5.6	41.5** ± 5.6 (*n* = 19)
Absolute, GDs 7–29	175.9 ± 5.7	167.2 ± 15.1	157.2** ± 17.5	155.5** ± 17.8 (*n* = 19)
Relative, GDs 7–29	46.7 ± 3.0	44.1* ± 2.6	42.2** ± 4.7	41.6** ± 4.5
Body weight gain (kg)				
GDs 7–20	0.27 ± 0.10	0.31 ± 0.06	0.24 ± 0.12	0.22 ± 0.14
GDs 20–29	0.24 ± 0.10	0.20 ± 0.08	0.21 ± 0.11	0.19 ± 0.09 (*n* = 19)
GDs 7–29	0.51 ± 0.12	0.51 ± 0.10	0.45 ± 0.16	0.44 ± 0.12 (*n* = 19)

bw: body weight; GD: gestation day; SD: standard deviation; **: $P \leq 0.01$

[a] *n* is given in parentheses wherever the value is not based on the total number of pregnant rabbits in the group.

Source: Hoberman (1995)

No statistically significant differences were observed in body weights or body weight gains for the entire dosing and post-dosing periods for treated rabbits compared with controls. However, a biologically significant reduction in body weight gain was noted during the dosing period (19%) and post-dosing period (21%) for does dosed at 900 mg/kg bw per day. Gravid uterine weights were not affected by administration of imazamox technical in any dose group. Gross necropsy findings for the does were considered unrelated to test substance intake.

Fetal litter evaluations for all remaining pregnant does occurred on day 29 of gestation following caesarean sectioning of the does. Litter averages for corpora lutea, implantations, litter sizes, live fetuses, early and late resorptions, fetal weights and per cent male fetuses were comparable among the four dose groups. The incidences of absent intermediate lung lobules and of vertebral malformations (thoracic hemivertebrae, cervical hemivertebrae) were increased in animals treated with 600 or 900 mg/kg bw per day (Table 11). The study report did not include a more detailed description of these findings. No further fetal gross external, soft tissue or skeletal malformations or variations observed were considered effects of the test substance.

Table 11. Incidence of selected findings in offspring

	Number of affected fetuses/number of affected litters			
	0 mg/kg bw per day	**300 mg/kg bw per day**	**600 mg/kg bw per day**	**900 mg/kg bw per day**
Number of litters evaluated	20	18	14	19
Number of live fetuses evaluated	160	148	116	160
Soft tissue alterations				
Lungs: absent intermediate lobe[a]	1/1 (0.6%/5.0%)	0/0	2/2 (1.7%/14.3%)	6**/4 (3.8%/21.0%)
Skeletal alterations				
Vertebral malformations	0/0	0/0	2/2 (1.7%/14.3%)	4/2 (2.5%/10.5%)
Vertebra, cervical hemivertebra	0/0	0/0	1/1 (0.9%/7.1%)	3/2 (1.9%/10.5%)
Vertebra, thoracic hemivertebra	0/0	0/0	1/1 (0.9%/7.1%)	2/1 (1.2%/5.3%)

bw: body weight; **: $P \leq 0.01$

[a] According to the report, the absence of other lung lobes was not observed in this study.

Source: Hoberman (1995)

Historical control data from the performing laboratory regarding the findings “lungs, one or more lobes partially or complete agenesis”, “vertebra, cervical hemivertebra” and “vertebra, thoracic hemivertebra” are summarized in Table 12. It should be noted that these historical control data on “lungs, one or more lobes partially or complete agenesis” also include findings in addition to the specific finding observed in fetuses in this study; hence, they may be of less relevance.

The NOAEL for adverse effects on maternal rabbits was 300 mg/kg bw per day, based on reduction of feed intake at 600 mg/kg bw per day, which was considered to be of equivocal toxicological relevance. The NOAEL for adverse effects on offspring was 300 mg/kg bw per day, based on an increased incidence of both absent intermediate lung lobes and hemivertebrae at 600 mg/kg bw per day (Hoberman, 1995).

Table 12. Historical control data on selected findings in rabbit fetuses

	Historical control data (range)		
	1990 to June 1992	**June 1992 to June 1995**	**June 1994 to June 1996**
Number of studies	49 (soft tissue and skeletal)	36 (soft tissue) 35 (skeletal)	17 (soft tissue) 18 (skeletal)
Number of litters examined	790 (soft tissue and skeletal)	593 (soft tissue) 586 (skeletal)	297 (soft tissue) 316 (skeletal)
Number of live fetuses examined	5 892 (soft tissue) 5 891 (skeletal)	4 479 (soft tissue) 4 436 (skeletal)	2 425 (soft tissue) 2 544 (skeletal)
Lungs: one or more lobes, partial or complete agenesis			
Number of litters affected	0–6 (0–35.3%)	0–5 (0–29.4%)	0–5 (0–29.4%)
Number of fetuses affected	0–12 (0–8.7%)	0–13 (0–6.9%)	0–9 (0–6.9%)
Vertebral malformations	Alteration not mentioned	Alteration not mentioned	Alteration not mentioned
Vertebra, cervical hemivertebra			
Number of studies with alteration	1	Alteration not mentioned	Alteration not mentioned
Number of litters affected	0–1 (0–5.3%)		
Number of fetuses affected	0–1 (0–0.6%)		
Vertebra, thoracic hemivertebra			
Number of studies with alteration	11	7	3
Number of litters affected	0–1 (0–7.1%)	0–1 (0–7.7%)	0–1 (0–5.9%)
Number of fetuses affected	0–2 (0–1.5%)	0–1 (0–1.1%)	0–1 (0–0.8%)

Source: Hoberman (1995)

2.6 Special studies

(a) Toxicity of metabolites

Submitted studies on the LD_{50} values determined in experimental animals and the genotoxicity studies conducted with metabolites CL 312622 (2-(4-isopropyl-4-methyl-5-oxo-3*H*-imidazol-2-yl)pyridine-3,5-dicarboxylic acid), CL 263284 (5-(hydroxymethyl)-2-(4-isopropyl-4-methyl-5-oxo-3*H*-imidazol-2-yl)pyridine-3-carboxylic acid) and CL 189215 (2-(4-isopropyl-4-methyl-5-oxo-3*H*-imidazol-2-yl)-5-({[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy}methyl)pyridine-3-carboxylic acid) are summarized in Tables 13, 14 and 15, respectively. The chemical structures of these metabolites are reproduced in Table 4. In the assessment of these three metabolites using Toxtree software (version 2.6.6), they were assigned to Cramer class III, which is the same class as for the parent compound.

In a 28-day dietary toxicity study, groups of five male and five female Wistar rats received diets containing metabolite CL 263284 at a concentration of 0, 1200, 4000 or 12 000 ppm (equal to 0, 102, 333 and 1004 mg/kg bw per day for males and 0, 104, 339 and 1028 mg/kg bw per day for females, respectively) (Buesen et al., 2013). Rats were observed daily for signs of overt toxicity, morbidity and mortality. Detailed clinical observations, individual body weights and cage feed consumption were recorded weekly. Drinking-water consumption was monitored daily by visual inspection. Towards the end of the administration period, a functional observational battery was

Table 13. Results of submitted toxicity studies with metabolite CL 312622 (M720H002; Reg. No. 4110542)

Study	Species/test system	Findings	Reference
Oral LD_{50}	Sprague-Dawley rats	> 5 000 mg/kg bw (males and females)	Bradley (1995c)
Microbial mutagenicity assay (Ames)	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA1538; *Escherichia coli* WP2 uvrA-	+S9: Negative −S9: Negative (at 0, 312.5, 625, 1 250, 2 500 and 5 000 µg/plate)	Mulligan (1995d)
In vitro/ micronucleus test	Chinese hamster lung V79 cells	+S9: Negative −S9: Negative (at 0, 4.5, 8.9, 17.9, 35.8, 71.6, 143.2, 286.4, 572.8, 1 145.5 and 2 291.0 µg/mL)	Bohnenberger (2013b)
Gene mutation assay (HPRT)	Chinese hamster ovary cells	+S9: Negative −S9: Negative (at 0, 218.8, 437.5, 875.0, 1 750.0 and 3 500.0 µg/mL and 0, 500.0, 1 000.0, 2 000.0 and 3 500.0 µg/mL [+S9 only])	Kapp & Landsiedel (2013)

bw: body weight; HPRT: hypoxanthine–guanine phosphoribosyltransferase; LD_{50}: median lethal dose; S9: 9000 × *g* supernatant fraction from rat liver homogenate

Table 14. Results of submitted toxicity studies with metabolite CL 263284 (M715H001; Reg. No. 4110773)

Study	Species/test system	Findings	Reference
Oral LD_{50}	CD-1 mice	> 5 000 mg/kg bw (males and females)	Bradley (1995b)
Microbial mutagenicity assay (Ames)	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA1538; *Escherichia coli* WP2 uvrA-	+S9: Negative −S9: Negative (at 0, 312.5, 625, 1 250, 2 500 and 5 000 µg/plate)	Mulligan (1995c)
In vitro/ micronucleus test	Chinese hamster lung V79 cells	+S9: Positive −S9: Negative (at 0, 5.0, 10.0, 20.1, 40.2, 80.3, 100.0, 160.6, 200.0, 300.0, 321.3, 400.0, 500.0, 600.0, 642.5, 800.0, 1 200.0, 1 285.0, 1 400.0, 1 600.0, 2 000.0 and 2 570.0 µg/mL)	Bohnenberger (2013a,d)
In vivo/ micronucleus test	NMRI mice (bone marrow)	Negative (at 0, 500, 1 000 and 2 000 mg/kg bw)	Schulz & Mellert (2013)
28-day dietary study	Wistar rats	NOAEL: 333 mg/kg bw per day (M) 1 028 mg/kg bw per day (F) LOAEL: 1 004 mg/kg bw per day (M, bw and bw gain ↓) > 1 028 mg/kg bw per day (F)	Buesen et al. (2013)

bw: body weight; F: female; LD_{50}: median lethal dose; LOAEL: lowest-observed-adverse-effect level; M: male; NOAEL: no-observed-adverse-effect level; S9: 9000 × *g* supernatant fraction from rat liver homogenate

Table 15. Results of submitted toxicity studies with metabolite CL 189215 (M715H002; Reg. No. 4110445)

Study	Species/test system	Findings	Source
Microbial mutagenicity assay (Ames)	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA1538; *Escherichia coli* WP2 uvrA-	+S9: Negative −S9: Negative (at 0, 312.5, 625, 1 250, 2 500 and 5 000 µg/plate)	Mulligan (1995b)
In vitro/ micronucleus test	Chinese hamster lung V79 cells	+S9: Negative −S9: Negative (at 0, 11.4, 22.8, 45.5, 91.1, 182.2, 364.4, 728.8, 1 457.5, 2 915.0 and 5 830.0 µg/mL)	Bohnenberger (2013c)

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

performed on all animals. Haematological, clinical chemistry and urine analysis parameters were determined in five animals of each sex per test group. At termination, all surviving rats were subjected to a gross necropsy, and selected organs were weighed. Samples of selected tissues from all rats in the 0 and 12 000 ppm groups only were submitted for histopathological evaluation.

Test material was distributed homogeneously within the diet and stable for 11 days at room temperature. The concentrations in diet achieved the expected range.

No mortalities or treatment-related clinical signs, including functional observational battery changes, were observed during the study period. Feed consumption values for treated males and females were generally similar to those of control rats at all measurement times. Body weights and body weight gains of females at all treatment levels were similar to those of control rats at all measurement times (Table 16). Body weights were decreased in males of the top-dose group on study days 21 and 28. Body weight gains were statistically significantly reduced among males of the top-dose group on study days 21 and 28. Body weight and body weight gain in males of the low- and mid-dose groups were similar to those of control rats at all measurement times.

Table 16. Selected body weight and body weight gains in rats treated with metabolite CL 263284 (M715H001; Reg. No. 4110773) in a 28-day study

	Body weight/body weight gain (g, mean ± SD)							
	Males				Females			
	0 ppm	1 200 ppm	4 000 ppm	12 000 ppm	0 ppm	1 200 ppm	4 000 ppm	12 000 ppm
Day 0	154.7 ± 5.7	154.7 ± 2.8	155.2 ± 4.8	155.2 ± 4.1	128.1 ± 3.2	125.9 ± 3.5	128.4 ± 1.3	125.9 ± 2.6
Day 28	288.1 ± 20.4	295.6 ± 6.7	292.1 ± 3.9	266.0 ± 21.4	185.5 ± 8.7	181.1 ± 8.3	188.5 ± 3.7	184.5 ± 5.8
Days 0–7	41.5 ± 4.8	43.4 ± 3.9	43.8 ± 2.8	38.4 ± 2.9	19.0 ± 2.4	16.1 ± 4.0	18.6 ± 1.1	20.9 ± 3.6
Days 0–15	84.3 ± 8.3	88.3 ± 6.6	84.9 ± 6.9	72.2 ± 8.3	32.8 ± 4.6	33.3 ± 6.8	29.0 ± 3.0	34.7 ± 8.0
Days 0–21	113.9 ± 13.8	120.5 ± 6.7	116.6 ± 7.8	93.6* ± 15.5	46.1 ± 8.1	46.1 ± 6.8	47.6 ± 2.7	48.7 ± 5.4
Days 0–28	133.4 ± 15.7	141.0 ± 8.8	136.9 ± 5.7	110.8* ± 18.5	57.4 ± 7.5	55.2 ± 5.9	60.1 ± 3.1	58.6 ± 5.1

ppm: parts per million; SD: standard deviation; *: $P < 0.05$

Source: Buesen et al. (2013)

Haematological, blood chemistry and urine analysis parameters either were comparable with those observed in control animals or did not show a dose–response relationship. Absolute and relative (to body weight) testes weights and absolute liver weights were statistically significantly increased for males in the 1200 ppm group only. These increases were not considered treatment related, given the absence of an effect at 4000 or 12 000 ppm. No adverse organ weight changes were reported in treated females. No gross pathological or histopathological changes were reported.

The NOAEL was 4000 ppm (equal to 333 mg/kg bw per day) for males, based on reduced body weights and body weight gains at 12 000 ppm (equal to 1004 mg/kg bw per day). The NOAEL was 12 000 ppm (equal to 1028 mg/kg bw per day) for females, based on the absence of adverse effects up to the highest dose level tested (Buesen et al., 2013).

(b) Other studies

Imazamox was tested for its ability to induce phototoxic effects in Balb/c 3T3 cells in vitro (Cetto & Landsiedel, 2012). The photo-cytotoxicity was estimated using the neutral red uptake (NRU) method. A single experiment was carried out, with and without irradiation with an ultraviolet A (UVA) light source. The test compound was tested at concentrations of 0, 4.6, 10.0, 21.5, 46.4, 100.0, 215.4, 464.2 and 1000.0 µg/mL.

In this study, no cytotoxicity in the absence or the presence of UVA irradiation was observed up to the highest required concentration indicated by the NRU method. The positive control chlorpromazine led to the expected cytotoxicity both with and without UVA irradiation, thus demonstrating the ability of the test system to detect phototoxicity.

Under the experimental conditions of this study, imazamox is considered not to be a phototoxic substance in the in vitro 3T3 NRU phototoxicity test using Balb/c 3T3 cells (Cetto & Landsiedel, 2012).

Technical imazamox was tested for its acute toxic potential in groups of three male and three female ICR mice when administered once intraperitoneally at a dose level of 0, 78.1, 313, 1250 or 5000 mg/kg bw (Futagawa, 1995). Additionally, groups of four male Japanese White rabbits were dosed once orally with technical imazamox at 0 or 5000 mg/kg bw. The behaviour of male and female mice was observed prior to administration, at 0.5, 1, 3 and 6 hours after administration and then once a day for 4 days. Clinical signs and cardiorespiratory parameters of male rabbits were measured before administration and at 0.5, 1, 3, 6 and 24 hours after administration.

Clinical signs in mice included decreases in awareness and motor activity, abnormal posture, lack of motor coordination, decreases in muscle tone and reflexes, and inhibitory abnormal autonomic signs at intraperitoneal doses of 1250 and 5000 mg/kg bw. These signs were noted 30 minutes following administration of the test material. Mortality occurred within 6 hours following administration for all mice in the 5000 mg/kg bw dose group. Animals in the 1250 mg/kg bw dose group recovered within 2 days after administration. No abnormal clinical signs were noted at a dose level of 313 mg/kg bw or lower.

Rabbits dosed orally with the test material at 5000 mg/kg bw exhibited no abnormal signs with regard to behaviour and somatic and autonomic profiles. No changes in respiration, blood pressure, electrocardiogram or heart rate were observed in male rabbits (Futagawa, 1995).

A comparison of the in vitro metabolism of imazamox in humans and those species that were used in in vivo toxicological testing was performed (draft report without signatures or quality assurance statement; Funk & Taraschewski, 2013) to investigate whether a metabolite occurs in human samples that might not be sufficiently covered by the animal testing.

^{14}C-^{15}N-labelled[3]/unlabelled imazamox mix was incubated with dog, rabbit, rat, mouse or human liver microsomes in the presence of a nicotinamide adenine dinucleotide phosphate (NADPH)–generating system. With 90% recovered radioactivity and above, only the parent molecule was detected in all test systems by high-performance liquid chromatographic analysis in fresh samples after the incubation. Under the conditions of the study, imazamox was not metabolized by liver microsomes of dogs, rabbits, rats, mice or humans. No unique human metabolite was detected. Under the conditions of the study, the positive control, testosterone, was metabolized by the microsome samples originating from different species (Funk & Taraschewski, 2013).

A summarizing article (Sipes et al., 2013) describes selected work conducted under the United States Environmental Protection Agency's (USEPA) ToxCast programme, but does not report primary data. According to the authors, two of 331 enzymatic and receptor signalling assays were affected by imazamox. In particular, human pregnane X receptor binding was indicated by the authors as being present at the lowest concentration tested.

In an in vitro study, male murine embryonic stem cells were treated with imazamox at a concentration of 0, 0.0125, 0.125, 1.25 or 12.5 µmol/L (Chandler et al., 2011). Cytotoxicity and changes in differentiation to cardiomyocytes were measured. The latter was assessed with antibodies against the α,β-cardiac myosin heavy chain. No change in differentiation was reported, whereas the top dose was cytotoxic to the cells (half maximal activity at 2.5 µmol/L).

In an in vitro study, human embryonic stem cells (WA09) were treated with imazamox at a concentration of 0, 1, 10 or 100 µmol/L (Kleinstreuer et al., 2011). Metabolomic changes were assessed by liquid chromatographic–mass spectrometric analysis. According to the authors, incubation did not induce cytotoxicity. Data were analysed with models to predict developmental toxicity, which were set up with data from (1) pharmaceutical agents or (2) selected pesticides from the USEPA's Toxicity Reference Database (ToxRefDB). Imazamox was predicted with a probability of approximately 0.7% to be a non-developmental toxicant. Considering the limited primary data included in the article, it is difficult to confirm the conclusions.

3. Observations in humans

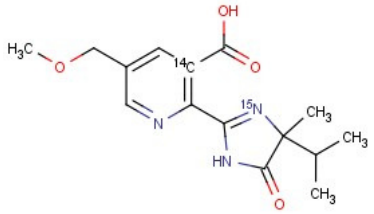
No information or data were provided on adverse health effects in workers involved in the manufacture or use of imazamox. No information on accidental or intentional poisoning in humans was submitted.

Comments

Biochemical aspects

In rats, imazamox was rapidly absorbed, and the oral absorption was approximately 75% of the administered dose. Urine was the major route of excretion (> 74%). Most of the elimination

[3] Chemical structure showing position of labels:



occurred within the first 24 hours after dosing, as unchanged parent compound. Smaller amounts of the test substance were excreted through faeces (> 19% in animals receiving 10 mg/kg bw and approximately 10–20% in animals receiving 1000 mg/kg bw). Only trace amounts of tissue residue were detected. Imazamox appears not to be metabolized. Trace levels of imazamox-related compounds detected in the urine and faeces were attributed to the presence of impurities in the dosing solution, not to rat metabolism.

A comparative in vitro metabolism study was performed in liver microsomes from mice, rats, rabbits, dogs and humans. Under the conditions of the study, no metabolites of imazamox were detected.

Toxicological data

Imazamox was of low acute toxicity after oral, dermal and inhalation exposure. The oral LD_{50} in rats was greater than 5000 mg/kg bw. The dermal LD_{50} in rabbits was greater than 4000 mg/kg bw, and the inhalation LC_{50} in rats was greater than 1.6 mg/L (value for respirable particles only). Imazamox was neither a skin irritant nor an eye irritant in rabbits. In a guinea-pig maximization test, no skin sensitization occurred.

In short-term toxicity studies in rats with dietary administration of imazamox over 28 and 90 days, no adverse effects were reported up to the top dose levels, which were at least 1500 mg/kg bw per day. Similarly, in 90-day and 1-year studies, no adverse effects were reported in dogs receiving imazamox in the diet up to the top dose levels, which were at least 1100 mg/kg bw per day. In long-term toxicity and carcinogenicity studies in mice and rats, no signs of systemic toxicity or treatment-related increases in neoplastic lesions were reported up to the highest dose levels tested, which were approximately 1000 mg/kg bw per day.

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

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

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

In a two-generation study in rats, there was no evidence of adverse effects on parental animals, offspring or reproduction up to the highest tested dietary imazamox concentration of 20 000 ppm (equal to 1554 mg/kg bw per day).

In a rat developmental toxicity study that tested imazamox doses of 0, 100, 500 and 1000 mg/kg bw per day, the NOAEL for maternal toxicity was 500 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 1000 mg/kg bw per day, the highest dose tested.

In a rabbit developmental toxicity study that tested imazamox doses of 0, 300, 600 and 900 mg/kg bw per day, the NOAEL for maternal toxicity was 300 mg/kg bw per day, for decreased feed intake during the dosing period at 600 mg/kg bw per day, which was of equivocal toxicological relevance. Effects on feed intake were not observed during the first days of dosing. The NOAEL for embryo and fetal toxicity was 300 mg/kg bw per day, based on an increased incidence of both absent intermediate lung lobes and hemivertebrae at 600 mg/kg bw per day.

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

Toxicological data on metabolites and/or degradates

The oral LD_{50}s of metabolites CL 312622 (2-(4-isopropyl-4-methyl-5-oxo-3*H*-imidazol-2-yl)pyridine-3,5-dicarboxylic acid) and CL 263284 (5-(hydroxymethyl)-2-(4-isopropyl-4-methyl-5-oxo-3*H*-imidazol-2-yl)pyridine-3-carboxylic acid) were greater than 5000 mg/kg bw in rats and mice, respectively. CL 312622 was tested for genotoxicity in an adequate range of assays in vitro.

No evidence of genotoxicity was observed. CL 263284 was tested for genotoxicity in an adequate range of assays in vitro and in vivo. It gave a positive response in the in vitro micronucleus assay, but was negative in the in vivo micronucleus assay. In a 28-day repeated-dose toxicity study in rats with CL 263284, which tested dietary concentrations of 0, 1200, 4000 and 12 000 ppm (equal to 0, 102, 333 and 1004 mg/kg bw per day for males and 0, 104, 339 and 1028 mg/kg bw per day for females, respectively), the NOAEL was 4000 ppm (equal to 333 mg/kg bw per day), based on lower body weights and significantly lower body weight gains observed in males treated with 12 000 ppm (equal to 1004 mg/kg bw per day). No effects were observed in females up to the highest tested dietary concentration of 12 000 ppm (equal to 1028 mg/kg bw per day). CL 189215 (2-(4-isopropyl-4-methyl-5-oxo-3*H*-imidazol-2-yl)-5-({[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy}methyl)pyridine-3-carboxylic acid) was tested for genotoxicity in a range of assays in vitro, and there was no evidence of genotoxicity.

Human data

No information was provided on the health of workers involved in the manufacture or use of imazamox. No information on accidental or intentional poisoning in humans is available.

The Meeting concluded that the existing database on imazamox 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 based on the NOAEL of 300 mg/kg bw per day for reduced feed intake in dams of equivocal toxicological relevance and an increased incidence of both absent intermediate lung lobes and hemivertebrae in the developmental toxicity study in rabbits, using a safety factor of 100.

The Meeting established an acute reference dose (ARfD) of 3 mg/kg bw based on the NOAEL of 300 mg/kg bw per day for an increased incidence of both absent intermediate lung lobes and hemivertebrae in the developmental toxicity study in rabbits, using a safety factor of 100. Considering the uncertainty as to whether the observed effects on prenatal bone development are also relevant for children's bone growth (bone remodelling), the ARfD is applicable to the whole population.

The plant metabolite CL 263284 is an *O*-demethylation product of imazamox and is a common metabolite with imazapic. Although there is some indication of slightly higher toxicity of this metabolite when compared with imazamox in a 28-day toxicity study in rats, the effects observed were mild changes in body weight gain in males only. Taking into account the close structural similarity to imazamox and the effects and effect levels observed in the developmental toxicity study in rats with imazamox, the Meeting concluded that CL 263284 is of similar toxicity to imazamox and would be covered by the ADI and ARfD for imazamox.

Levels relevant to risk assessment of imazamox

Species	Study	Effect	NOAEL	LOAEL
Mouse	Eighteen-month study of carcinogenicity[a]	Toxicity	7 000 ppm, equal to 1 053 mg/kg bw per day[b]	–
		Carcinogenicity	7 000 ppm, equal to 1 053 mg/kg bw per day[b]	–

Species	Study	Effect	NOAEL	LOAEL
Rat	Ninety-day study of toxicity[a]	Toxicity	20 000 ppm, equal to 1 550 mg/kg bw per day[b]	–
	Two-year study of toxicity and carcinogenicity[a]	Toxicity	20 000 ppm, equal to 1 068 mg/kg bw per day[b]	–
		Carcinogenicity	20 000 ppm, equal to 1 068 mg/kg bw per day[b]	–
	Two-generation reproductive toxicity study[a]	Parental toxicity	20 000 ppm, equal to 1 554 mg/kg bw per day[b]	–
		Reproductive toxicity	20 000 ppm, equal to 1 554 mg/kg bw per day[b]	–
		Offspring toxicity	20 000 ppm, equal to 1 554 mg/kg bw per day[b]	–
	Developmental toxicity study[c]	Maternal toxicity	500 mg/kg bw per day	1 000 mg/kg bw per day
		Embryo and fetal toxicity	1 000 mg/kg bw per day[b]	–
Rabbit	Developmental toxicity study[c]	Maternal toxicity	300 mg/kg bw per day	600 mg/kg bw per day
		Embryo and fetal toxicity	300 mg/kg bw per day	600 mg/kg bw per day
Dog	Ninety-day and 1-year studies of toxicity[a,d]	Toxicity	40 000 ppm, equal to 1 333 mg/kg bw per day[b]	–

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

Estimate of acceptable daily intake (ADI)

0–3 mg/kg bw

Estimate of acute reference dose (ARfD)

3 mg/kg bw

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

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

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

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rats: T_{max} = 0.5–1 hour; extensive, ~75%
Dermal absorption	No data
Distribution	Widespread tissue distribution
Potential for accumulation	No evidence of accumulation
Rate and extent of excretion	Excretion mainly via urine within 48 hours
Metabolism in animals	No metabolism
Toxicologically significant compounds in animals and plants	Parent compound
Acute toxicity	
Rat, LD_{50}, oral	> 5 000 mg/kg bw
Rabbit, LD_{50}, dermal	> 4 000 mg/kg bw
Rat, LC_{50}, inhalation	> 1.6 mg/L (respirable particles)
Rabbit, dermal irritation	Not irritating
Rabbit, ocular irritation	Not irritating
Guinea-pig, dermal sensitization	Not sensitizing (maximization test)
Short-term studies of toxicity	
Target/critical effect	No adverse effects
Lowest relevant oral NOAEL	> 1 000 mg/kg bw per day, highest dose tested (rat and dog)
Lowest relevant dermal NOAEL	1 000 mg/kg bw per day, highest dose tested (rat)
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	No adverse effects
Lowest relevant NOAEL	~1000 mg/kg bw per day, highest dose tested (rat and mouse)
Carcinogenicity	Unlikely to pose a carcinogenic risk to humans
Genotoxicity	
	Unlikely to be genotoxic
Reproductive toxicity	
Target/critical effect	No evidence of reproductive toxicity (rat)
Lowest relevant parental NOAEL	1 554 mg/kg bw per day, highest dose tested
Lowest relevant offspring NOAEL	1 554 mg/kg bw per day, highest dose tested
Lowest relevant reproductive NOAEL	1 554 mg/kg bw per day, highest dose tested
Developmental toxicity	
Target/critical effect	Increased incidence of both absent intermediate lung lobes and hemivertebrae at maternally toxic doses (rabbits)
Lowest relevant maternal NOAEL	300 mg/kg bw per day (rabbit)
Lowest relevant embryo/fetal NOAEL	300 mg/kg bw per day (rabbit)

Neurotoxicity	
Acute neurotoxicity NOAEL	No data
Subchronic neurotoxicity NOAEL	No data
Developmental neurotoxicity NOAEL	No data
Toxicological studies on CL 312622 (plant metabolite)	
Rat LD_{50}, oral	> 5 000 mg/kg bw
Genotoxicity	Unlikely to be genotoxic
Toxicological studies on CL 263284 (plant metabolite)	
Mouse LD_{50}, oral	> 5 000 mg/kg bw
Genotoxicity	Unlikely to be genotoxic in vivo
Twenty-eight day, rat	NOAEL: 333 mg/kg bw per day (based on reduced body weight and body weight gain in males)
Toxicological studies on CL 189215 (plant metabolite)	
Genotoxicity	Unlikely to be genotoxic
Medical data	
	No data

Summary

	Value	Study	Safety factor
ADI	0–3 mg/kg bw	Developmental toxicity study (rabbit)	100
ARfD	3 mg/kg bw	Developmental toxicity study (rabbit)	100

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MESOTRIONE

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Explanation

Mesotrione is the International Organization for Standardization (ISO)–approved common name for 2-(4-mesyl-2-nitrobenzoyl)cyclohexane-1,3-dione (International Union of Pure and Applied Chemistry), with Chemical Abstracts Service number 104206-82-8. It is a new triketone herbicide with a 4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibition mode of action.

Mesotrione 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) or good clinical practice and the Declaration of Helsinki, as appropriate.

Evaluation for acceptable daily intake

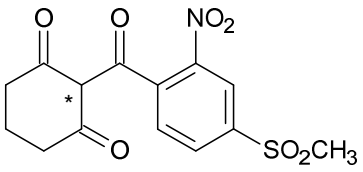
1. Biochemical aspects

The absorption, distribution, metabolism and excretion, as well as the toxicokinetics, of mesotrione have been investigated in CD-1:Crl(ICR)BR mice and Alpk:APfSD rats. Summaries of the relevant data are presented below.

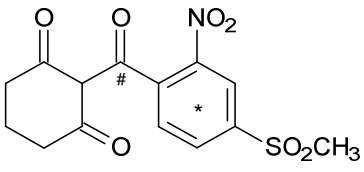
1.1 Absorption, distribution and excretion

The absorption, distribution, metabolism and excretion (ADME) of mesotrione in CD-1:Crl(ICR)BR mice and Alpk:APfSD rats were first investigated using the active substance radiolabelled in the cyclohexane and phenyl rings (Fig. 1). Upon confirmation in biliary elimination and biotransformation studies that there were no pronounced differences in the metabolism of the two labelled forms, subsequent ADME studies were performed with [^{14}C-aromatic]mesotrione (Gledhill, 1996). This finding was confirmed in the qualitative whole-body autoradiographic study in the rat, in that there were no clear differences in tissue distribution profiles between [^{14}C-dione]mesotrione and [^{14}C-aromatic]mesotrione (Prescott & Bennet, 1995). The test item was a mixture of ^{14}C-labelled mesotrione (phenyl or dione label; Fig. 1) and unlabelled mesotrione. Radiolabelled mesotrione was administered via oral gavage in a water/sodium bicarbonate vehicle. The study design is summarized in Table 1.

Fig. 1. Structure of mesotrione with radiolabel positions for the ADME studies



(* = ^{14}C position)



(* = ^{14}C position)
(# = The exocyclic carbonyl group was enriched with ^{13}C for some studies)

Source: Gledhill (1996)

The data generated with mesotrione (including intravenous dosing) indicated that mesotrione is rapidly and extensively absorbed, with almost complete bioavailability.

In mice, total recovery of radioactivity at 72 hours following oral administration ranged from 90.76% to 95.06% of the administered dose, with the main route of excretion being the urine in males and females at 1 or 100 mg/kg body weight (bw) (Table 2). In low-dose groups, the majority of excretion was in the first 6 hours. In high-dose groups, the majority was excreted in the urine in hours 0–6; however, a significant amount of radioactivity was excreted between hours 6 and 12. The majority of radioactivity excreted in the faeces in mice was excreted in the first 12 hours. In the low-dose groups, 14–15% of the administered dose was recovered in the tissues and carcass. In the high-dose groups, less than 0.5% of the administered dose was recovered in the tissues and carcass. Mesotrione was therefore extensively absorbed (40–70% of the administered dose) and rapidly excreted following oral administration.

Following termination at 72 hours, the carcass and select tissues of the animals were examined. Other than the amounts in the gastrointestinal tract and carcass (Table 2), residual radioactivity was found in the liver (13–14% in the low-dose groups and 0.2–0.3% in the high-dose groups) and kidney (0.2% and 1.0% in low-dose males and females, respectively, and 0.003% and 0.015% in high-dose males and females, respectively). Trace amounts of residual radioactivity (< 0.005%) were found in the brain, gonads, heart, lungs and spleen (Gledhill, 1997).

Table 1. Dosing groups for balance/excretion experiments with [^{14}C]mesotrione

Test group	Dose of labelled material (mg/kg bw)	Number of animals of each sex	Remarks	Reference
Single oral low dose in the mouse – Excretion and tissue retention	1	4 mice	A single nominal dose of 1 mg/kg bw (4 mL of 0.25 mg/kg bw) of [^{14}C-aromatic]mesotrione (specific activity for 4 mL: 4 MBq/kg) was administered by oral gavage. Killed after 72 h.	Gledhill (1997)
Single oral high dose in the mouse – Excretion and tissue retention	100	4 mice	A single nominal dose of 100 mg/kg bw (4 mL of 25.4 mg/kg bw) of [^{14}C-aromatic]mesotrione (specific activity for 4 mL: 8 MBq/kg) was administered by oral gavage. Killed after 72 h.	Gledhill (1997)
Single oral low dose in the rat – Excretion and tissue retention	1	5 rats	A single nominal dose of 1 mg/kg bw (4 mL of 0.25 mg/kg bw) of [^{14}C-aromatic]mesotrione (specific activity for 4 mL: 4 MBq/kg) was administered by oral gavage. Killed after 72 h.	Macpherson (1996a)
Single intravenous low dose in the rat – Excretion and tissue retention	1	8 rats	A single nominal dose of 1 mg/kg bw (4 mL of 0.22 mg/kg bw) of [^{14}C-aromatic]mesotrione (specific activity for 4 mL: 4.185 MBq/kg) was administered by injection. Killed after 72 h.	Macpherson (1996b)
Repeated oral low dose in the rat	1	8 rats	*Unlabelled*: Fourteen daily doses of 1 mg/kg bw (4 mL of 0.25 mg/mL) of mesotrione. *Labelled*: A single nominal dose of 1 mg/kg bw (4 mL of 0.26 mg/kg bw) of [^{14}C-aromatic]mesotrione (specific activity for 4 mL: 4.185 MBq/kg) was administered by oral gavage. Killed after 72 h.	Macpherson (1996c)
Single oral high dose in the rat	100	5 rats	A single nominal dose of 100 mg/kg bw (4 mL of 24.94 mg/kg bw) of [^{14}C-aromatic]mesotrione (specific activity for 4 mL: 6 MBq/kg) was administered by oral gavage. Killed after 72 h.	Macpherson (1996d)
Whole-body autoradiography in the rat	5	2 rats per radiolabel	A single nominal dose of 5 mg/kg bw (4 mL/kg bw) of [^{14}C-dione]mesotrione (specific activity for 4 mL/kg bw: 6 MBq/kg) was administered by oral gavage. Killed at 24 and 48 h after dosing. A single nominal dose of 5 mg/kg bw (4.25 mL/kg bw) of [^{14}C-aromatic]mesotrione (specific activity for 4.25 mL/kg bw: 6 MBq/kg) was administered by oral gavage. Killed at 24 and 48 h after dosing.	Prescott & Bennet (1995)
Biotransformation in the rat	50 [^{14}C-dione]-mesotrione 50 or 100 [^{14}C-aromatic]-mesotrione	2 rats per dose per radiolabel	A single nominal dose of 50 mg/kg bw (4 mL/kg bw) of [^{14}C-dione]mesotrione (specific activity of dosing solution 4.15 MBq/g) was administered by oral gavage. A single nominal dose of 50 mg/kg bw (4 mL/kg bw) of [^{14}C-aromatic]mesotrione (specific activity of dosing solution 1.59 MBq/g) was administered by oral gavage. A single nominal dose of 100 mg/kg bw (4 mL/kg bw) of [^{14}C-aromatic]mesotrione (specific activity of dosing solution 3.61 MBq/g) was administered by oral gavage.	Gledhill (1996)

Table 1 (continued)

Test group	Dose of labelled material (mg/kg bw)	Number of animals of each sex	Remarks	Reference
Single oral low dose in the rat – Excretion and distribution	1	4 rats	A single nominal dose of 1 mg/kg bw (4 mL/kg bw) of [^{14}C-aromatic]mesotrione (specific activity of dosing solution 4.13 MBq/kg) was administered by oral gavage. Killed after 7 days.	Duerden (2005a)
Single oral low dose in the rat – Pharmacokinetics and tissue depletion	1	9 rats per phase	A single nominal dose of 1 mg/kg bw (4 mL/kg bw) of [^{14}C-aromatic]mesotrione (specific activity of dosing solution 4.13 MBq/kg) was administered by oral gavage. Serial blood collection at 0.5, 1.5, 1, 2, 3, 4, 6, 8, 10, 12, 18, 24, 30, 48, 72 and 96 h. A single nominal dose of 1 mg/kg bw (4 mL/kg bw) of [^{14}C-aromatic]mesotrione (specific activity of dosing solution 4.13 MBq/kg) was administered by oral gavage. Killed at 1, 6, 12, 24, 48 and 96 h.	Duerden (2005b)
Single oral low dose in the rat – Pharmacokinetics and tissue depletion	100	9 rats per phase	A single nominal dose of 1 mg/kg bw (4 mL/kg bw) of [^{14}C-aromatic]mesotrione (specific activity of dosing solution 4.13 MBq/kg) was administered by oral gavage. Serial blood collection at 0.5, 1.5, 1, 2, 3, 4, 6, 8, 10, 12, 18, 24, 30, 48, 72 and 96 h. A single nominal dose of 1 mg/kg bw (4 mL/kg bw) of [^{14}C-aromatic]mesotrione (specific activity of dosing solution 4.13 MBq/kg) was administered by oral gavage. Killed at 1, 6, 12, 24, 48 and 96 h.	Duerden (2005b)

bw: body weight

In rats, total recovery of radioactivity at 72 hours ranged from 92.02% to 97.06% of the administered dose (Table 3). In all dose groups, excretion in the urine comprised over 50% of the administered dose. Excretion in the faeces constituted 23–30% of the administered dose in orally dosed groups and 2–7% in the intravenously dosed group, indicating that there is a significant amount of the administered dose excreted unabsorbed and that bile excretion is not a major route of excretion in the rat. However, oral absorption was greater than 60% in all dose groups. Residual radioactivity in the tissues and carcass comprised 11–12% in the oral low-dose group, 5% in the oral repeated-dose group and 10% in the intravenous low-dose group. In the oral high-dose group, residual radioactivity in the tissues and carcass comprised 0.7–1% of the administered dose. Results of the single oral low-dose study were confirmed in a second study (Duerden, 2005a), which extended the recovery period to 168 hours (Macpherson, 1996a,b,c,d).

Following termination at 72 hours, the carcass and select tissues of the animals were examined. Other than residual radioactivity in the gastrointestinal tract (0.009–0.031% of the administered dose) and carcass (0.180–1.353% of the administered dose), the only quantifiable residual radioactivity was found in the kidney and liver. In the single oral and intravenous low-dose groups, 9–12% of the administered radioactivity was found in the liver. In the same groups, 0.3% and 0.8–0.9% of the administered dose were found in the kidneys of males and females, respectively. In the single oral high-dose group, 0.2% of the administered dose was found in the liver and 0.01% in the kidneys. In the repeated low-dose group, 4% of the administered dose was found in the liver of males and females and 0.1% and 0.4% in the kidneys of males and females, respectively. In all dose groups, radioactivity

Table 2. Excretion balance in mice at 72 hours post-dosing

	% of administered dose			
	Single oral low dose (1 mg/kg bw)		Single oral high dose (100 mg/kg bw)	
Route and time (h)	**Males**	**Females**	**Males**	**Females**
Urine				
0–6	31.35 ±24.21	51.08 ± 6.64	44.54 ± 17.63	42.68 ± 9.21
6–12	3.07 ± 1.62	4.40 ± 2.27	13.38 ± 5.91	22.20 ± 9.03
12–24	2.55 ± 1.76	1.74 ± 0.64	1.09 ± 0.08	3.37 ± 1.69
24–36	2.23 ± 1.98	0.67 ± 0.34	2.16 ± 2.09	0.99 ± 0.37
36–48	0.86 ± 0.82	0.44 ± 0.14	1.60 ± 2.16	0.39 ± 0.05
48–72	0.56 ± 0.17	0.28 ± 0.20	0.14 ± 0.11	0.08 ± 0.01
Subtotal	**40.64**	**58.61**	**62.90**	**69.82**
Faeces				
0–12	24.88 ± 2.14	16.63 ± 3.37	22.01 ± 2.72	18.06 ± 1.53
12–24	5.41 ± 4.44	2.61 ± 1.44	1.81 ± 1.39	4.53 ± 1.04
24–36	2.60 ± 2.29	0.84 ± 0.24	0.57 ± 0.54	0.98 ± 0.33
36–48	0.63 ± 0.57	0.22 ± 0.03	2.48 ± 3.38	0.62 ± 0.16
48–72	4.13 ± 3.82	0.58 ± 0.15	0.40 ± 0.33	0.28 ± 0.01
Subtotal	**37.66**	**20.88**	**27.27**	**24.46**
Tissues	13.94	13.84	0.18	0.28
Carcass	0.842	0.300	0.096	0.126
Cage wash	0.62	0.90	0.31	0.37
Total	**93.70**	**94.53**	**90.76**	**95.06**

bw: body weight

Source: Gledhill (1997)

in the brain, gonads, heart, lungs or spleen was below 0.001% of the administered dose (Macpherson, 1996a,b,c,d).

Whole-body autoradiography confirmed that the major route of excretion was the urine and that both the kidneys and the liver were subject to tagging by the radiolabelled compounds or their respective metabolites. The gastrointestinal tract and contents appeared to contain the greatest amounts of radiolabelled compound, which would be expected in association with faecal elimination (Prescott & Bennet, 1995).

Pharmacokinetics was generally similar between low and high doses and between sexes. The peak plasma concentrations (C_{max}) were 0.27 and 0.25 µg equivalents (eq) per gram in the low-dose groups of males and females, respectively. In the high-dose groups, the C_{max} values were 40.4 µg eq/g in males and 19.9 µg eq/g in females. The times to reach C_{max} (T_{max}) were 0.5 hour in the low-dose groups and 1.5 hours in the high-dose groups. The half-lives in blood were 1.6 and 1.4 hours in low-dose males and females, respectively, and 1.7 and 1.8 hours in high-dose males and females, respectively (Duerden, 2005b).

Table 3. Recovery of radioactivity in tissues and excreta of rats

	% of administered dose							
	Single low dose[a]		Single high dose[b]		Intravenous dose[c]		Repeated low dose[d]	
	Males	Females	Males	Females	Males	Females	Males	Females
Expired air	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Tissues	12.21	10.17	0.26	0.21	10.01	9.85	4.58	3.98
Carcass	0.25	1.07	0.44	0.90	0.33	0.18	0.53	1.35
Cage wash	0.90	1.36	0.73	0.97	0.46	0.53	0.24	0.76
Urine	54.15	55.88	61.54	63.02	79.40	84.14	60.84	66.97
Faeces	24.51	23.80	30.49	28.77	6.76	2.36	30.27	23.11
Total	**92.02**	**92.28**	**93.46**	**93.87**	**96.96**	**97.06**	**96.46**	**96.17**

N/A: not available

[a] *Source*: Macpherson (1996a).
[b] *Source*: Macpherson (1996d).
[c] *Source*: Macpherson (1996b).
[d] *Source*: Macpherson (1996c).

1.2 Biotransformation

The urinary and faecal samples from the Gledhill (1997) study were subjected to high-performance liquid chromatographic (HPLC) analysis to determine the metabolic fate of mesotrione. The vast majority of mesotrione was excreted as unchanged parent via the urine (43–64%), with 0–8% excreted in the faeces. Proportionately, there was a greater amount of unknown metabolites in the faeces, indicating metabolism of mesotrione by the intestinal flora (0–12% of the administered dose). The chemical structures and retention times of metabolites are provided in Table 4 (Gledhill, 1996).

The proposed metabolic pathway in the rat is shown in Fig. 2 and proceeds by either hydroxylation of the dione ring or, following biliary excretion of unchanged mesotrione, by cleavage of the molecule into its two constituent rings and reduction by the gut microflora (Gledhill, 1996).

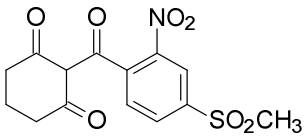
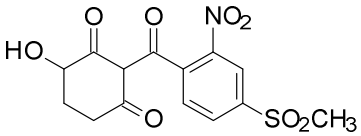
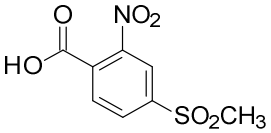
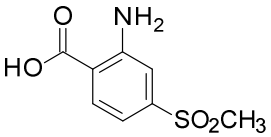
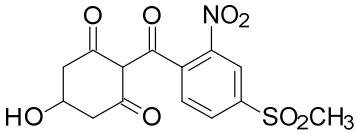
2. Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

Mesotrione is of low acute toxicity in rats via the oral, dermal and inhalation routes (Table 5). All tests were performed at the limit dose. Clinical signs in the oral toxicity study were limited to staining around the nose and lack of body weight gain in females from days 3 to 8. In the dermal toxicity study, staining about the nose was noted in three males, and salivation and urinary incontinence were noted in 3–4 females. Signs of irritation, scabbing, desquamation, oedema and yellow staining were noted at the test site. There were no changes noted at gross necropsy in the oral or dermal studies, and all animals in the dermal toxicity study gained weight throughout the study period. In the inhalation toxicity study, clinical signs of toxicity consisted of hunched posture, piloerection, irregular breathing and/or abnormal respiratory noise, salivation, wet fur, decreased activity, head and paw flicking, decreased response to sound, shaking, reduced righting reflex, mucous secretion from the nose, reduced stability, ptosis, absence of pinna reflex, stains around the nose, splayed gait and chromodacryorrhoea. All animals gained weight following day 2. Pinpoint red spots were noted in the lungs of one male (Robinson, 1994a,b; Rattray, 1995).

Table 4. Chemical structures of identified metabolites

Metabolite designation (code)	Retention time (min)	Structure	Urine	Faeces
Mesotrione	21		+	+
4-Hydroxy mesotrione	15		+	+
MNBA (2-nitro-4-(methylsulfonyl)-benzoic acid)	6		+	+
AMBA (4-(methylsulfonyl)-2-aminobenzoic acid)	9		+	+
5-Hydroxy mesotrione	–		+	+

–: not given

Source: Gledhill (1996)

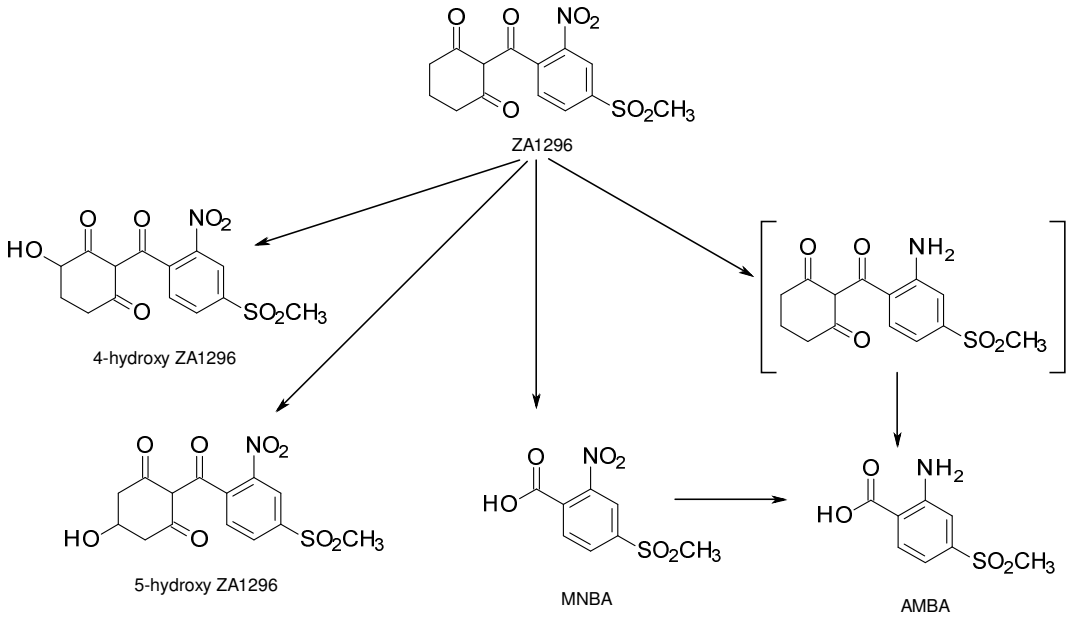
(b) Ocular irritation

In an eye irritation study, 0.1 g mesotrione (purity 95.1%; batch no. WRC4845-32-2) was applied into the conjunctival sac of the left eye of nine female New Zealand White rabbits. In six animals, the eyes were left unwashed, and in a subsequent group of three animals, the eyes were washed immediately following instillation. Animals were then observed for 3 or 4 days. Irritation was scored by the method of Draize. In the non-irrigation study, mild corneal opacity and iritis were noted at 1 hour following instillation, and mild to moderate conjunctivitis was noted from hours 1 to 72. In the irrigation study, there were no signs of corneal opacity or iritis, and mild to moderate conjunctivitis was noted from hours 1 to 48. Mesotrione would be considered mildly irritating to the eyes of rabbit due to continued irritation at 72 hours in the non-irrigation study (Robinson, 1994c).

(c) Dermal irritation

In a dermal irritation study, six adult male New Zealand White rabbits were exposed to 0.5 g mesotrione (purity 95.1%; batch no. WRC14845-32-2) applied to 6.25 cm^2 of body surface area with occlusion for 4 hours. Following exposure, the test site was washed with tap water, and the animals

Fig. 2. Proposed metabolic pathway of mesotrione in rat



Source: Gledhill (1996)

Table 5. Acute toxicity of mesotrione

Species	Strain	Sex	Route	Purity (%)	Result	Reference
Rat	Alpk:APfSD (Wistar-derived)	Male and female	Oral	95.1	LD_{50} > 5 000 mg/kg bw	Robinson (1994a)
Rat	Alpk:APfSD (Wistar-derived)	Male and female	Dermal	95.1	LD_{50} > 2 000 mg/kg bw	Robinson (1994b)
Rat	Alpk:APfSD (Wistar-derived)	Male and female	Inhalation	97.3	LC_{50} > 4.75 mg/L	Rattray (1995)

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

were observed for 3 days. At 1 hour, 2/6 animals exhibited slight erythema and oedema, which cleared by 24 hours. No oedema was observed (Robinson, 1994d).

(d) Dermal sensitization

The skin sensitization potential of mesotrione (purity 95.1%; batch no. WRC14845-32-2) was investigated using the guinea-pig maximization test. Twenty female albino guinea-pigs were assigned to the test group, and an additional 20 female albino guinea-pigs were assigned to the vehicle control group. Test animals were treated with three injections consisting of Freund's Complete Adjuvant plus deionized water (1:1), a 3% weight per volume (w/v) preparation of test material in deionized water and a 3% w/v preparation of test material in a 1:1 preparation of Freund's Complete Adjuvant plus deionized water. Vehicle control animals were treated with three injections of Freund's Complete Adjuvant plus deionized water in a 1:1 ratio, deionized water and Freund's Complete Adjuvant plus deionized water in a 1:1 ratio. The following week, 0.3 g of test substance in a 75% w/v preparation in deionized water for test animals or deionized water for vehicle control animals was applied to an area of the scapular region clipped free of fur. The preparation was kept under occlusive dressing for 2 days. Two weeks following the topical induction application, preparations of 75% w/v mesotrione in

deionized water and 30% w/v mesotrione in deionized water were applied to clipped skin of the left flank of both test and vehicle control animals, and two gauze patches containing only deionized water were applied to the clipped skin of the right flank for 24 hours under occlusive dressing. Irritation was then scored according to Draize.

No irritation was noted in the test animals during the induction phase, and no irritation was noted in the test or vehicle control group following the challenge application. The response in the positive control animals validated the test. It was concluded that mesotrione does not have a sensitizing effect on the skin in the guinea-pig maximization test (Robinson, 1994e).

2.2 Short-term studies of toxicity

(a) Oral administration

Mice

In a 90-day study, groups of 20 (40 control) C57BL/10JfCD-1 mice of each sex per dose received mesotrione (purity 96.8%) in the diet at a dose level of 0, 10, 50, 350 or 7000 parts per million (ppm) (equal to 0, 1.7, 8.4, 61.5 and 1212.4 mg/kg bw per day for males and 0, 2.4, 12.4, 80.1 and 1537.1 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 a week, and an ophthalmoscopic examination was performed just prior to termination. Haematological and clinical chemistry parameters were measured at study termination. At necropsy, the weights of selected organs were recorded, assessed by gross examination and examined histopathologically.

There were no treatment-related effects on mortality, feed consumption, haematological or clinical chemistry parameters, ophthalmoscopic parameters, organ weights or gross pathological and histopathological parameters. There were no adverse effects on clinical signs, body weight or body weight gain. No neurotoxicity battery or cholesterol measurements were conducted in this study.

The no-observed-adverse-effect level (NOAEL) was 7000 ppm (equal to 1212.4 mg/kg bw per day), the highest dose tested (Pinto, 1997a).

Rats

In a 90-day study, groups of 12 Alpk:APfSD rats of each sex per dose received mesotrione (purity 93.3%) in the diet at a dose level of 0, 1, 125, 1250 or 12 500 ppm (equal to 0, 0.09, 10.96, 112.09 and 1110.86 mg/kg bw per day for males and 0, 0.10, 12.81, 125.58 and 1212.53 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. An ophthalmoscopic examination was performed prior to treatment and during the week prior to termination. Clinical chemistry, haematological and urine analysis parameters were measured at study termination. At necropsy, the weights of selected organs were recorded, assessed by gross examination and examined histopathologically.

There were no effects on mortality. Body weights were decreased by 8%, 7% and 16% and body weight gains were decreased by 12%, 11% and 23% compared with controls in males at 125, 1250 and 12 500 ppm, respectively. In females, body weight was decreased by 10% and body weight gain was decreased by 22% at 12 500 ppm. Feed efficiency was decreased in males at doses of 125 ppm and above.

Clinical signs of toxicity were limited to purple and/or yellow staining on the tray papers at doses of 1250 ppm and higher. In the ophthalmological examination, there was an increase in corneal opacity and vascularization in males (0/12, 0/12, 10/12, 10/12, 9/12) at doses of 125 ppm and higher and in females (0/12, 0/12, 0/12, 4/12, 9/12) at doses of 1250 ppm and higher.

The NOAEL was 1 ppm (equal to 0.09 mg/kg bw per day) in males and 125 ppm (equal to 12.81 mg/kg bw per day) in females. The lowest-observed-adverse-effect level (LOAEL) was 125 ppm in males (equal to 10.96 mg/kg bw per day), based on increased corneal opacity and

vascularization and decreased body weight and feed efficiency, and 1250 ppm in females (equal to 125.58 mg/kg bw per day), based on increased corneal opacity and vascularization (Horner, 1995).

In a 13-week study, groups of 12 Alpk:APfSD rats of each sex per dose received mesotrione (purity 96.8%) in the diet at a dose level of 0, 2.5, 5.0, 7.5 or 150 ppm (equal to 0, 0.21, 0.41, 0.63 and 12.46 mg/kg bw per day for males and 0, 0.23, 0.47, 0.71 and 14.48 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. An ophthalmoscopic examination was performed prior to treatment and during the week prior to termination. Clinical chemistry, haematological and urine analysis parameters were measured at study termination. At necropsy, the weights of selected organs were recorded, assessed by gross examination and examined histopathologically.

There were no effects on mortality or body weight. Clinical signs of toxicity consist of an increase in cloudy eyes in males at doses of 7.5 ppm and higher. There was one female with cloudy eyes at 150 ppm.

Under ophthalmoscopic examination, hazy corneal opacities, corneal opacities and vascularization were increased in males at doses of 7.5 ppm and higher, and corneal opacity was increased in females at 150 ppm.

The NOAEL was 5.0 ppm (equal to 0.41 mg/kg bw per day). The LOAEL was 7.5 ppm (equal to 0.63 mg/kg bw per day), based on increased cloudy eyes, corneal opacities and vascularization of the eyes in males (Pinto, 1997b).

Dogs

In a 13-week oral toxicity study, four Beagle dogs of each sex per dose were administered mesotrione (purity 96.8%) in gelatine capsules at a concentration of 0, 100, 600 or 1000 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, at weeks 4 and 8 and prior to termination. Select organs were weighed at necropsy, assessed by gross observations and examined histopathologically.

There was no effect on mortality, and there were no adverse clinical signs of toxicity. Body weight gains were decreased compared with controls in males at 1000 mg/kg bw per day. Histopathologically, there was an increase in minimal/slight focal mesothelial proliferation of the atrium of the heart in two males at 1000 mg/kg bw per day.

The NOAEL was 600 mg/kg bw per day. The LOAEL was 1000 mg/kg bw per day, based on decreased body weights and increased mesothelial proliferation of the atrium of the heart in males (Brammer, 1997a).

In a short-term oral toxicity study, four Beagle dogs of each sex per dose were administered mesotrione (purity 97.6%) in capsules for 1 year at a concentration of 0, 10, 100 or 600 mg/kg bw per day. 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 weeks 13, 26 and 39 and prior to termination. Blood samples were withdrawn for haematology and plasma clinical chemistry, and urine samples were collected from all animals predosing, at approximately weeks 4, 13 and 26 and prior to termination. Urine analysis was performed from samples collected prior to study initiation and during weeks 26 and 52. The animals were killed 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. No measure of blood clotting potential was taken, and the spleen and uterus were not weighed.

Clinical signs of irritation and toxicity consisted of interdigital cysts at doses of 100 mg/kg bw per day and above in males and 600 mg/kg bw per day in females, dry sores or abrasions in the paws or limbs in all treated groups of males and yellow staining of the fur in all treated animals of both sexes. However, the findings were attributed to the presence of irritating metabolites of tyrosine in the urine (as confirmed by the presence of free phenolic acids in the urine of all treated dogs) and were not considered toxicologically relevant. Additionally, lime-green discoloration of the urine was noted in all treated animals, although the finding was not considered adverse.

Body weights were decreased in females at 600 mg/kg bw per day, and body weight gains were decreased in females at doses of 100 mg/kg bw per day and higher. There were no effects on body weight or body weight gain in males (Table 6).

Table 6. Mean body weight and body weight gain of dogs administered mesotrione for approximately 12 months

	Males (*n* = 6)				**Females (*n* = 6)**			
	0 mg/kg bw per day	**10 mg/kg bw per day**	**100 mg/kg bw per day**	**600 mg/kg bw per day**	**0 mg/kg bw per day**	**10 mg/kg bw per day**	**100 mg/kg bw per day**	**600 mg/kg bw per day**
Body weight (g)[a]								
Week 1	10.53 ± 0.76	10.18 ± 0.93	10.18 ± 0.89	10.38 ± 1.86	8.45 ± 1.05	8.35 ± 0.39	8.65 ± 0.73	8.60 ± 1.01
Week 53	13.13 ± 1.28	12.13 ± 1.68	12.40 ± 0.34	13.68 ± 1.62	10.80 ± 1.19	10.60 ± 0.54	10.40 ± 0.65	9.83 ± 1.10
Body weight relative to controls (%)	–	↓8	↓6	↑4	–	↓2	↓4	↓9
Overall body weight gain (g)	2.60	1.95	2.22	3.30	2.35	2.25	1.75	1.23
Overall body weight gain relative to controls (%)	–	↓25	↓15	↑27	–	↓4	↓26	↓48

bw: body weight; *: $P \leq 0.05$ (analysis of covariance)

[a] Mean ± standard deviation.

Source: Brammer (1997b)

There was a single incidence of lenticular opacity at the end of the treatment period in both high-dose males and females. There were also five observations in one animal in the control males; however, this was attributed to the remnants of a hyaline blood vessel, and the observation at the high dose was attributed to treatment (Table 7).

Plasma tyrosine levels were increased in all treated groups. In males, the increases were 2.6-fold, 8-fold and 10-fold in the low-, mid- and high-dose groups, respectively. In females, the increases were 2.5-fold, 13-fold and 16-fold in the low-, mid- and high-dose groups, respectively. The changes were considered adverse in the presence of treatment-related changes to the eye at 600 mg/kg bw per day.

At histopathological examination, there were single incidences of unilateral keratitis and periorbital haemorrhage in high-dose males and unilateral corneal erosion in high-dose females (Table 8).

The LOAEL was 600 mg/kg bw per day, based on keratitis and lenticular/corneal opacities and increased plasma tyrosine in males and females and decreased body weight in females only. The NOAEL was 100 mg/kg bw per day (Brammer, 1997b).

Table 7. Ophthalmoscopic observations in dogs administered mesotrione for approximately 12 months

Observation	Males				Females			
	0 mg/kg bw per day	10 mg/kg bw per day	100 mg/kg bw per day	600 mg/kg bw per day	0 mg/kg bw per day	10 mg/kg bw per day	100 mg/kg bw per day	600 mg/kg bw per day
Lenticular opacity								
No. of animals affected	1/4[a]	0/4	0/4	1/4	0/4	0/4	0/4	1/4
Total no. of observations	5	0	0	1	0	0	0	1
Range of first incidence (weeks)	Pre-52	–	–	52	–	–	–	47

bw: body weight

[a] Attributed to the remnant of hyaline blood vessel.

Source: Brammer (1997b)

Table 8. Incidence of selected histopathological lesions in dogs administered mesotrione for 1 year

Histopathological lesion	Males (n = 4)				Females (n = 4)			
	0 mg/kg bw per day	10 mg/kg bw per day	100 mg/kg bw per day	600 mg/kg bw per day	0 mg/kg bw per day	10 mg/kg bw per day	100 mg/kg bw per day	600 mg/kg bw per day
Eye								
Unilateral keratitis	0	0	0	1	0	0	0	0
Periorbital haemorrhage	0	0	0	1	0	0	0	0
Unilateral corneal erosion	0	0	0	0	0	0	0	1
Skin								
Interdigital dermatitis/folliculitis	0	1	2	2	0	0	0	2

bw: body weight

Source: Brammer (1997b)

(b) Dermal application

Rabbits

In a 21-day dermal toxicity study, groups of five New Zealand White rabbits of each sex per dose were treated dermally with mesotrione (purity 96.8%) applied to an approximately 15 cm × 13 cm area of the body surface at a dose of 0, 10, 500 or 1000 mg/kg bw per day for 3 weeks. The duration of treatment was 6 hours daily, with occlusion, 5 days/week. The animals were observed for clinical signs and skin irritation. Animals were weighed daily, and feed consumption was monitored daily and calculated as a weekly mean. An ophthalmoscopic examination was performed prior to study initiation and 2 days prior to study termination. Pretreatment and terminal blood samples were subjected to haematological and plasma chemistry analysis. Following termination, animals were subjected to necropsy and postmortem examination of major organs and tissues, including organ weights and histopathology of control and high-dose animals and any gross lesions.

There were no systemic signs of toxicity at the highest dose tested. Slight erythema was noted at 500 and 1000 mg/kg bw per day in males and females.

The NOAEL in males and females for systemic toxicity was 1000 mg/kg bw per day, the highest dose tested (Lees, 1997).

2.3 Long-term studies of toxicity and carcinogenicity

Mice

In a chronic toxicity study in mice, mesotrione (purity 96.8%) was administered in the diet to 60 C57BL/10Jf CD-1 mice of each sex per dose at 0, 10, 50, 350 or 7000 ppm (equal to 0, 1.5, 7.8, 56.2 and 1114 mg/kg bw per day for males and 0, 2.1, 10.3, 72.4 and 1495 mg/kg bw per day for females, respectively) for up to 1 year. Twenty animals of each sex per dose were terminated after 3 and 6 months of treatment, with the 3-month results reported separately (see section 2.2(a); Pinto, 1997a). Detailed clinical signs were recorded weekly, and animals were observed daily for signs of overt toxicity and twice daily for morbidity and mortality. Body weights were recorded prior to treatment, weekly for the first 14 weeks of the study and monthly thereafter. Feed consumption was recorded weekly for 13 weeks of the study, in week 16 and then once every 4 weeks thereafter. Ophthalmological examinations were performed in males the week prior to termination in the control and high-dose animals in the 3- and 12-month study groups and in females the week prior to termination in all dose groups at 12 months. Haematological and clinical chemistry parameters were determined in all animals of each sex per dose at 14, 27 or 53 weeks via cardiac puncture at termination. Urine was collected over a period of 10–18 hours from each cage of surviving mice during weeks 13, 26 and 52. All animals were subjected to necropsy, postmortem examination and tissue preservation. Organ weights were recorded for 10 animals of each sex per dose from terminations at weeks 14 and 53. Lungs, liver, gallbladder and kidneys from both sexes and adrenal glands from males only from all groups from the 12-month scheduled termination were submitted for histology.

Body weight and body weight gain were decreased in males at 7000 ppm throughout treatment and in females at 7000 ppm in weeks 1–4. There were no effects on mortality, clinical signs of toxicity, ophthalmoscopy, haematology, clinical chemistry, urine analysis or postmortem findings. There was no evidence of oncogenicity.

The LOAEL for chronic toxicity was 7000 ppm (equal to 1114 mg/kg bw per day), based on decreased body weight and body weight gain in males. The NOAEL for chronic toxicity was 350 ppm (equal to 56.2 mg/kg bw per day) (Pinto, 1997c).

In an 18-month combined chronic toxicity and carcinogenicity study in mice, mesotrione (purity 96.8%) was administered in the diet to 55 C57BL/10Jf CD-1 Alpk mice of each sex per dose at 0, 10, 350 or 3500/7000 ppm (equal to 0, 1.4, 49.7 and 897.7 mg/kg bw per day for males and 0, 1.8, 63.5 and 1103 mg/kg bw per day for females, respectively) for 80 weeks. Animals received 3500 ppm mesotrione for the first 7 weeks of the study, but that dose was increased to 7000 ppm for the remainder of the study because of a lack of effects on body weight or feed consumption. Detailed clinical signs were recorded weekly, and animals were observed daily for signs of overt toxicity and twice daily for morbidity and mortality. Body weights were recorded prior to treatment, weekly for the first 12 weeks of the study and once every 2 weeks thereafter. Feed consumption was recorded for each cage of five mice weekly for 12 weeks of the study and then once every 4 weeks thereafter. Haematological parameters were determined in all animals of each sex per dose at 53 weeks from the tail vein and at study termination via cardiac puncture. All animals were subjected to necropsy, postmortem examination and tissue preservation. Organ weights were recorded for liver, kidneys, testes, brain and adrenal glands.

There was no evidence of carcinogenicity. There were no effects on mortality, clinical observations, haematology or postmortem findings. Body weight and body weight gain were decreased by 7% and 18%, respectively, in males given a dose of 3500 ppm. Feed efficiency was decreased in the same group by 16%. There were no adverse effects on body weight or feed efficiency in females.

The LOAEL for toxicity was 3500 ppm (equal to 897.7 mg/kg bw per day), based on decreased body weight, body weight gain and feed efficiency in males. The NOAEL was 350 ppm (equal to 49.7 mg/kg bw per day). The NOAEL for carcinogenicity was 3500/7000 ppm (equal to 897.7 mg/kg bw per day), the highest dose tested (Rattray, 1997).

Rats

In a combined chronic toxicity and carcinogenicity study in rats, mesotrione (purity 96.8%) was administered in the diet to 64 Alpk:APfSD (Wistar-derived) rats of each sex per dose at 0, 7.5, 100 or 2500 ppm (equal to 0, 0.48, 6.48 and 159.9 mg/kg bw per day for males and 0, 0.57, 7.68 and 189.5 mg/kg bw per day for females, respectively) for a period of up to 105 weeks. A satellite group of 12 preselected animals of each sex per dose was terminated at 52 weeks to assess chronic toxicity. In addition, 20 rats of each sex per group were assigned to dose levels of 1.0 or 2.5 ppm (equal to 0.06 and 0.16 mg/kg bw per day for males and 0.08 and 0.19 mg/kg bw per day for females, respectively) for a period of up to 105 weeks, to aid in the assessment of ocular toxicity only. 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 and biweekly thereafter. Feed consumption was measured for each cage of four rats on a weekly basis for 14 weeks, in week 16 and once every 4 weeks thereafter. An ophthalmological examination was conducted on all main study animals in all groups, including the 1.0 and 2.5 ppm groups, prior to study initiation, during study weeks 26, 52 and 78 and during the week prior to study termination. Blood samples taken from 13 predesignated animals of each sex dosed at 0, 7.5, 100 and 2500 ppm at weeks 14, 27, 53 and 79 were subjected to haematological and clinical chemistry analyses. Urine analysis was performed during study weeks 13, 26, 52, 78, 97 (males only) and 104 (females only) from the same predesignated animals as above. All animals dosed at 0, 7.5, 100 and 2500 ppm, including decedents, were subjected to necropsy, postmortem examination and tissue preservation. Select organs of all interim kill animals and terminal animals were weighed. Major organs and tissues from the control and high-dose groups and 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 uterus and ovaries were weighed together, and only from animals killed at 24 months. The pharynx and larynx were not examined histopathologically. Eyes from the 1.0 and 2.5 ppm groups were preserved in Davidson's fixative and examined histopathologically.

There was no evidence of mortality, and there were no adverse changes in haematological, clinical chemistry or urine parameters. There were no adverse effects on organ weights in the interim or terminal kill animals.

Signs of clinical toxicity consisted of cloudy eyes at doses of 7.5 ppm and higher in males and of 100 ppm and higher in females, yellow and/or purple staining of tray papers at 2500 ppm in both sexes and increased incidence of dry sore in males and urine staining in both sexes at doses of 100 ppm and higher. Body weights and body weight gains were decreased in males at doses of 7.5 ppm and above; there were no adverse effects on body weight or body weight gain in females. Feed consumption was decreased in males and females at 2500 ppm.

In males, there was an increase in hazy corneal opacities, corneal opacities, vascularization and ghost vascularization at doses of 7.5 ppm and above. In females, vascularization and ghost vascularization were increased at doses of 100 ppm and above, and opacities were comparable with those in controls. In interim kill animals, there was an increase in opaque eyes at doses of 100 ppm and above in males and at 2500 ppm in females. In terminal animals, opaque and/or cloudy and/or vascularized corneal lesions were increased in males at doses of 7.5 ppm and above and in females at doses of 100 ppm and above. Additional findings noted for males at doses of 7.5 ppm and higher were enlarged adrenals, reduced seminal vesicles, pale livers, renal cysts and roughened renal surface. For females, there was an increased incidence of pale kidneys at 100 and 2500 ppm and an increased incidence of pale livers at 2500 ppm.

Histopathological examination revealed an increased incidence of keratitis in the 100 and 2500 ppm groups (both sexes) and in the 7.5 ppm group (males only); hepatocyte fatty vacuolation in the 100 and 2500 ppm groups (both sexes) and in the 7.5 ppm group (males only); thyroid follicular cysts for males in the 100 and 2500 ppm groups; follicular cysts with hyperplastic epithelium for males at all dose levels and for females in the 2500 ppm group; and thyroid squamous cysts for females in the 100 and 2500 ppm groups. At 2500 ppm, there was an increase in follicular cell adenomas in females. Follicular cell carcinomas were also increased, but were increased above the historical control range in all dose groups, including concurrent controls, and the increase was not considered related to treatment (Table 9).

Table 9. Incidence of selected histopathological lesions in rats administered mesotrione for 2 years

	Males (n = 4)				Females (n = 4)			
Histopathological lesion	**0 ppm**	**7.5 ppm**	**100 ppm**	**2 500 ppm**	**0 ppm**	**7.5 ppm**	**100 ppm**	**2 500 ppm**
Liver								
Hepatocyte fatty vacuolation	1/64	4/64	2/64	7/64	0/64	0/64	2/64	6/64
Thyroid								
Follicular cysts	1/64	0/63	3/64	5/64	0/64	0/64	1/62	0/64
Follicular cysts with hyperplastic epithelium	1/64	5/63	7/64	5/64	0/64	0/64	1/62	3/64
Thyroid squamous cysts	5/64	8/63	4/64	6/64	0/64	1/64	2/62	5/64
Follicular cell adenomas	0/64	1-63	3/64	1/64	0/64	1/64	1/64	4/64
Follicular cell carcinomas	0/64	1/63	0/64	1/64	5/64	4/64	2/64	6/64
Total thyroid tumours	2/64	2/63	3/64	2/64	5/64	5/64	3/64	10/64

ppm: parts per million

Source: Brammer (1997c)

The LOAEL was identified as 7.5 ppm (equal to 0.48 mg/kg bw per day), the lowest dose tested, based on an increased incidence of ocular effects noted at clinical, ophthalmological, gross or histopathological examinations, body weight changes and histopathological changes noted in the liver and thyroid in males. A NOAEL could not be determined There was no evidence of carcinogenicity (Brammer, 1997c).

2.4 Genotoxicity

A range of GLP-compliant studies of the genotoxicity of mesotrione was conducted to assess its potential for inducing chromosomal aberration, gene mutation and reverse gene mutation. There was no evidence for genotoxicity or mutagenicity in the available studies in the presence of metabolic activation (+S9) (summarized in Table 10); however, in the absence of metabolic activation (−S9), the in vitro mammalian chromosomal aberration test was equivocal. Overall, mesotrione did not demonstrate any genotoxic potential.

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

Mice

In a two-generation reproductive toxicity study in mice, mesotrione (purity 96.8%) was administered in the diet to 26 CD-1 mice of each sex per dose at a concentration of 0, 10, 50, 350, 1500 or 7000 ppm (equal to 0, 2.1, 10.2, 71.4, 311.8 and 1472 mg/kg bw per day for males and 0, 2.1, 10.0, 71.3, 301.6 and 1439 mg/kg bw per day for females; Table 11). After 8 weeks, the animals were mated and allowed to rear the ensuing F_{1A} litters to weaning. The breeding programme was repeated

Table 10. Genotoxicity studies with mesotrione

End-point	Test object	Concentration	Purity (%)	Results	Reference
In vitro					
Reverse mutation	*Salmonella typhimurium* and *Escherichia coli*	0, 100, 200, 500, 1 000, 2 500 or 5 000 µg/plate ±S9	98.1	Negative	Callander (1993)
Mammalian cell cytogenetics	Human lymphocytes	0, 250, 1 000 or 2 000 µg/mL +S9	98.1	Negative	Griffiths (1994a)
		0, 250, 1 000 or 1 500/2 000 µg/mL –S9		Equivocal	
Mammalian cell gene mutation	L5178Y mouse lymphoma cells, *TK* locus	125, 250, 500 or 1 000 µg/mL	98.1	Negative	Clay (1994)
In vivo					
Mouse micronucleus	CD-1 mouse bone marrow, males and females	500 mg/kg bw Harvest time: 24 and 48 h	98.1	Negative	Griffiths (1994b)

bw: body weight; S9: 9000 × *g* supernatant fraction from liver homogenate from Aroclor-treated rats; TK: thymidine kinase

Table 11. Mean intake of mesotrione in the two-generation reproductive toxicity study in mice

	Dose (mg/kg bw per day)									
	F_0 generation					F_1 generation				
	10 ppm	50 ppm	350 ppm	1 500 ppm	7 000 ppm	10 ppm	50 ppm	350 ppm	1 500 ppm	7 000 ppm
Males premating	2.1	10.2	71.4	311.8	1 472	2.1	10.0	71.3	301.6	1 439
Females premating	2.4	12.0	84.4	371.6	1 632	2.4	11.4	80.5	353.8	1 673
Females gestation period	2.1	10.0	72.9	300.1	1 430	2.0	9.8	73.5	302.9	1 491
Females lactation period	13.4	70.1	481.6	2 001	8 726	13.2	65.8	422.4	1 879	8 260

bw: body weight; F_0: parental generation; F_1: first filial generation; ppm: parts per million

Source: Moxon (1997)

with the F_1 adults selected from the F_{1A} offspring to produce the F_{2A} litters after a premating period of at least 8 weeks. Selected F_{2A} pups were retained post-weaning (F_2 adults) for the measurement of preputial separation only. Diets containing mesotrione were fed continuously throughout the study. The following investigations were undertaken in the adults: clinical observations, body weights (including during pregnancy and lactation for females), feed consumption (including during pregnancy and lactation for females) and utilization, reproductive performance, preputial separation (F_1 and F_2 adults only), postmortem examination, organ weights, histopathology and plasma tyrosine levels (some F_1 adults). The following investigations were undertaken in the litters/pups: numbers of pups at birth and up to/including day 29, pup survival, pup and litter weights, clinical condition, postmortem examination of selected pups, organ weights and plasma tyrosine levels (some F_{2A} pups) for 8 weeks prior to mating and through lactation and weaning of the F_1 offspring. Groups of 26 male and 26 female F_1 generation offspring were then similarly treated through 21 days of lactation of the F_2 offspring.

There were no effects on mortality. Clinical signs of toxicity were limited to a single incidence of cloudy eye in F_0 high-dose males and multiple incidences in F_1 high-dose males and females (Table 12). This was corroborated by findings in the gross necropsy and increases in unilateral cataractous changes in 7000 ppm males in the F_0 generation and unilateral/bilateral cataractous changes in 7000 ppm males and females in the F_1 generation. There were no effects on body weight or body weight gain in adult animals. Body weights were lower in high-dose animals in the F_1 generation; however, this is considered to be a result of lower pup body weights. There were no treatment-related or adverse effects on feed consumption or feed efficiency.

Table 12. Summary of eye-related changes in parental mice from the two-generation reproductive toxicity study

	Incidence of finding					
Finding	**0 ppm**	**10 ppm**	**50 ppm**	**350 ppm**	**1 500 ppm**	**7 000 ppm**
Males F_0						
Clinical signs of toxicity						
Cloudy eyes	0/26	0/26	0/26	0/26	0/26	1/26
Gross pathology						
Opaque/cloudy eyes	0/26	0/26	0/26	1/26	0/26	3/26
Histopathological examination						
Unilateral cataractous change	0/26	0/22	0/25	0/26	0/26	3/26
Males F_1						
Clinical signs of toxicity						
Cloudy eyes	0/26	1/26	0/26	1/26	0/26	4/26
Gross pathology						
Opaque/cloudy eyes	0/26	2/26	0/26	2/26	1/26	5/26
Histopathological examination						
Unilateral cataractous change	0/26	0/26	0/25	0/24	0/26	7/25
Bilateral cataractous change	0/26	0/26	0/25	0/24	0/26	1/25
Females F_1						
Clinical signs of toxicity						
Cloudy eyes	0/26	0/26	0/26	0/26	1/26	6/26
Gross pathology						
Opaque/cloudy eyes	1/26	0/26	0/26	0/26	1/26	5/26
Histopathological examination						
Unilateral cataractous change	0/26	0/26	0/26	0/26	0/26	4/26
Bilateral cataractous change	0/26	0/26	0/26	0/26	0/26	2/26

F_0: parental generation; F_1: first filial generation; ppm: parts per million
Source: Moxon (1997)

Estrous cycle parameters and sperm parameters were not measured. There was a decrease in successful matings (i.e. the number of females producing at least one live pup) in F_0 females at and above 350 ppm (Table 13). The incidence of successful matings in all groups, including controls, was lower than expected in the F_1 animals, and therefore the change in the F_0 animals was of unknown significance.

Table 13. Successful matings in mice treated with mesotrione

	Incidence of successful matings					
	0 ppm	**10 ppm**	**50 ppm**	**350 ppm**	**1 500 ppm**	**7 000 ppm**
F_0 females						
Dams with at least one live pup	24/26	25/26	23/24	20/26	22/26	20/26
F_1 females						
Dams with at least one live pup	21/26	20/26	21/25	20/24	21/26	19/26

F_0: parental generation; F_1: first filial generation; ppm: parts per million

Source: Moxon (1997)

Clinical signs of toxicity in the offspring were limited to an increased incidence of opaque eyes observed in one F_{2A} litter at 7000 ppm (Table 14). There were no effects on viability. Mean pup body weight was lower in the 1500 and 7000 ppm groups (both generations and both sexes) on lactation days 22 and 29 and slightly lower for F_{2A} pups in the 7000 ppm group on lactation day 8. F_{1A} males in the 10 ppm dose group had decreased body weights, but the finding was not consistent across sexes or generations; therefore, the change was not considered treatment related. In the F_{1A} generation, there was an increase in unilateral and bilateral cataractous changes at 7000 ppm in both sexes. In the F_{2A} generation, changes at necropsy consisted of an increase in opaque/cloudy eyes in the 1500 and 7000 ppm groups of both sexes and an increased incidence of unilateral/bilateral cataractous change in the 7000 ppm group in both sexes and in the 1500 ppm group in males only. Plasma tyrosine levels were increased by factors of 3.7 in males and 2.6 in females at 10 ppm. At 7000 ppm, plasma tyrosine levels were increased by factors of 6.9 in males and 6.4 in females.

The LOAEL for parental toxicity in CD-1 mice was 7000 ppm (equal to 1439 mg/kg bw per day), based on clinical, gross and histopathological changes to the eyes (opaque/cloudy eyes; cataractous change) and increased plasma tyrosine levels. The NOAEL for parental toxicity was 1500 ppm (equal to 301.6 mg/kg bw per day).

The NOAEL for reproductive toxicity in CD-1 mice was 7000 ppm (equal to 1439 mg/kg bw per day), the highest dose tested.

The LOAEL for offspring toxicity in CD-1 mice was 1500 ppm (equal to 301.6 mg/kg bw per day), based on decreased body weight and body weight gain, clinical, gross and histopathological changes to the eyes (opaque/cloudy eyes; cataractous change) and increased plasma tyrosine levels. The NOAEL was 350 ppm (equal to 71.3 mg/kg bw per day) (Moxon, 1997).

Rats

In a three-generation reproductive toxicity study in rats, mesotrione (purity 96.8%) was administered in the diet to 26 Alpk:APfSD rats of each sex per group at a concentration of 0, 2.5, 10, 100 or 2500 ppm (equal to 0, 0.3, 1.1, 11.6 and 278.1 mg/kg bw per day for males and 0, 0.3, 1.1, 11.7 and 297.2 mg/kg bw per day for females, respectively). After 10 weeks, the animals were mated and allowed to rear the ensuing F_{1A} litters to weaning. The breeding programme was repeated with the

Table 14. Summary of eye-related changes in offspring from the two-generation reproductive toxicity study in mice

	Incidence of finding					
Finding	**0 ppm**	**10 ppm**	**50 ppm**	**350 ppm**	**1 500 ppm**	**7 000 ppm**
F_{1A} litters						
Clinical signs of toxicity						
No. of pups (no. of litters)	*282 (25)*	*321 (25)*	*300 (23)*	*252 (20)*	*279 (21)*	*236 (20)*
Discharge from eyes	0 (0)	4 (3)	1 (1)	4 (3)	5 (3)	11 (5)
Histopathological examination (no. affected/no. examined)						
Unilateral cataractous change						
- Males	0/37	0/24	0/11	0/12	0/14	2/30
- Females	0/40	0/21	0/17	0/13	0/13	2/30
Bilateral cataractous change						
- Males	0/37	0/24	0/11	0/12	0/14	2/30
- Females	0/40	0/21	0/17	0/13	0/13	0/30
F_{2A} litters						
Clinical signs of toxicity						
No. of pups (no. of litters)	*249 (21)*	*248 (20)*	*249 (21)*	*222 (20)*	*242 (21)*	*228 (19)*
Discharge from eyes	0 (0)	3 (3)	0 (0)	3 (3)	2 (2)	11 (5)
Opaque eyes	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	6 (1)
Macroscopic examination (no. affected/no. examined)						
Opaque/cloudy eyes						
- Males	0/30	1/28	0/31	0/30	4/30	10/33
- Females	1/31	0/29	0/31	0/30	3/30	3/31
Histopathological examination (no. affected/no. examined)						
Unilateral cataractous change						
- Males	0/30	0/16	0/11	0/11	2/18	3/33
- Females	0/32	0/18	0/13	0/12	0/18	2/31
Bilateral cataractous change						
- Males	0/30	0/16	0/11	0/11	0/18	8/33
- Females	0/32	0/18	0/13	0/12	0/18	1/31

F_0: parental generation; F_1: first filial generation; ppm: parts per million

Source: Moxon (1997)

F_1 adults selected from the F_{1A} offspring to produce the F_{2A} litters after a 10-week premating period. A further generation (F_2) was then selected from the F_{2A} litters in order to clarify findings seen in the first two generations. In the F_2 generation, all animals were fed experimental diet until week 14, when approximately half of the animals in all groups continued with the same treatment while the remainder of the animals were assigned to a recovery subgroup and fed control diet. At week 18, the subgroups were mated to produce the F_{3A} litters. Ophthalmoscopic examinations were included in all generations. Test diets were fed continuously throughout the study with the exception of the recovery animals in the third generation referred to above (F_2 adults and F_{3A} offspring). The following investigations were undertaken in the adults: clinical observations, body weights (including during pregnancy and lactation for females), feed consumption (including during pregnancy and lactation for females) and utilization, reproductive performance, preputial separation (F_1 and F_2 adults only), postmortem examination and organ weights. Estrous cycle length and periodicity and sperm parameters were not measured. The following investigations were undertaken in the litters/pups: numbers of pups at birth and up to/including day 29, pup survival, pup and litter weights, clinical condition, postmortem examination of selected pups, organ weights (except F_{3A} pups) and ophthalmoscopy (F_{3A} pups only).

In the parental animals, there were no effects on body weight gain, feed intake, feed efficiency or organ weights. Eye lesions were observed in the clinical observations, ophthalmoscopic examination and gross and histopathological examinations at 10 ppm in males and in both sexes at and above 100 ppm. Changes to the eyes consisted of cloudy/opaque eyes, corneal opacity and/or keratitis with corneal vascularization. Recovery animals were noted to have ghost vascularization indicating healed lesions in both sexes at 2500 ppm and in males at 100 ppm. At gross necropsy, there was an increase in bilateral hydronephrosis in the F_1 generation at 2500 ppm in males and females and in the F_2 adults at 2500 ppm in males only following continuous treatment and in both sexes in the recovery group. Plasma tyrosine levels were increased to a toxicologically significant extent at doses of 10 ppm and above in F_1 animals. In the recovery F_2 adults, plasma tyrosine levels were unchanged from control values.

Litter size and pup survival to day 22 were decreased at 2500 ppm in the F_{1A}, F_{2A} and F_{3A} continuous treatment litters. In addition, whole litter loss was increased and the percentage of pups born live was decreased in the F_{2A} and F_{3A} continuous treatment litters. There were no other treatment-related effects on reproductive function or reproductive performance.

Effects on offspring consisted of increased incidence of cloudy/opaque eyes, keratitis and/or corneal vascularization in all treated groups of males and at doses of 100 ppm and above in females in litters exposed to mesotrione in utero. Cataractous change was noted in both sexes at 2500 ppm in the F_{1A}, F_{2A} and F_{3A} continuous treatment litters. Grossly, there were increased incidences of bilateral renal pelvic dilatation in both sexes at 2500 ppm in continuously treated F_{3A} litters. Histopathologically, there was an increase in bilateral hydronephrosis at 2500 ppm in F_{1A} and F_{2A} litters and at 2500 ppm in F_{3A} continuous treatment litters. There were no effects of treatment in the absence of mesotrione exposure in utero. Plasma tyrosine levels were increased in F_{3A} pups under continuous treatment at all dose levels tested. Plasma tyrosine levels in F_{3A} pups in the recovery groups were similar to the control values at all dose levels.

The LOAEL for parental toxicity was 10 ppm (equal to 1.1 mg/kg bw per day), based on clinical, ophthalmological, gross and pathological changes in the eyes and increased plasma tyrosine levels in males and females. The NOAEL was 2.5 ppm (equal to 0.3 mg/kg bw per day).

The LOAEL for reproductive toxicity was 2500 ppm (equal to 297.2 mg/kg bw per day), based on decreased litter size, decreased pup survival to day 22, decreased percentage of pups born live and an increase in whole litter loss. The NOAEL was 100 ppm (equal to 11.7 mg/kg bw per day).

The LOAEL for offspring toxicity was 2.5 ppm (equal to 0.3 mg/kg bw per day), the lowest dose tested, based on clinical, ophthalmological, gross and histopathological changes to the eyes and increased plasma tyrosine levels. A NOAEL for offspring toxicity was not identified (Milburn, 1997b).

(b) Developmental toxicity

Mice

In a developmental toxicity study, mesotrione (purity 96.8%) was administered to mated Alpk:APfCD-1 female mice via gavage from days 5 to 18 of gestation at a dose level of 0 (two control groups), 10, 60, 150 or 600 mg/kg bw per day at a dosing volume of 10 mL/100 g bw in water. The animals were killed on day 19 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 were no clinical signs of toxicity, no effects on body weight or feed consumption and no treatment-related changes noted during the gross necropsy. There were no changes to the caesarean section parameters.

There was a slight increase in fetuses with minor external anomalies in the 600 mg/kg bw per day dose group; however, there was no increase in minor anomalies as a function of litters (Table 15). There were no other treatment-related changes to fetal parameters.

Table 15. External/visceral examinations in a developmental toxicity study in mice

	Litter (fetal) incidence					
	0 mg/kg bw per day	**0 mg/kg bw per day**	**10 mg/kg bw per day**	**60 mg/kg bw per day**	**150 mg/kg bw per day**	**600 mg/kg bw per day**
No. of litters (fetuses) examined	*24 (299)*	*26 (315)*	*24 (302)*	*25 (315)*	*25 (313)*	*23 (294)*
Total malformations	3 (3)	1 (6)	2 (2)	2 (2)	2 (2)	3 (4)
Total minor anomalies	11 (12)	9 (17)	10 (16)	11 (17)	12 (21)	12 (26*)
Total variants	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

bw: body weight, *: $P \leq 0.05$ (Student's *t*-test)

Source: Moxon (1999a)

A LOAEL for maternal toxicity could not be identified. The NOAEL for maternal toxicity was 600 mg/kg bw per day, the highest dose tested.

A LOAEL for embryo and fetal toxicity could not be identified. The NOAEL for embryo and fetal toxicity was 600 mg/kg bw per day, the highest dose tested (Moxon, 1999a).

Rats

In a developmental toxicity study, mesotrione (purity 96.8%) was administered to mated female Alpk:APfSD (Wistar-derived) rats (25 per dose) via gavage from day 7 to day 16 of gestation at a dose level of 0, 100, 300 or 1000 mg/kg bw per day at a dosing volume of 1.0 mL/100 g bw in deionized water. The animals were terminated on day 22 after mating for reproductive assessment and fetal examination. Clinical signs, body weight and feed consumption were recorded. Adult females were examined macroscopically at necropsy on day 20 after mating, and all fetuses were examined macroscopically at maternal necropsy and subsequently by detailed internal visceral or skeletal examination. Maternal body weight and feed consumption were not analysed statistically, and the homogeneity and stability of the test substance in the vehicle were not determined.

There were no maternal deaths. There was a dose-related decrease in body weight gain in all treated animals during the dosing period and throughout the study period in 1000 mg/kg bw per day dams (Table 16). Feed consumption was decreased during dosing and decreased in the first 3 days of the post-dosing period in all treated animals and was comparable with control values thereafter.

Table 16. Maternal body weight and body weight gain in pregnant rats (n = 24 per dose) given mesotrione

	Body weight/body weight gain (g)			
	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**
Initial body weight	268.3 ± 16.9	271.0 ± 16.2	269.6 ± 17.2	261.6 ± 17.8
Pretreatment gain				
Days 1–7	28	26.6	27.5	28.0
Treatment gain				
Days 7–10	16.0	13.3 (↓17)	9.6 (↓40)	8.1 (↓49)
Days 10–13	17.8	14.3 (↓20)	11.9 (↓33)	11.1 (↓38)
Days 13–16	22.7	21.2 (↓7)	21.2 (↓7)	19.4 (↓15)
Total gain, days 7–16	56.5	48.8 (↓14)	42.7 (↓24)	38.6 (↓32)
Post-treatment gain				
Days 16–20	40.1	40.8	38.7	40.1
Days 20–22	31.8	36.8	39.5	32.9
Final body weight	424.7 ± 22.6	424.0 ± 29.2	418.0 ± 30.0	401.3 ± 31.8* (↓6)
Gravid uterine weight	96.3 ± 14.5	94.2 ± 20.5	91.6 ± 19.2	90.0 ± 20.6
Corrected final body weight	328.4 ± 21.5	329.8 ± 27.1	326.4 ± 24.6	310.4 ± 26.5* (↓5)
Body weight gain				
Days 1 to 22	156.4	153.0	148.4 (↓5)	139.7 (↓11)
Days 7–22	128.4	126.4	120.9 (↓6)	111.7 (↓13)
Corrected body weight gain				
Days 1 to 22	60.1	58.8	56.8 (↓5)	49.7 (↓17)
Days 7–22	32.1	32.2	29.3 (↓9)	20.8 (↓35)

bw: body weight; *: $P < 0.05$ (analysis of covariance)

Source: Moxon (1999c)

Fetal weights were decreased in the 1000 mg/kg bw per day dose group; however, there were no other treatment-related effects on the caesarean section parameters.

Although there were no effects on external and skeletal malformations and all litters showed at least one minor skeletal anomaly and/or variant in control and treated dams, there was a dose-related and statistically significant increase in the number of fetuses exhibiting minor skeletal anomalies in all treated groups. These anomalies were generally related to a lack of ossification or supernumerary ribs and were determined to be a result of decreased body weight gain in the dams.

The LOAEL for maternal toxicity was 100 mg/kg bw per day, based on decreased body weight gain and feed consumption at the lowest dose tested. A NOAEL for maternal toxicity could not be identified.

The LOAEL for embryo and fetal toxicity was 100 mg/kg bw per day, based on decreased ossification at the lowest dose tested. A NOAEL for embryo and fetal toxicity could not be identified (Moxon, 1999b).

Rabbits

In a developmental toxicity study in rabbits, mesotrione (purity 96.8%) was administered to time-mated female New Zealand White rabbits (20 per dose) via gavage, from day 8 to day 20 of gestation, at a dose level of 0, 100, 250 or 500 mg/kg bw per day at a dosing volume of 10 mL/kg bw in deionized water. The animals were killed on day 30 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 30 after mating, and all fetuses were examined macroscopically at maternal necropsy and subsequently by detailed examination for external and visceral variations and abnormalities and skeletal variations and abnormalities, including ossification of the manus and pes.

Clinical signs of toxicity consisted of an increased incidence of red/brown urine at all dose levels and decreased defecation in the 250 and 500 mg/kg bw per day dose groups. Body weight gain was decreased by 34% at 500 mg/kg bw per day during the dosing period, although there were no changes in corrected final body weight or body weight gain.

In the caesarean section parameters, there was a slight increase in abortions in the 250 and 500 mg/kg bw per day dose groups (0/20, 1/20, 2/20, 2/20). Other than a large, but non-treatment-related, increase in preimplantation loss at 500 mg/kg bw per day, all other caesarean section parameters were comparable with control values.

Fetal examination revealed no increase in external or visceral malformations, anomalies or variants. There was no increase in skeletal malformations, and there was a decrease in minor skeletal anomalies. Although all litters, including controls, contained fetuses with delays in ossification, the number of fetuses with delayed ossification increased in all treated litters, with statistically significant increases at doses of 250 mg/kg bw per day and higher. These delays were also seen in the manus and pes assessment. Mean scores were comparable among all groups, but examination of individual scores indicated a dose-related reduction in the degree of ossification in all treated groups. Although the changes were considered treatment related, they were also considered transient and reversible and not considered to be adverse.

The LOAEL for maternal toxicity was 250 mg/kg bw per day, based on increased abortions and decreased defecation. The NOAEL for maternal toxicity was 100 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 500 mg/kg bw per day, the highest dose tested (Moxon, 1999c).

2.6 Special studies

(a) Neurotoxicity

In an acute neurotoxicity study, mesotrione (purity 97.6%) was administered to young adult Alpk:APfSD rats (10 of each sex per dose) via gavage at a dosing volume of 10 mL/kg bw in deionized water at a dose level of 0, 20, 200 or 2000 mg/kg bw and then maintained for a 15-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, 2 hours post-dosing and on days 8 and 15. Five animals of each sex in the control and high-dose groups 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 and spinal cord.

No premature deaths occurred. There were no clinical signs of toxicity and no effects on body weight or feed consumption. There were no treatment-related findings observed at the functional

observational battery or motor activity testing conducted at 2 hours, 8 days and 15 days post-dosing. Gross examination of the brain and histopathological examination of the central nervous system and the peripheral nervous system did not reveal any treatment-related findings.

The NOAEL for systemic toxicity and acute neurotoxicity was 2000 mg/kg bw, the highest dose tested (Horner, 1997a).

In a 13-week neurotoxicity study, mesotrione (purity 97.6%) was administered in the diet to groups of 10 male and 10 female Sprague-Dawley rats at a concentration of 0, 2.5, 100 or 5000 ppm (equal to 0, 0.20, 8.25 and 402.8 mg/kg bw per day for males and 0, 0.23, 9.29 and 466.6 mg/kg bw per day for females, respectively) for 3 months. 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 during weeks 5, 9 and 14 post-dosing. Five animals of each sex in the control and high-dose groups 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, skeletal muscle and gross lesions. In addition, an ophthalmological examination was performed during week 13.

There were no treatment-related mortalities. Clinical signs were limited to an increase in ocular opacity at doses of 100 ppm and above in males and at 5000 ppm in females. There was a slight decrease in final body weights at doses of 100 ppm and higher in males and females; however, the extent of the impact was not considered adverse. There were no effects on feed consumption, functional observational battery evaluations or motor activity measurements. There were no neurological effects noted in the postmortem examinations, and postmortem effects were limited to corneal opacity at 100 ppm and above in males and at 5000 ppm in females. In the ophthalmological examination, there were increases in corneal opacities, hazy corneal opacities and vascularization at doses of 100 ppm and higher in males and at 5000 ppm in females. Ghost vascularization was noted in females at doses of 100 ppm and above.

The LOAEL for systemic toxicity was 100 ppm (equal to 8.25 mg/kg bw per day), based on corneal opacity, hazy corneal opacity and vascularization in males and ghost vascularization in females. The NOAEL for systemic toxicity was 2.5 ppm (equal to 0.20 mg/kg bw per day). The NOAEL for neurotoxicity was 5000 ppm (equal to 402.8 mg/kg bw per day), the highest dose tested (Horner, 1997b).

(b) Mode of action studies

A number of studies have been conducted with mesotrione to investigate the ocular and systemic toxicity in rats and the differences in the systemic toxicity between rats and mice and to facilitate an understanding of the mode of action (MOA) of mesotrione in mammals. Appendix 1 contains further summaries of these mode of action studies within the human relevance framework developed by the International Life Sciences Institute (ILSI)/Health and Environmental Sciences Institute (HESI) workgroup.

In a 28-day dietary toxicity study, mesotrione (purity 100%) was administered in the diet to five Alpk:AP rats and five AP Swiss albino mice of unspecified sex per dose at 0, 1000, 7000 or 16 000 ppm (equivalent to 0, 100, 700 and 1600 mg/kg bw per day, respectively, for males and females) to rats and at 0, 1000, 3000 or 7000 ppm (equivalent to 0, 150, 450 and 1050 mg/kg bw per day, respectively, for males and females) to mice for 28 days. Clinical observations, body weights and feed consumption were recorded throughout the study. Blood samples were taken at termination and analysed for various clinical chemistry parameters. Livers were removed at termination and weighed, and portions were processed for liver biochemistry, routine histopathology and electron microscopy.

As a positive control, groups of three Alpk:AP rats and four AP Swiss albino mice were administered β-naphthoflavone, phenobarbitone, dexamethasone or methylclofenapate in standard inducer studies in which control animals received corn oil.

Mesotrione caused minimal cytochrome P450 induction at high doses and slight centrilobular hypertrophy in rats at the high dose of 16 000 ppm and slight liver hypertrophy at doses of 3000 ppm and above in mice. There were increases in proliferation of the smooth endoplasmic reticulum, but no evidence of peroxisome proliferation in either mice or rats. Ethoxycoumarin *O*-deethylation, pentoxyresorufin *O*-dependentylation and methoxyresorufin *O*-demethylation were increased in rats, whereas benzoxyresorufin *O*-debenzylation was increased in mice. However, as there was no evidence of hepatotoxicity, hepatomegaly or peroxisome proliferation, mesotrione was considered to be unlikely to cause liver tumours in rats or mice (Odum, 1997).

In a non-guideline dietary study to investigate the effects of L-tyrosine on the eye, eight male Alpk:APfSD weanling rats were fed a low-protein diet supplemented with 0%, 0.5%, 1%, 2.5% or 5% L-tyrosine for up to 21 days. Ophthalmoscopy was undertaken on days 2, 3, 4, 5, 6, 7, 8, 11, 12, 14, 18 and 21. Rats were killed when the majority of animals in each group showed corneal lesions. All remaining rats were killed on day 21. All rats were necropsied, and the eyes and Harderian glands were examined histopathologically.

There was no evidence of corneal lesions up to 21 days in animals given 1% L-tyrosine or less. In the 2.5% dose group, 2/8 animals showed evidence of corneal lesions by day 4, and 6/8 animals exhibited lesions by day 6. In the 5% dose group, 5/8 animals exhibited lesions on day 3, and 6/8 animals exhibited lesions on day 4. At histopathology, there was a slight increase in retinal rosettes and increased porphyrin at doses of 1% L-tyrosine and above; however, the changes were considered unrelated to tyrosine administration. In the animals dosed at 2.5% L-tyrosine and above, there were increases in minimal and slight keratitis and polymorphic filtration angle. At 5% L-tyrosine, there was an increase in minimal epithelial disorganization.

In conclusion, an increase in L-tyrosine was considered to induce the same corneal changes in Alpk:APfSD rats in the performing laboratory as seen in the literature regarding changes induced by triketones (Robinson, 1995).

In a non-guideline dietary study, eight female Alpk:APfSD (Wistar-derived) rats were fed diet containing 0 or 100 ppm mesotrione (purity 96.8%) with 0%, 0.5%, 1.0% or 2.5% tyrosine for 28 consecutive days (Table 17). Clinical observations, body weights and feed consumption were monitored throughout the study. Urine samples were taken for assessment of ketones after 24 hours, after 1 week and prior to scheduled termination. Blood samples were taken for assessment of plasma tyrosine after 24 hours, after 1 week and at scheduled termination. At the end of the scheduled period, the animals were killed and subjected to a postmortem examination. Liver and kidneys were weighed, and samples of kidney and liver were fixed and stored. Liver samples taken at termination were assayed for the activities of the enzymes tyrosine aminotransferase (TAT) and HPPD.

There was a statistically significant decrease in body weight and body weight gain in animals given 100 ppm mesotrione in conjunction with 2.5% tyrosine. There was a 6% decrease in body weight in animals given 100 ppm mesotrione in conjunction with 1.0% tyrosine, but the change was not statistically significant. Feed consumption was decreased in animals given 100 ppm mesotrione and 2.5% tyrosine. There were decreases in both parameters at other doses and time points, but there was no dose–response relationship, and the changes were considered adverse only in animals given both mesotrione and 2.5% tyrosine.

Corneal lesions were seen in all groups given mesotrione. There was a treatment-related increase in the severity of lesions with the addition of increasing amounts of tyrosine to the point where all animals given 100 ppm mesotrione and 2.5% tyrosine exhibited marked to moderate corneal

Table 17. Dose groups for the 28-day non-guideline study in female Alpk:APfSD (Wistar-derived) rats

Dose group	Mesotrione (ppm)	Tyrosine (%)
1	0	0
2	100	0
3	100	0.5
4	100	1.0
5	100	2.5
6	0	0.5
7	0	1.0
8	0	2.5

ppm: parts per million

Source: Milburn (1997a)

opacity and vascularization. In the absence of mesotrione, there were no changes to the eyes in any of the groups treated with tyrosine.

Plasma tyrosine levels were increased in all treated animals. In the presence of 100 ppm mesotrione, plasma tyrosine levels were at least an order of magnitude higher than those in animals given equivalent amounts of tyrosine in the absence of mesotrione (Table 18).

Table 18. Plasma tyrosine levels in female rats given mesotrione and/or tyrosine in diet

	Plasma tyrosine level (nmol/mL)							
Study period	**Control diet**	**100 ppm M**	**100 ppm M and 0.5% T**	**100 ppm M and 1.0% T**	**100 ppm M and 2.5% T**	**Control diet and 0.5% T**	**Control diet and 1.0% T**	**Control diet and 2.5% T**
24 hours	112.5 ± 12.53	1 503 ± 122.8	2 129 ± 98.99	2 579 ± 276.8	3 517 ± 872.3	136.4 ± 15.61	142.5 ± 12.85	268.9 ± 38.27
1 week	141.5 ± 5.019	1 243 ± 246.6	1 733 ± 323.7	2 291 ± 355.0	3 729 ± 756.1	160.5 ± 29.59	183.7 ± 29.56	244.6 ± 52.64
Termination	106.9 ± 8.790	1 189 ± 107.8	1 519 ± 65.69	2 803 ± 388.8	2 576 ± 866.2	129.5 ± 11.86	139.3 ± 27.58	187.6 ± 37.70

M: mesotrione; ppm: parts per million; T: tyrosine

Source: Milburn (1997a)

TAT activity was increased in all groups given mesotrione and in the group given 2.5% tyrosine in the absence of mesotrione. HPPD activity was decreased in all groups given mesotrione and not increased in groups given tyrosine in the absence of mesotrione.

At necropsy, relative liver weights were increased in all groups given mesotrione and not increased in groups given tyrosine in the absence of mesotrione. Relative kidney weights were increased only in the presence of mesotrione and 2.5% tyrosine. All animals given mesotrione exhibited cloudy eyes. There were no other treatment-related findings.

In conclusion, the administration of 100 ppm mesotrione in combination with tyrosine in the diet to female rats resulted in marked tyrosinaemia and associated ocular effects and changes in liver enzyme activities. There was some evidence of exacerbation of these effects with increasing tyrosine

levels. Generally, the effects seen were more marked than those observed when either 100 ppm mesotrione or tyrosine was given alone (Milburn, 1997a).

In a non-guideline dietary study to investigate dose–response patterns, 16 young adult male Alpk:APfSD (Wistar-derived) rats were fed a diet containing mesotrione (purity 96.8%) at a concentration of 0, 0.5, 1, 3, 4, 5, 7.5, 10 or 100 ppm (equal to 0, 0.04, 0.09, 0.27, 0.35, 0.44, 0.67, 0.89 and 8.96 mg/kg bw per day, respectively) for 90 consecutive days. Clinical observations, body weights and feed consumption were measured, and the eyes of all animals were examined by indirect ophthalmoscopy. Blood samples were taken for plasma tyrosine analysis, and overnight urine samples were collected for biochemical analyses. At the end of the scheduled period, the animals were killed and subjected to a limited postmortem examination. Selected organs were weighed, and specified tissues were taken for subsequent histopathological, electron microscopic or biochemical examinations.

There were no effects on mortality. Clinical signs of toxicity consisted of cloudy eyes at doses of 5 ppm and higher and red/brown staining on the tray paper of one 5 ppm cage; as it is consistent with the toxicity of mesotrione in other studies, this change was considered treatment related. Body weight was decreased at 100 ppm, but there were no clear effects on feed consumption. Under ophthalmological examination, changes to the cornea started at 7.5 ppm, with hazy to complete opacity of the cornea with or without vascularization. One animal in the 5 ppm dose group showed evidence of slight hazy opacity, but this was considered a random occurrence. Plasma tyrosine levels were increased at doses of 1 ppm and higher after 24 hours of treatment and at 0.5 ppm at weeks 1 and 14. There was an increase in urinary 4-hydroxyphenylpyruvate (HPPA), 4-hydroxyphenyllactate (HPLA) and 4-hydroxyphenylacetate (HPAA) phenyl acids in all tested animals at week 13, with a dose-related decrease in conjugates to free phenolic acids. There was a treatment-related increase in kidney weights at doses of 10 ppm and above and a treatment-related increase in liver weights at doses of 5 ppm and above. However, there were no gross pathological or histopathological changes noted in either organ at any dose in this study. Gross pathological changes were limited to increased opacity of the cornea at doses of 7.5 ppm and higher.

In the tissue biochemistry analysis, TAT was increased at doses of 3 ppm and higher, and HPPD was inhibited at doses of 0.5 ppm and above (Table 19).

In conclusion, in young male rats, there was a treatment-related inhibition of HPPD and increases in plasma tyrosine levels down to the lowest dose tested of 0.5 ppm. Adverse outcomes of tyrosinaemia were apparent at doses of 5 ppm and above, with increases in corneal opacity (Brammer, 1997d).

In a non-guideline dietary study to investigate dose–response relationships, 12 young adult female Alpk:APfSD (Wistar-derived) rats were fed a diet containing mesotrione (purity 96.8%) at a concentration of 0, 1, 5, 10, 50, 100, 1000 or 2500 ppm (equal to 0, 0.09, 0.48, 0.95, 4.82, 9.54, 94.83 and 236.75 mg/kg bw per day, respectively) for 90 consecutive days. An additional eight females per group were designated as satellites for interim kills on day 8 or 29. Clinical observations, body weights and feed consumption were measured, and the eyes of all animals were examined by indirect ophthalmoscopy. Overnight urine samples were collected for biochemical analyses. At the end of the scheduled period, the animals were killed, cardiac blood samples were taken for plasma tyrosine analysis and organs were removed, weighed and taken for biochemical analyses.

There were no effects on mortality. Clinical signs of toxicity were limited to cloudy eyes at doses of 1000 ppm and above. Body weights and feed consumption were decreased compared with controls in the 2500 ppm females, but unaffected at lower doses. Under ophthalmoscopic examination, there were increases in corneal opacity at doses of 100 ppm and higher and increases in vascularization and ghost vascularization at doses of 1000 ppm and above.

Table 19. Correlation of effects seen in rats administered mesotrione in the diet (maximum effect seen in each parameter)

	Dietary level of mesotrione (ppm)								
Parameter	**0**	**0.5**	**1**	**3**	**4**	**5**	**7.5**	**10**	**100**
Dose received (mg/kg bw per day)	–	0.04	0.09	0.27	0.35	0.44	0.67	0.89	8.96
Plasma tyrosine levels, week 13 (nmol/mL)	113	228*	431*	915**	1 241**	1 482**	1 934**	1 771**	2 772**
Kidney weight (% difference from control)	–	−1	1	1	1	4*	3	8**	8**
Liver weight (% difference from control)	–	−1	−1	4	5*	10**	12**	12**	15**
Corneal opacity (% affected)	0	0	0	0	0	6	25	31	94
Body weight, week 14 (% difference from control)	–	6	−1	3	0	4	−4	1	−4*
TAT activity (% difference from control)	100	110	125	151**	149**	148*	161**	140*	135
HPPD activity (% difference from control)	100	32	18*	20	18*	8*	10*	7*	3*
Urinary total phenolic acids (mg eq)	1.0	2.21	2.46	2.14	5.62	3.48	2.83	6.12	13.47

bw: body weight; eq: equivalents; HPPD: 4-hydroxyphenylpyruvate dioxygenase; ppm: parts per million; TAT: tyrosine aminotransferase; *: $P < 0.05$; **: $P < 0.01$ (Student's *t*-test, two-sided)

Source: Brammer (1997d)

Plasma tyrosine levels were increased to a statistically significant extent at doses of 5 ppm and higher and increased, but not statistically significantly, at 1 ppm. At doses of 1000 ppm and above, plasma tyrosine levels were between 1500 and 1600 nmol/mL, compared with control values of 112–127 nmol/mL, and remained relatively constant from weeks 1 to 13. At dose levels of 100 ppm and higher, there were increases in the levels of conjugated and free phenolic acids in the urine. As the dose increased, the proportion of free to conjugated acids decreased. The acids were identified as HPPA, HPLA and HPAA.

Liver weights were increased to a statistically significant extent at doses of 50 ppm and higher; however, the magnitude of the change was not biologically significant at any dose. There were no clear effects on kidney weights.

TAT induction occurred in all treated groups after the first week, but was statistically significant at doses of 5 ppm and above. By weeks 5 and 14, there was no change from control in TAT activity at doses of 100 ppm or less. HPPD inhibition occurred in all treated groups at all time points (Table 20).

In conclusion, in young female rats, inhibition of HPPD, a temporary activation of TAT and increases in plasma tyrosine levels occurred at doses of 1 ppm and higher. Adverse outcomes of tyrosinaemia occurred at doses of 100 ppm and above, with increases in corneal opacity (Brammer, 1997e).

In a non-guideline study investigating ocular toxicity development and reversibility, a group of 40 male Alpk:APfSD (Wistar-derived) rats were fed a diet containing 2500 ppm (equal to 272 mg/kg bw per day) mesotrione (purity 96.8%) for 35 consecutive days, with a concurrent control

Table 20. Correlation of effects seen in female rats administered mesotrione in the diet (maximum effect seen in each parameter)

	Dietary level of mesotrione (ppm)							
Parameter	**0**	**1**	**5**	**10**	**50**	**100**	**1 000**	**2 500**
Dose received (mg/kg bw per day)	–	0.09	0.48	0.95	4.82	9.54	94.83	236.75
Plasma tyrosine levels, week 13 (nmol/mL)	127	147	219**	249**	620**	836**	1 593**	1 534**
Liver weight, week 14 (% difference from control)	–	0	0	3	6	5	6*	7*
Corneal opacity (animals affected of 12)	0	0	0	0	0	2	11	10
Body weight, week 14 (% difference from control)	–	0	1	0	0	3	0	4*
TAT activity, week 2 (% of control)	100	132	165**	183**	200**	197**	233**	253**
HPPD activity, week 2 (% of control)	100	44**	12**	11**	12**	7**	2**	1**
Urinary total phenolic acids (mg eq)	0	0	0	0	0	1.82	7.0	23.0

bw: body weight; eq: equivalents; HPPD: 4-hydroxyphenylpyruvate dioxygenase; ppm: parts per million; TAT: tyrosine aminotransferase; *: $P < 0.05$; **: $P < 0.01$ (Student's *t*-test, two-sided)

Source: Brammer (1997e)

group consisting of 16 males fed untreated diet. Clinical observations, body weights and feed consumption were recorded, and eyes were examined by ophthalmoscopy. Blood samples were taken for measurement of plasma tyrosine levels, and eye and Harderian gland were taken for subsequent histopathological examination. At the end of the treatment period, all animals in the treated group without eye lesions and half of the animals in the control group were subjected to a postmortem examination. Approximately half of the animals that had eye lesions in the treated group were also given a postmortem examination, and the remainder were retained for a recovery period of 8 weeks prior to postmortem examination.

There were no effects on mortality. Body weight was decreased in the first 4 weeks of treatment, but not to a biologically significant degree. There were no changes to body weight thereafter.

Ophthalmological lesions were seen in treated animals starting at week 1 and were seen in 28/40 (70%) animals by the end of the treatment period. Two weeks into the recovery period, 9/15 (60%) animals exhibited corneal lesions. The vast majority of the changes at the end of the recovery period consisted of ghost vascularization.

Plasma tyrosine levels were increased starting at week 1 until week 14. The times for peak plasma tyrosine levels were weeks 2 and 6, with a substantial drop at week 7 and levels almost returning to control levels by week 14.

Microscopic changes in the eyes of animals killed at the end of the treatment period consisted of various severity grades of keratitis characterized by polymorphonuclear leukocytic infiltration of the outer corneal stroma with or without corneal epithelial disorganization and polymorphonuclear leukocytic infiltration of the corneal epithelium. The epithelial disorganization often took the form of a "V"-shaped area with basal cells present at the more superficial levels of the epithelium. Recovery animals showing no remaining changes at ophthalmoscopic evaluation showed no evidence of keratitis and no histopathological changes. Those recovery animals that exhibited ghost

vascularization at ophthalmoscopic evaluation exhibited histological evidence of remaining corneal vessels, and minimal or slight degrees of corneal fibroblasts were present in the subepithelial stroma. There was minimal or slight epithelial disruption in 3/15 rats. This was characterized by a slightly altered growth pattern of the epithelium so that the regular nature of the epithelial layers was disrupted. In some areas, there was hyaline material in a small number of basal epithelial cells.

In conclusion, corneal lesions associated with dietary administration of mesotrione for 5 weeks undergo ophthalmoscopic and histopathological recovery following cessation of treatment (Tinston, 1997).

In a non-guideline dietary study to investigate non-ocular end-points, 12 young adult male Alpk:APfSD rats per dose were fed diets containing mesotrione (purity 95.1%) at a concentration of 0, 10, 20, 50 or 125 ppm (equal to 0, 0.9, 1.7, 4.3 and 10.7 mg/kg bw per day, respectively) for 13 weeks. Body weight and feed consumption were monitored during this time. The eyes of all surviving animals were examined by ophthalmoscopy just prior to termination. At the end of the dosing period, the rats were killed, and blood samples were taken and stored. Liver and kidneys were weighed, and kidneys were processed to blocks and stored.

There were no treatment-related effects on mortality. Body weights were slightly decreased in the 125 ppm dose group at week 14, along with feed consumption from weeks 9 to 13. Corneal lesions occurred in all treated dose groups, with a dose-responsive increase in incidence. Although adjusted liver weights were increased in all treated groups, there was no dose–response relationship. Likewise, kidney weights were increased in all treated groups without a dose–response relationship.

In conclusion, under the limited investigations of this non-guideline study, body weight and feed consumption were the only signs of toxicity when ocular effects were excluded. These first signs of toxicity occurred at 125 ppm (equal to 10.7 mg/kg bw per day) (Brammer, 1995).

In a non-guideline dietary toxicity study to investigate reversibility, groups of 40 young adult male Alpk:APfSD (Wistar-derived) rats were fed diets containing mesotrione (purity 96.8%) at a concentration of 0 (two control groups), 5, 100 or 2500 ppm (equal to 0, 0.37, 7.52 and 192 mg/kg bw per day, respectively) for 90 consecutive days. These groups were subdivided into groups of eight rats and subjected to recovery periods of 0, 2, 4, 6 or 9 weeks for the 0 (first control group), 5 and 100 ppm dose groups and 0, 1, 2, 4 or 9 weeks for the 0 (second control group) and 2500 ppm dose groups. Clinical observations, body weights and feed consumption were measured, and the eyes of all animals were examined by indirect ophthalmoscopy. Blood samples were taken by tail venipuncture for plasma tyrosine analysis, and eight animals per group were killed at the end of each scheduled period and subjected to a postmortem examination. Additionally, cardiac blood samples were taken for plasma tyrosine analysis; liver and kidneys were weighed and were taken for subsequent examination by light and electron microscopy (control and 2500 ppm animals only) and for biochemical analyses.

There were no effects on mortality. Clinical signs of toxicity were limited to cloudy eyes in animals dosed at and above 5 ppm, and all signs of eye changes had resolved by week 17 in all dose groups. Body weights in animals dosed at 2500 ppm were decreased by 9% compared with controls during the last week of treatment and decreased by 10% in weeks 19–21 and by 8–10% at 23 weeks. Feed consumption was decreased sporadically in the 2500 ppm dose group during treatment. Effects in dose groups of 100 ppm and below were below levels considered adverse, although there was also evidence of recovery in the 5 and 100 ppm groups.

Under ophthalmoscopic examination, 63–70% of animals dosed at 100 and 2500 ppm exhibited corneal change by the end of the treatment period. Animals dosed at 5 ppm showed a lower incidence of lesions, including lower degrees of hazy or complete opacity with or without vascularization. Upon removal of mesotrione from the diet, corneal lesions began to reverse until only ghost vascularization persisted until the end of the recovery period.

Plasma tyrosine level was increased in 2500 ppm animals starting at 24 hours after the commencement of dosing and remained elevated until week 14. However, levels at 14 weeks were 1995 nmol/mL compared with 2917 nmol/mL, indicating a certain amount of adaptation. At the end of the recovery period, levels were within 21–32% of the control values, although the increase was statistically significant at week 23. At lower doses, plasma tyrosine concentrations were increased at week 1 and comparable with control values by week 18 (Table 21). Following homogenization of the liver and kidney samples, tyrosine concentrations were increased in the 5 and 100 ppm groups immediately following 90 days of treatment, but were comparable with control values following 9 weeks of recovery.

Table 21. Intergroup comparison of plasma tyrosine levels in rats

	Plasma tyrosine level (nmol/mL)				
	Part I			**Part II**	
Time point	**0 ppm (control)**	**5 ppm**	**100 ppm**	**0 ppm (control)**	**2 500 ppm**
24 hours	–	–	–	126	2 917**
Week 1	129.6	1 190.1**	2 021.6**	–	–
Week 14	155.4	1 283.4**	2 142.3**	192	1 995**
Week 15 + 1-week recovery	–	–	–	120	408**
Week 16 + 2-week recovery	105.1	128.1**	152.0**	113	423**
Week 18 + 4-week recovery	154.7	156.2	164.4	111	146
Week 20 + 6-week recovery	106.5	124.1*	129.6**	–	–
Week 23 + 9-week recovery	180.9	193.4	190.7	111	134**

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Student's *t*-test, two-sided)

Source: Brammer (1997f)

In animals killed at the end of the treatment period, there were increases in liver and kidney weights following 90 days of mesotrione administration at doses of 5, 100 and 2500 ppm. Kidney and liver weights were increased compared with controls after 1 week of recovery at 2500 ppm, and liver weights were increased until and including 9 weeks of recovery at the same dose, although the magnitude of the increase was lower than immediately following treatment. In animals dosed at 5 and 100 ppm, there was no evidence of increased liver or kidney weights at 2–9 weeks of recovery.

TAT induction occurred at all doses during treatment and was similar to control values after 9 weeks of recovery. HPPD was inhibited following treatment at 5, 100 and 2500 ppm. There was recovery in the 2500 ppm group, although complete recovery did not seem to occur in the 5 and 100 ppm dose groups. However, there was a large variation in control values between the two parts of the study, and the finding is of limited value (Table 22).

In conclusion, mesotrione exhibited a potential for recovery from treatment-related effects on body weight and feed consumption, plasma and tissue tyrosine concentrations, enzyme induction and inhibition and ocular effects (Brammer, 1997f).

In a non-guideline dietary toxicity study in mice to investigate dose–response relationships, groups of 10 young adult C57BL/10JfAP/Alpk mice of each sex per dose were fed diets containing

Table 22. Intergroup comparison of HPPD activity in rats administered mesotrione in the diet

	HPPD activity (µL oxygen/min/mg protein)				
	Part I			Part II	
Week	0 ppm (control)	5 ppm	100 ppm	0 ppm (control)	2 500 ppm
14	0.880	0.099**	0.031**	0.141	0.005**
23 + 9-week recovery	1.030	0.664**	0.690**	0.207	0.161

HPPD: 4-hydroxyphenylpyruvate dioxygenase; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Student's *t*-test, two-sided)

Source: Brammer (1997f)

mesotrione (purity 96.8%) at a concentration of 0, 1, 10, 50, 100, 350, 1000, 3500 or 7500 ppm (equal to 0, 0.16, 1.69, 8.49, 18.0, 58.5, 179.3, 599.9 and 1222.5 mg/kg bw per day for males and 0, 0.19, 1.94, 10.8, 20.5, 72.7, 214.9, 714.8 and 1436.4 mg/kg bw per day for females) for 90 consecutive days. An additional 10 males and 10 females per group were included as satellite groups for interim kills after 1 and 4 weeks of administration of mesotrione. Clinical observations, body weights and feed consumption were measured, and overnight urine samples were collected for biochemical analyses. At the end of the scheduled period, the animals were killed, cardiac blood samples were taken for plasma tyrosine analysis, liver and kidney were weighed, and samples were submitted for subsequent biochemical examinations and stored for possible histopathological examination.

There were no effects on mortality or clinical signs of toxicity. Body weights were decreased by up to 5% in males at week 8 and by up to 6% in females at week 7 at 7000 ppm. There were no effects on body weight at doses of 3500 ppm or lower. There were no effects on feed consumption at any dose tested.

Plasma tyrosine levels were increased at all time points in males and females at doses of 10 ppm and above. At doses of 50 ppm and higher in males, peak plasma tyrosine levels occurred at week 4. In the 1 ppm dose group, plasma tyrosine levels were increased in the first week following the start of treatment, but were comparable with control values thereafter in males. In females, plasma tyrosine levels were increased to a statistically significant extent at the 4-week time point, but were comparable with control values at weeks 1 and 14. In females at all doses, plasma tyrosine levels peaked at week 1 and, although still elevated compared with controls, decreased thereafter.

There was no evidence of conjugated phenolic acids in the urine of male or female mice at any dose. Free phenolic acids were increased in the urine of males at 10 ppm and above, but peaked at 100 ppm and decreased thereafter. In females, free phenolic acids were increased at all treatment doses, peaked at 100–350 ppm and decreased thereafter. The predominant phenolic acid in the urine samples was HPPA in both males and females, although HPLA and HPAA were also present.

There were no effects on liver or kidney weights.

TAT induction in male mice did not show consistent dose-responsiveness, but there was a trend towards increased induction from 50 to 1000 ppm at week 1, from 50 to 350 ppm at week 4 and from 100 to 3500 ppm at week 14. In females, there was a greater amount of statistical significance for this effect, as induction was increased at 50–3500 ppm at week 1, 1–7000 ppm at week 4 and 50–7000 ppm at week 14. HPPD inhibition was consistent in all males throughout the treatment period. In females, there was a statistically significant inhibition of HPPD at all doses in weeks 1 and 4 and at 100 ppm and above in week 14.

In conclusion, administration of mesotrione down to 1 ppm induced elevated plasma tyrosine levels in male and female mice. There was a correlation between the degree of tyrosinaemia, HPPD inhibition and excretion of phenolic acids in the urine in both sexes and a correlation with the induction of TAT in females. There were no significant clinical effects and no organ weight, gross

pathological or histopathological effects in the liver or kidney. There was a slight decrease in body weights in males and females at 7000 ppm (Brammer, 1997g).

In a non-guideline reproductive toxicity study in rats to investigate the effects of mesotrione in conjunction with dietary tyrosine, 20 time-mated female Alpk:APfSD (Wistar-derived) rats were fed diets containing 0 ppm mesotrione/0% tyrosine, 0 ppm mesotrione/0.5% tyrosine, 0 ppm mesotrione/1% tyrosine, 0 ppm mesotrione/2% tyrosine, 2500 ppm mesotrione/0% tyrosine, 2500 ppm mesotrione/0.5% tyrosine, 2500 ppm mesotrione/1% tyrosine or 2500 ppm mesotrione/2% tyrosine from day 1 of gestation until termination on day 29 postpartum. Clinical observations, body weights and feed consumption were recorded for the parent females. In addition, plasma tyrosine concentration was determined on two occasions. The females were allowed to litter, and the pups were sexed, counted, examined and weighed during the lactation period. Terminal blood samples were analysed for plasma tyrosine, and kidneys were examined for bilateral pelvic dilatation.

One dam dosed with 0 ppm mesotrione/2% tyrosine was found dead. There was a treatment-related increase in whole litter losses in dams dosed with 2500 ppm mesotrione and 0.5% tyrosine and above. All the dams in the 2500 ppm/2% tyrosine dose group were killed for humane reasons by day 11. Clinical signs of toxicity consisted of opaque eyes in all groups treated with mesotrione, but not in groups treated with tyrosine in the absence of mesotrione; hunched posture in at least one dam in each of the treated groups, but in two and five dams of the 2500 ppm mesotrione with 1% and 2% tyrosine groups, respectively; and piloerection in all of the groups treated with mesotrione and a slight increase in the dams treated with 2% tyrosine in the absence of mesotrione. During gestation, body weights were decreased in all dams given mesotrione without a dose–response trend dependent on the proportion of tyrosine. During lactation, body weights were decreased in dams given both mesotrione and tyrosine, with increased effects with increased tyrosine. Feed consumption was decreased in the first week of gestation in all groups given mesotrione and thereafter in the two groups given mesotrione and tyrosine. Plasma tyrosine levels were increased by an order of magnitude in all groups given mesotrione, with little effect of additional dietary tyrosine. In the groups given tyrosine in the absence of mesotrione, there was a slight increase in plasma tyrosine levels at 2% tyrosine at day 3 and at 1% and 2% tyrosine at day 51.

In the litters, pups showed an increase in opaque and cloudy eyes in all groups subjected to mesotrione, but in none of the groups exposed only to dietary tyrosine. The percentage of pups born live was decreased in the 2500 ppm mesotrione/1% tyrosine group, and the percentage of pups live at day 22 was decreased in both the 2500 ppm mesotrione/0.5% tyrosine and 2500 ppm mesotrione/1% tyrosine groups. Whole litter losses did not occur in the controls, but occurred at incidences of 1, 2 and 2 in the 0.5%, 1% and 2% dietary tyrosine groups and at incidences of 1, 6 and 10 in the 0%, 0.5% and 1% tyrosine with 2500 ppm mesotrione groups. Pup body weights were decreased by 7% in males and by 5% in females dosed with 2500 ppm mesotrione/1% tyrosine. Plasma tyrosine levels were increased in all dose groups; however, the increase was up to 1.8-fold in pups dosed with dietary tyrosine compared with increases of 16-fold in pups dosed with mesotrione or a combination of mesotrione and dietary tyrosine. There were no effects on the kidneys in pups dosed with dietary tyrosine, but there were increases in bilateral pelvic dilatation in all groups exposed to mesotrione and dietary tyrosine (36%, 45% and 52% in males and 37%, 45% and 33% in females at 0.5%, 1% and 2% tyrosine, respectively).

In conclusion, tyrosine and mesotrione caused increased plasma tyrosine levels and increases in whole litter losses, although the effect of mesotrione was greater than that of dietary tyrosine. Dietary tyrosine did not seem to have an effect on bilateral pelvic dilatation, whereas mesotrione with or without dietary tyrosine did increase kidney pathology and increase clinical signs in dams (Williams, 2000).

In a non-guideline developmental toxicity study to investigate the effects of tyrosinaemia on the ossification of the fetal skeleton and occurrence of abortions, 20 time-mated female New Zealand

White rabbits per dose were treated with diet containing 1% tyrosine from gestation day (GD) 8 to GD 21, 500 mg/kg bw per day mesotrione via gavage from GD 8 to GD 20 or 500 mg/kg bw per day mestrione via gavage from GD 8 to GD 20 along with 1% tyrosine in the diet from GD 8 to GD 21. A control group of 20 time-mated female New Zealand White rabbits was fed untreated diet and gavaged with water on GDs 8–20. The day of mating was designated day 1 of gestation. The rabbits were killed on day 30 of gestation. The following observations and measurements were made in the dams: clinical observations, body weights, feed consumption, ophthalmoscopy, plasma tyrosine concentration, HPPD and TAT activities in liver and kidney, postmortem examination (macroscopic), number of corpora lutea, gravid uterine weight and number and position of implantations in the uterus. The following observations and measurements were made in the fetuses/litters: number and position of live fetuses, number and position of intrauterine deaths (early and late), percentage preimplantation loss, percentage post-implantation loss, fetal weight, fetal sex, external and visceral variations and abnormalities and skeletal variations and abnormalities, including evaluation of bone and cartilage and ossification of the manus and pes.

There was one abortion in the mesotrione and tyrosine treatment following decreased feed consumption from day 17 and decreased body weight from day 19. One control female was found dead on day 19 (with live pups).

There were no biologically significant effects on adjusted mean body weights, and there was no evidence of effects on feed consumption. There were no changes at ophthalmoscopic examination.

Maternal plasma tyrosine levels increased starting at 12 hours post-dosing. At this point, dams dosed with tyrosine only had a 2.9-fold increase in tyrosine level, mesotrione-only dams had an 8-fold increase and dams dosed with both had a 17-fold increase. Values were similar at 12 hours post-dosing on day 14, but at 24 hours following dosing on day 14, tyrosine levels were increased by 1.5-, 2.6- and 3.8-fold in tyrosine-only dams, mesotrione-only dams and dams dosed with both tyrosine and mesotrione, respectively. Twenty-four hours following dosing on day 14, plasma tyrosine values were increased 1.5-fold in tyrosine-only dams, 2.6-fold in mesotrione-only dams and 3.8-fold in dams treated with both. By GD 29, plasma tyrosine values were practically comparable with control values. In the groups dosed with mesotrione and with mesotrione and tyrosine, there were comparable inhibitions of HPPD. In groups dosed with tyrosine alone, HPPD activity was comparable with control values. TAT activity was unaffected in all groups in the liver, but was inhibited in the kidneys in both mesotrione-dosed groups to similar extents. There were no macroscopic findings at necropsy. There were no treatment-related findings in the caesarean section parameters.

In the offspring, there was an increase in a minor defect of extra vessels arising from the aortic arch in both groups dosed with mesotrione; however, this type of finding was not seen in previous rabbit studies, and its relevance to treatment is unknown. In the skeletal examination, there were no major or minor defects, and changes were limited to delays in ossification. There was a distinct trend for delays to be more prevalent in groups exposed to both mesotrione and tyrosine over mesotrione alone and more prevalent in both compared with the tyrosine-only groups; however, delays occurred in all treated groups compared with controls.

In conclusion, although mesotrione had a greater effect than tyrosine, there was evidence that the effects were cumulative and a result of the increases in plasma tyrosine levels caused by the exposure to mesotrione. The delays in ossification were determined to be related to the increase in plasma tyrosine levels and a result of the inhibition of HPPD activity. The abortion in the mesotrione and tyrosine group was the only one in the study, and its relationship to treatment is unknown (Moxon, 2000).

(c) Studies on metabolites

Absorption, excretion and biotransformation

In a metabolism study on MNBA to investigate biotransformation in the rat, four male Alpk:APfSD rats were given a single oral [^{14}C]MNBA dose of 75 mg/kg bw. The excretion of radioactivity in urine and faeces was monitored for 12 hours after dosing. After this period, the rats

were killed, and the residual radioactivity was measured in the excreta, gastrointestinal tract, gastrointestinal tract contents and residual carcass. The metabolites present in urine and solvent extract of the gastrointestinal tract contents were identified and quantified by HPLC and HPLC with mass spectrometry.

Twelve hours following dosing, the largest proportion of the administered dose (43.6%) was found in the gastrointestinal tract, with 16.1% and 26.6%, respectively, found in the urine and faeces. Of the radioactivity recovered in the urine, 25% of the radioactivity recovered at 6 hours and 91% of the radioactivity recovered at 12 hours were characterized as AMBA. At 12 hours following dosing, 100% of the radioactivity in the gastrointestinal tract was characterized as AMBA. According to the study author, the molecular weights of both MNBA and AMBA are lower than the biliary elimination cut-off, and so it is assumed that radioactivity in faeces represents unabsorbed dose.

In conclusion, MNBA is minimally absorbed and excreted in the urine. The majority is converted to AMBA in the gut, where it is excreted unabsorbed (Gledhill, 2000).

Acute studies on metabolites

Acute toxicity studies on the metabolites are summarized in Table 23.

Table 23. Acute toxicity of mesotrione metabolites

Test substance	Species	Strain	Sex	Route	Purity (%)	Result	Reference
MNBA	Rat	Alpk:APfSD (Wistar-derived)	Male and female	Oral	97	LD_{50} > 5 000 mg/kg bw	Robinson (1996)
AMBA	Rat	Alpk:APfSD (Wistar-derived)	Male and female	Oral	99	LD_{50} > 5 000 mg/kg bw	Lees (1996)

AMBA: 4-(methylsulfonyl)-2-aminobenzoic acid; bw: body weight; LD_{50}: median lethal dose; MNBA: 2-nitro-4-(methylsulfonyl)-benzoic acid

Short-term studies of toxicity on metabolites

In a short-term oral toxicity study, groups of five young adult Alpk:APfSD (Wistar-derived) rats of each sex per dose were given MNBA (purity 97.1%) in corn oil via gavage at 0, 15, 150 or 1000 mg/kg bw per day for 28 consecutive days. Clinical observations, body weights and feed consumption were measured throughout the study. In the fourth week of the study, the following tests were assessed: sensory reactivity to stimuli, grip strength and motor activity. At the end of the scheduled period, the animals were killed and subjected to a postmortem examination. Cardiac blood samples were taken for clinical pathology, selected organs were weighed and specified tissues were taken for subsequent histopathological examination.

There was no treatment-related mortality, and there were no clinical signs of toxicity. There were no effects on body weight, feed consumption, detailed clinical parameters or functional observational battery parameters. There were no effects on haematological parameters, and the only change in clinical chemistry was a slight, non-adverse decrease in alanine aminotransferase activity. Spleen weights were decreased by 12% in high-dose females, and testes weights were increased by 13% in high-dose males. However, as there were no findings in the gross necropsy or histopathological examination, the changes were of unknown adversity.

The NOAEL for MNBA was 1000 mg/kg bw per day, the highest dose tested (Milburn, 1998).

In a short-term oral toxicity study, groups of 12 young adult Alpk:APfSD (Wistar-derived) rats of each sex per dose were given diets containing MNBA (purity 98.3%) at 0, 100, 650 or 3000 ppm (equal to 0, 7.7, 50.6 and 231.0 mg/kg bw per day for males and 0, 8.8, 56.9 and 263.7 mg/kg bw

per day for females, respectively) for 90 consecutive days. Clinical observations, body weights and feed consumption were measured throughout the study, and urine samples were taken for clinical pathology during week 13 of the study. In addition, detailed clinical observations, including quantitative assessments of landing foot splay, sensory perception and muscle weakness, and assessment of motor activity were performed during week 12 of the study. At the end of the scheduled period, the animals were killed and subjected to a postmortem examination. Cardiac blood samples were taken for clinical pathology, selected organs were weighed and specified tissues were taken for subsequent histopathological examination.

There were no mortalities or treatment-related clinical effects. There was an 8% decrease in body weight in high-dose males and a sporadic, non-adverse decrease in feed consumption at the same dose. There were no changes to ophthalmological parameters and no changes in the detailed observations of the functional observational battery. There were no changes in haematological parameters. There were 1.2- and 1.9-fold increases in plasma tyrosine levels in males only at 650 and 3000 ppm, respectively; however, the increase was not associated with any adverse changes in the animals. In females, triglyceride levels were increased 36% over those of concurrent controls in the 3000 ppm dose group. There were no changes in urine analysis parameters, and no phenolic acids were detected in the urine. There were no changes noted at necropsy or in the histopathological examination.

The LOAEL for MNBA was 3000 ppm (equal to 231.0 mg/kg bw per day), based on decreased body weight in males and increased triglyceride levels in females. The NOAEL was 650 ppm (equal to 50.6 mg/kg bw per day) (Rattray, 2000).

Genotoxicity studies on metabolites

Genotoxicity studies on mesotrione metabolites are summarized in Table 24.

Special studies on metabolites

In a non-guideline study to investigate the inhibition of HPPD by MNBA, excised livers from untreated male Alpk:APfSD rats were exposed to 0.02 or 20 µmol/L of MNBA as well as 0.02 and 20 µmol/L concentrations of mesotrione and 2-(2-nitro-4-fluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) (known inhibitors of HPPD).

The positive control substances, mesotrione and NTBC, inhibited HPPD at both 0.02 and 20 µmol/L. At the lower dose, mesotrione inhibited HPPD by 78%, and NTBC inhibited HPPD by 70.3%. For MNBA, there was no inhibition at 0.02 µmol/L, and HPPD was inhibited by 7.2% at 20 µmol/L.

In conclusion, MNBA is a very weak inhibitor of HPPD compared with mesotrione and NTBC (Elcombe & Meadowcroft, 1998a).

In a non-guideline study to investigate the inhibition of HPPD by AMBA, excised livers from untreated male Alpk:APfSD rats were exposed to 0.02 or 20 µmol/L of AMBA as well as 0.02 and 20 µmol/L concentrations of mesotrione and NTBC (known inhibitors of HPPD).

The positive control substances, mesotrione and NTBC, inhibited HPPD at both 0.02 and 20 µmol/L. At the lower dose, mesotrione inhibited HPPD by 78%, and NTBC inhibited HPPD by 70.3%. For AMBA, there was no inhibition at 0.02 µmol/L, and HPPD was inhibited by 18.7% at 20 µmol/L.

In conclusion, AMBA is a very weak inhibitor of HPPD compared with mesotrione and NTBC (Elcombe & Meadowcroft, 1998b).

Table 24. Genotoxicity studies with mesotrione metabolites

Test substance	End-point	Test object	Concentration	Purity (%)	Results	Reference
In vitro						
MNBA	Reverse mutation	*Salmonella typhimurium* and *Escherichia coli*	0, 100, 200, 500, 1 000, 2 500 or 5 000 µg/plate (±S9)	97	Negative	Callander (1996a)
MNBA	Mammalian cell cytogenetics	Human lymphocytes	0, 250 or 2 451 µg/mL (+S9)	98.8	Negative	Fox (2000a)
			0, 250, 1 000/1 500 or 2 000/2 451 µg/mL (–S9)		Negative	
AMBA	Reverse mutation	*S. typhimurium* and *E. coli*	0, 100, 200, 500, 1 000, 2 500 or 5 000 µg/plate (±S9)	99	Negative	Callander (1996b)
AMBA	Mammalian cell cytogenetics	Human lymphocytes	250, 1 000 or 2 150 µg/mL (+S9)	100	Negative	Fox (2000b)
			250, 1 000 or 2 150 µg/mL (–S9)		Positive	
In vivo						
MNBA	Rat liver unscheduled DNA synthesis	Alpk:APfSD rat liver, males	1 000 or 2 000 mg/kg bw Harvest time: 2, 6 or 16 h	98.8	Negative	Clay (2000)
MNBA	Rat micronucleus	Alpk:APfSD rat bone marrow, males	0 or 2 000 mg/kg bw Harvest time: 28 and 48 h	98.9	Negative	Fox (2000c)

AMBA: 4-(methylsulfonyl)-2-aminobenzoic acid; bw: body weight; DNA: deoxyribonucleic acid; MBNA: 2-nitro-4-(methylsulfonyl)-benzoic acid; S9: 9000 × *g* supernatant fraction from liver homogenate from Aroclor-treated rats

3. Observations in humans

In an acute oral capsule human volunteer study to identify suitable urinary markers for worker exposure, three groups of six male human volunteers were administered a single oral dose of 0.1, 0.5 or 4 mg/kg bw. Volunteers were fed controlled diet to provide a consistent intake of tyrosine 72 hours prior to dosing and in the 96-hour period following dosing. Plasma and urine samples were collected in the predosing and post-dosing intervals and were analysed for tyrosine and test substance. Samples from the volunteers given a 4 mg/kg bw dose were monitored for tyrosine metabolites. Volunteers were examined for changes to ophthalmoscopy, clinical chemistry, haematology and urine analysis, and vital signs and symptomatology were monitored post-dosing.

Plasma tyrosine levels peaked within 24 hours of administration in all three dose groups. In the 0.1 and 0.5 mg/kg bw dose groups, plasma tyrosine levels had returned to predosing levels at the end of the 24-hour period. In the 4 mg/kg bw group, plasma tyrosine levels had returned to predosing levels by the 48-hour time period. Peak tyrosine concentrations occurred between 91.0 and 160, 121 and 210, and 241 and 420 nmol/mL plasma in the 0.1, 0.5 and 4 mg/kg bw groups, respectively. There was no statistical difference in elevations in the 0.1 and 0.5 mg/kg bw groups. In comparison, in a study with a strong HPPD inhibitor, NTBC, plasma tyrosine levels peaked at a mean of 1155 ± 121.2 nmol/mL following a single NTBC administration of 1 mg/kg bw (Stevens, 1998). There were no adverse signs noted, and peak concentrations of test substance were reached within 1 hour of dosing. A significant proportion of the administered dose was excreted in the urine as test substance, whereas tyrosine metabolites were detected in the urine following dosing with mesotrione at 4 mg/kg bw (Hall, 1998a).

In an acute dermal spray human volunteer study to determine urinary concentrations of mesotrione and effects on plasma tyrosine level following dermal exposure, three groups of six male human volunteers were administered single dermal doses of 5 µg of 100 g/L mesotrione per square centimetre (group 1), 5 µg of 480 g/L mesotrione per square centimetre (group 2) or 32 µg of 480 g/L mesotrione per square centimetre (group 3), representing two formulations and two concentrations of the 480 g/L formulation. The test substance was applied to a total area of 800 cm^2 and left unoccluded for 10 hours before the site was washed. Tape stripping of the washed sites was performed on a 6 cm^2 area in three sessions: post-initial washing, after 24 hours of wearing a T-shirt and following showering. Plasma and urine samples were collected in the predosing and post-dosing intervals and analysed for tyrosine and test substance. Urine samples were monitored for known tyrosine metabolites. Volunteers were examined for changes to ophthalmoscopy, clinical chemistry, haematology and urine analysis, the test site was monitored for signs of irritation, and vital signs and symptomatology were monitored post-dosing.

Dermal reactions consisted of an increase in mild transient itching in participants in group 3, a mild, transient burning sensation 30 minutes after application in one participant in group 1 and a mild, transient stinging sensation 9 hours following application in one participant in group 2. Erythema was noted in all groups, although the study authors attributed the reaction to the procedure or tape stripping. Symptomatology designated as "possibly related" to administration of the test substance consisted of mild tingling at the dose site 9 hours following application in one group 1 member and mild headache experienced 4 hours following application in one group 3 member.

There were no changes in plasma tyrosine level in any of the groups and no markers of mesotrione in the urine in the two lower-dose groups. In group 3, mesotrione was detected at slightly above the level of quantification in five out of six volunteers in up to five of the 13 samples collected from each participant. There were no quantifiable concentrations of mesotrione isolated in the plasma in any of the groups. Tape stripping indicated that mesotrione was located in the stratum corneum following initial washing, but was removed by washing and/or contact with clothing soon thereafter (Hall, 1998b).

Comments

Biochemical aspects

Excretion and tissue retention studies were performed in mice and rats. In addition, a full set of metabolism studies was performed in rats. Radiolabelled mesotrione was administered by gavage in both species. In mice, mesotrione was extensively absorbed (> 60%) and primarily excreted in the urine, constituting 41–59% of the administered low dose (1 mg/kg bw) and 63–70% of the high dose (100 mg/kg bw). Faecal elimination comprised 21–38% of the administered dose. Elimination was essentially complete within the first 24 hours; by 72 hours following dosing, elimination comprised 79–95% of the administered dose. In rats, mesotrione was rapidly and extensively absorbed (> 60%), metabolized to a limited extent and excreted primarily in the urine after single low (54–56% at 1 mg/kg bw) or high doses (62–63% at 100 mg/kg bw) or repeated low doses (61–67% at 1 mg/kg bw per day) over 14 days to rats. Biliary excretion was minimal. Most of the radioactivity was excreted as the parent compound within the first 12 hours post-dosing. Highest levels were found in liver, kidneys and gastrointestinal tract in both species, with 10–12% present in tissues following a low dose and less than 0.3% following a high dose.

In studies performed only in rats, there was no evidence of accumulation. C_{max} values were 0.27 and 0.25 µg eq/g in male and female rats, respectively, at the low dose (1 mg/kg bw) and 40.4 and 19.9 µg eq/g, respectively, at the high dose (100 mg/kg bw). The T_{max} was 0.5 hour at the low dose and 1.5 hours at the high dose. Half-lives in blood were less than 2 hours, regardless of sex or dose. There were no notable differences in absorption or excretion between the sexes. Mesotrione and its metabolites were not excreted in expired air. Parent compound accounted for more than 43–64% of the administered dose in the urine and 0–8% of the administered dose in the faeces.

In rats, the metabolites produced from hydroxylation of the dione ring include 4-hydroxy-mesotrione, 5-hydroxy-mesotrione, MNBA and AMBA. There is also a proposed cleavage of the molecule into constituent rings and reduction by the gut microflora, resulting in a number of unidentified metabolites, accounting for a total of approximately 0–12% of the administered dose in the faeces.

Toxicological data

In the rat, mesotrione is of low acute oral toxicity (LD_{50} > 5000 mg/kg bw), low acute dermal toxicity (LD_{50} > 2000 mg/kg bw) and low acute inhalation toxicity (LC_{50} > 4.75 mg/L). In the rabbit, mesotrione was non-irritating to the skin and mildly irritating to the eyes. Mesotrione was not a dermal sensitizer in guinea-pigs (maximization test).

The primary effect of mesotrione in mammals is the inhibition of HPPD, a key enzyme of the tyrosine catabolic pathway. Inhibition of HPPD by mesotrione results in raised plasma tyrosine levels, which appear to be responsible for the critical effects observed (ocular, kidney, liver and thyroid toxicity). The plateau levels of plasma tyrosine after mesotrione administration are higher in rats (males > females) than in mice. The difference in sensitivity between male and female rats as well as between rats and mice can be attributed to differences in tyrosine catabolism. If the activity of TAT is low, as it is in the male rat, tyrosine cannot convert quickly to HPPA; when HPPD is inhibited, the resultant increase in plasma tyrosine levels leads to toxicity.

The critical effect (ocular toxicity) associated with the administration of mesotrione is mediated by these increased systemic levels of tyrosine and occurs only when plasma tyrosine levels exceed about 1000 nmol/mL. The ocular sensitivity of the various species to tyrosine plasma levels seems to be similar; the difference in overall toxicity of mesotrione among the species is attributable to the different levels of plasma tyrosine achieved after HPPD inhibition by mesotrione.

Although the rat is the most sensitive species for assessing tyrosine-mediated mesotrione toxicity, the mouse is a better model for such effects in humans. Humans and mice have similar TAT activities and do not experience the adverse effects associated with the same degree of HPPD inhibition in rats. The effects on the eyes, kidneys, liver and thyroid seen in the rat are unlikely to occur in humans exposed to mesotrione owing to differences in tyrosine metabolism. As all the relevant studies normally performed in the rat were also performed in the mouse, it was determined that the risk assessment would be based on toxicity in the mouse, rabbit and dog.

In a 90-day oral toxicity study in mice, animals were given diets containing mesotrione at a concentration of 0, 10, 50, 350 or 7000 ppm (equal to 0, 1.7, 8.4, 61.5 and 1212.4 mg/kg bw per day for males and 0, 2.4, 12.4, 80.1 and 1537.1 mg/kg bw per day for females, respectively). The NOAEL was 7000 ppm (equal to 1212.4 mg/kg bw per day), the highest dose tested.

In a 90-day oral toxicity study in rats, animals were given diets containing mesotrione at a concentration of 0, 1, 125, 1250 or 12 500 ppm (equal to 0, 0.09, 10.96, 112.09 and 1110.86 mg/kg bw per day for males and 0, 0.10, 12.81, 125.58 and 1212.53 mg/kg bw per day for females, respectively). At 125 ppm (equal to 10.96 mg/kg bw per day), male rats showed evidence of increased corneal opacity and vascularization and decreased body weight and feed efficiency.

In a 13-week oral toxicity study in rats, animals were given diets containing mesotrione at a concentration of 0, 2.5, 5.0, 7.5 or 150 ppm (equal to 0, 0.21, 0.41, 0.63 and 12.46 mg/kg bw per day for males and 0, 0.23, 0.47, 0.71 and 14.48 mg/kg bw per day for females, respectively). There were no non-ocular findings in either male or female rats in this study. At 7.5 ppm and above, males showed evidence of cloudy eyes.

In a 13-week oral capsule toxicity study in dogs, animals were exposed to 0, 100, 600 or 1000 mg/kg bw per day. At 1000 mg/kg bw per day, body weights were decreased in males compared with controls and there was an increase in minimal/slight focal mesothelial proliferation of the atrium of the heart in two males. The NOAEL was 600 mg/kg bw per day.

In a 1-year oral capsule toxicity study in dogs, animals were exposed to 0, 10, 100 or 600 mg/kg bw per day. At the high dose, body weights were decreased in females, and lenticular opacity was observed in one male and one female. In the male, the lenticular opacity was associated with unilateral keratitis and periorbital haemorrhage; in the female, it was associated with unilateral corneal erosion. The NOAEL was 100 mg/kg bw per day.

In a 1-year oral toxicity study in mice, animals were given diets containing mesotrione at a concentration of 0, 10, 50, 350 or 7000 ppm (equal to 0, 1.5, 7.8, 56.2 and 1114 mg/kg bw per day for males and 0, 2.1, 10.3, 72.4 and 1495 mg/kg bw per day for females, respectively). At the highest dose tested, males exhibited decreased body weight and body weight gains. There were no effects in females at the highest dose tested. The NOAEL was 350 ppm (equal to 56.2 mg/kg bw per day).

In an 18-month oral toxicity and carcinogenicity study in mice, animals were given diets containing mesotrione at a concentration of 0, 10, 350 or 3500/7000 ppm (equal to 0, 1.4, 49.7 and 897.7 mg/kg bw per day for males and 0, 1.8, 63.5 and 1103 mg/kg bw per day for females, respectively). As seen in the 1-year study, body weight, body weight gains and feed efficiency were decreased in males at the highest dose tested, and there were no effects in females at the highest dose tested. There was no evidence of carcinogenicity. The NOAEL was 350 ppm (equal to 49.7 mg/kg bw per day).

In a 2-year carcinogenicity study in rats, animals were given diets containing mesotrione at a concentration of 0, 7.5, 100 or 2500 ppm (equal to 0, 0.48, 6.48 and 159.9 mg/kg bw per day for males and 0, 0.57, 7.68 and 189.5 mg/kg bw per day for females, respectively). There was no evidence of carcinogenicity. In males, changes at all doses consisted of cloudy eyes, corneal opacities, vascularization and keratitis in the clinical, ophthalmological and histopathological examinations, decreased body weights, hepatocyte fatty vacuolation in the liver and thyroid follicular cysts.

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

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

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

In a two-generation reproductive toxicity study in mice, animals were given diets containing mesotrione at a concentration of 0, 10, 50, 350, 1500 or 7000 ppm (equal to 0, 2.1, 10.2, 71.4, 311.8 and 1472 mg/kg bw per day for males and 0, 2.1, 10.0, 71.3, 301.6 and 1439 mg/kg bw per day for females, respectively). At the highest dose tested, F_1 adults and pups showed evidence of cataractous changes at clinical, gross and histopathological examination. Pups at the next lower dose also exhibited decreased body weight and body weight gain, clinical, gross and histopathological changes to the eyes (opaque/cloudy eyes, cataractous change) and increased plasma tyrosine levels. The NOAEL for parental toxicity was 1500 ppm (equal to 301.6 mg/kg bw per day). The NOAEL for reproductive toxicity was 7000 ppm (equal to 1439 mg/kg bw per day), the highest dose tested. The NOAEL for offspring toxicity was 350 ppm (equal to 71.3 mg/kg bw per day).

In a three-generation reproductive toxicity study in rats, animals were given diets containing mesotrione at a concentration of 0, 2.5, 10, 100 or 2500 ppm (equal to 0, 0.3, 1.1, 11.6 and 278.1 mg/kg bw per day for males and 0, 0.3, 1.1, 11.7 and 297.2 mg/kg bw per day for females, respectively), with an F_2 recovery group in which the dams were not treated through gestation. Effects in the parental generations consisted of ocular changes in clinical, ophthalmological, gross and histopathological examinations at dietary concentrations of 10 ppm and above, along with increased plasma tyrosine levels. In pups, cloudy/opaque eyes, keratitis and/or corneal vascularization were observed in all treated groups in males and at 100 and 2500 ppm in females in litters exposed to mesotrione in utero. Plasma tyrosine levels were measured in pups in the F_{3A} groups and were increased in all treatment groups in the continuous treatment animals; levels were comparable with

those of controls in all the recovery groups. Decreased litter size, decreased survival, decreased percentage of pups born live and increased whole litter loss were observed at the highest dose tested.

A mode of action study in rats was performed to determine the link between tyrosinaemia and the changes noted in the rat reproductive toxicity study. In a modified one-generation reproductive toxicity study, animals were exposed to 0 ppm mesotrione with 0%, 0.5%, 1% or 2% tyrosine or to 2500 ppm mesotrione with 0%, 0.5%, 1% or 2% tyrosine from day 1 of gestation until termination on day 29 postpartum. Tyrosine and mesotrione increased plasma tyrosine levels and caused increases in whole litter losses, although the effect of mesotrione was greater than that of dietary tyrosine. The Meeting concluded that the reproductive effects observed in rats were likely a consequence of the elevated levels of tyrosine.

In a developmental toxicity study in mice, pregnant females were dosed at 0, 10, 60, 150 or 600 mg/kg bw per day. There were no signs of maternal or embryo/fetal toxicity up to 600 mg/kg bw per day, the highest dose tested.

In a developmental toxicity study in rats, pregnant females were dosed at 0, 100, 300 or 1000 mg/kg bw per day. Maternal body weight and feed consumption were decreased at all doses. In fetuses, delays in ossification were increased at all doses.

In a developmental toxicity study in rabbits, pregnant females were dosed at 0, 100, 250 or 500 mg/kg bw per day. At 250 and 500 mg/kg bw per day, there were increases in abortions and decreased defecation. The NOAEL for maternal toxicity was 100 mg/kg bw per day, based on increased abortions and decreased defecation at 250 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 500 mg/kg bw per day, the highest dose tested.

An investigative study was performed with pregnant female rabbits treated as follows: control (no tyrosine or mesotrione), tyrosine (1% dietary), mesotrione (500 mg/kg bw per day by gavage) and tyrosine and mesotrione (1% dietary tyrosine and 500 mg mesotrione/kg bw per day by gavage). Plasma tyrosine levels were increased in all groups treated with mesotrione, tyrosine or a combination of the two. In groups treated with both mesotrione and tyrosine, the plasma tyrosine levels were highest, followed by mesotrione-only treated dams and, lastly, tyrosine-only treated dams. Likewise, delays in ossification were most prevalent in the fetuses of dams treated with both mesotrione and tyrosine, followed by mesotrione-only and tyrosine-only treated dams; however, delays were prevalent in all treated groups at rates higher than those in the concurrent controls. There was only one abortion, which occurred in the group treated with both mesotrione and tyrosine. As such, the Meeting concluded that delays in ossification were related to the increase in plasma tyrosine levels. There was insufficient information to enable a conclusion to be reached with regard to abortions.

The Meeting concluded that mesotrione is not teratogenic.

In an acute neurotoxicity study in rats, no neurotoxic effects were seen at 2000 mg/kg bw, the highest dose tested.

In a 13-week dietary neurotoxicity study in rats, ophthalmoscopic findings were observed at 100 ppm (equal to 8.25 mg/kg bw per day). No neurotoxicity was observed up to 5000 ppm (equal to 402.8 mg/kg bw per day), the highest dose tested.

The Meeting concluded that mesotrione is not neurotoxic.

Toxicological data on metabolites and/or degradates

For MNBA, a plant and livestock metabolite, studies of metabolism, acute toxicity, short-term toxicity, genotoxicity and HPPD inhibition were performed.

When given to rats as a single oral dose of 75 mg/kg bw, [^{14}C]MNBA was minimally absorbed and excreted in the urine. The majority was converted to AMBA in the gut, which was excreted unabsorbed.

MNBA is of low acute oral toxicity, with an LD_{50} of greater than 5000 mg/kg bw.

In a 28-day gavage study in rats, MNBA was given in corn oil at a dose of 0, 15, 150 or 1000 mg/kg bw per day. The NOAEL was 1000 mg/kg bw per day, the highest dose tested.

In a 90-day study in rats, animals were given MNBA in the diet at a concentration of 0, 100, 650 or 3000 ppm (equal to 0, 7.7, 50.6 and 231.0 mg/kg bw per day for males and 0, 8.8, 56.9 and 263.7 mg/kg bw per day for females, respectively). At 3000 ppm, body weights were decreased statistically significantly (by 8%) in males, and triglyceride levels were increased (by 36%) in females. The NOAEL was 650 ppm (equal to 50.6 mg/kg bw per day), based on equivocal effects on body weight and increased triglyceride levels.

MNBA was tested in an adequate range of genotoxicity assays. No evidence of genotoxicity was observed.

MNBA was a very weak inhibitor of HPPD compared with mesotrione.

For AMBA, a plant and livestock metabolite, studies of acute toxicity, genotoxicity and HPPD inhibition were performed.

AMBA is of low acute oral toxicity, with an LD_{50} of greater than 5000 mg/kg bw.

AMBA showed no evidence of genotoxicity in a reverse mutation assay or in a mammalian cell cytogenetic assay in the presence of metabolic activation and gave positive results in the mammalian cell cytogenetic assay in the absence of metabolic activation.

AMBA was a very weak inhibitor of HPPD compared with mesotrione.

As there was insufficient information to determine the toxicological profile of MNBA and AMBA, their toxicological relevance was assessed using JMPR's metabolite assessment scheme included in the guidance document for WHO monographers.[1] On the basis of this assessment, the Meeting concluded that these metabolites are unlikely to be a safety concern.

Human data

In a study in which human volunteers were exposed to a single oral dose of mesotrione of 0.1, 0.5 or 4 mg/kg bw in capsules, there were no symptoms, clinical signs or changes on ophthalmological examination. In volunteers given 4 mg/kg bw, plasma tyrosine levels were increased up to 48 hours following dosing, with a peak tyrosine concentration of up to 420 nmol/mL plasma; unchanged mesotrione was found in the urine.

There are no reports of poisoning cases with mesotrione.

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

Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) of 0–0.5 mg/kg bw on the basis of the NOAEL of 49.7 mg/kg bw per day, based on decreased body weight, body weight gain and feed efficiency in male mice in an 18-month toxicity and carcinogenicity study. A safety factor of 100 was applied.

The Meeting considered the mode of action of the HPPD-dependent effects of mesotrione and concluded that the rat was not an appropriate model on which to base the toxicological evaluation.

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

[1] http://www.who.int/entity/foodsafety/areas_work/chemical-risks/jmpr_Guidance_Document_FINAL.pdf

Levels relevant to risk assessment of mesotrione

Species	Study	Effect	NOAEL	LOAEL
Mouse	Eighteen-month study of toxicity and carcinogenicity[a]	Toxicity	350 ppm, equal to 49.7 mg/kg bw per day	3 500/7 000 ppm, equal to 897.7 mg/kg bw per day
		Carcinogenicity	3 500/7 000 ppm, equal to 897.7 mg/kg bw per day[b]	–
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	7 000 ppm, equal to 1 439 mg/kg bw per day[b]	–
		Parental toxicity	1 500 ppm, equal to 301.6 mg/kg bw per day	7 000 ppm, equal to 1439 mg/kg bw per day
		Offspring toxicity	350 ppm, equal to 71.3 mg/kg bw per day	1 500 ppm, equal to 301.6 mg/kg bw per day
	Developmental toxicity study[c]	Maternal toxicity	600 mg/kg bw per day[b]	–
		Embryo and fetal toxicity	600 mg/kg bw per day[b]	–
Rat	Two-year study of toxicity and carcinogenicity[a]	Carcinogenicity	159.9 mg/kg bw per day[b]	–
Rabbit	Developmental toxicity study[c]	Maternal toxicity	100 mg/kg bw per day	250 mg/kg bw per day
		Embryo and fetal toxicity	500 mg/kg bw per day[b]	—
Dog	One-year study of toxicity[d]	Toxicity	100 mg/kg bw per day	600 mg/kg bw per day

[a] Dietary administration.
[b] Highest dose tested.
[c] Gavage administration.
[d] Capsule administration.

Estimate of acceptable daily intake (ADI)

0–0.5 mg/kg bw

Estimate of acute reference dose (ARfD)

Unnecessary

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

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

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

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid; extensive (> 60%)
Dermal absorption	No data
Distribution	Rapidly eliminated; highest residues in carcass, gastrointestinal tract, liver and kidneys
Potential for accumulation	No evidence of accumulation
Rate and extent of excretion	Largely complete within 24 hours; primarily via urine (41–70% in mice and 54–84% in rats), with 21–38% in faeces (rats and mice)
Metabolism in animals	Mostly excreted unchanged
Toxicologically significant compounds in animals and plants	Mesotrione, MNBA and AMBA
Acute toxicity	
Rat, LD_{50}, oral	> 5 000 mg/kg bw
Rat, LD_{50}, dermal	> 2 000 mg/kg bw
Rat, LC_{50}, inhalation	> 4.75 mg/L
Rabbit, dermal irritation	Not irritating
Rabbit, ocular irritation	Mildly irritating
Guinea-pig, dermal sensitization	Not sensitizing (maximization test)
Short-term studies of toxicity	
Target/critical effect	Body weight
Lowest relevant oral NOAEL	100 mg/kg bw per day (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	Body weight
Lowest relevant oral NOAEL	49.7 mg/kg bw per day (mouse)
Carcinogenicity	Unlikely to pose a carcinogenic risk to humans
Genotoxicity	
	Unlikely to be genotoxic
Reproductive toxicity	
Target/critical effect	Decreased body weight, clinical, gross and histopathological changes to the eye
Lowest relevant parental NOAEL	301.6 mg/kg bw per day (mouse)
Lowest relevant offspring NOAEL	71.3 mg/kg bw per day (mouse)
Lowest relevant reproductive NOAEL	1 439 mg/kg bw per day (mouse)
Developmental toxicity	
Target/critical effect	Abortions and decreased faecal output
Lowest relevant maternal NOAEL	100 mg/kg bw per day (rabbit)
Lowest relevant embryo/fetal NOAEL	500 mg/kg bw per day, highest dose tested (rabbit)

Neurotoxicity	
Acute neurotoxicity NOAEL	2 000 mg/kg bw, highest dose tested
Subchronic neurotoxicity NOAEL	402.8 mg/kg bw per day, highest dose tested
Developmental neurotoxicity NOAEL	No data
Other toxicological studies	
Studies on toxicologically relevant metabolites	*MNBA*: Metabolism: Minimally absorbed, excreted primarily in urine, majority in gut at 12 hours converted to AMBA HPPD inhibition: very weak compared with mesotrione Oral LD_{50}: > 5 000 mg/kg bw NOAEL: 1 000 mg/kg bw per day, highest dose tested (4-week gavage study in rats) NOAEL: 50.6 mg/kg bw per day, based on equivocal decreases in body weight and increased triglyceride levels (90-day study in rats) Unlikely to be genotoxic *AMBA*: HPPD inhibition: very weak compared with mesotrione Oral LD_{50}: > 5 000 mg/kg bw Unlikely to be genotoxic
Medical data	
	No studies submitted

Summary

	Value	Study	Safety factor
ADI	0–0.5 mg/kg bw	Eighteen-month study of toxicity and carcinogenicity (mouse)	100
ARfD	Unnecessary	–	–

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Appendix 1: Mode of action

The following analysis is based on the methodology developed by an ILSI/HESI workgroup and is based on the decision logic outlined by Seed et al. (2005). The human relevance framework is based on a four-part analysis:

1) Is the weight of evidence sufficient to establish the MOA in animals?
2) Are the key events in the animal MOA plausible in humans?
3) Taking into account kinetic and dynamic factors, is the animal MOA plausible in humans?
4) Statement of confidence; analysis; implications.

Mesotrione has been reviewed below, using the human relevance framework principles.

Is the weight of evidence sufficient to establish the MOA in animals?

Inhibition of HPPD

Mesotrione is a triketone herbicide and exerts its MOA via inhibition of the enzyme HPPD (Lee et al., 1997). HPPD occurs in plants and animals, the 52 active site amino acid residues being similar across phyla and highly conserved within mammalian species (Table A-1).

Table A-1. HPPD amino acid sequence comparisons across phyla

	Arabidopisis	**Maize**	**Rat**	**Mouse**	**Pig**	**Human**
Arabidopisis		60%[1]	32%	31%	30%	32%
Maize	**6**[2]		31%	30%	29%	31%
Rat	**13**	**13**		96%	77%	90%
Mouse	**14**	**13**	**0**		77%	90%
Pig	**14**	**14**	**1**	**1**		
Human	**14**	**13**	**1**	**1**	**2**	

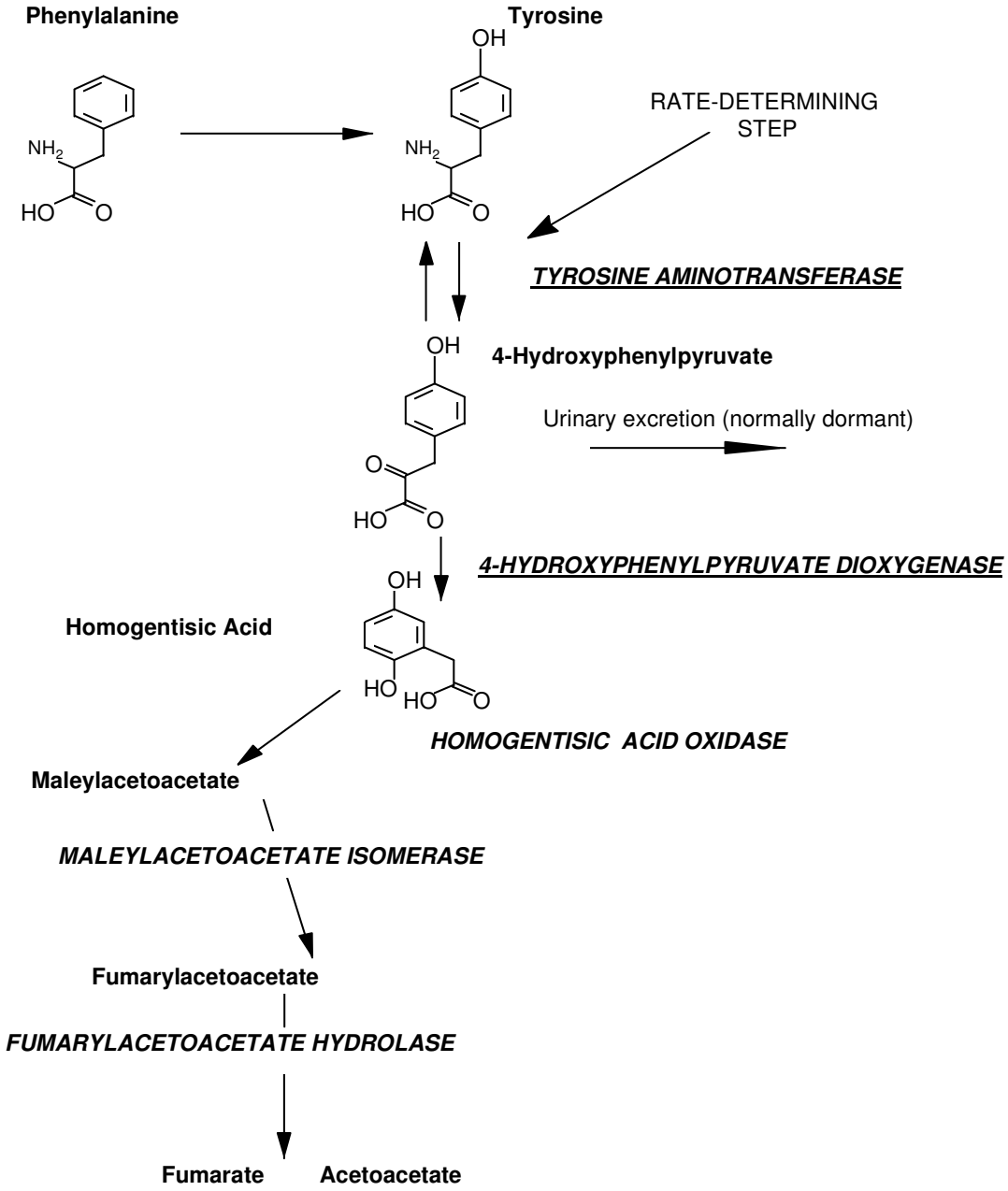
[1]% numbers are the overall % sequence similarity.
[2]Bold numbers are the number of differing active site residues (out of 52 total). From (Yang, et al., 2004)

Source: Syngenta (2014)

It can be concluded that mesotrione will inhibit HPPD in both plants and animals, and this is supported by direct measurements of hepatic and renal enzyme activity in rats and mice, which confirm that mesotrione inhibits HPPD in both species and that this inhibition is reversible (Lock et al., 1994).

HPPD is the second enzyme in the catabolic cascade of tyrosine (Fig. A-1).

Fig. A-1. Catabolic pathway of tyrosine

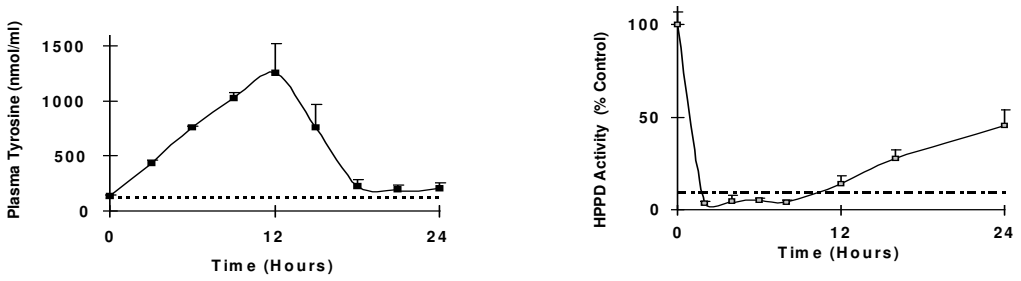


Source: Syngenta (2014)

Increase in plasma tyrosine concentration

The consequence of HPPD inhibition is a dose- and species-dependent elevation in plasma tyrosine levels. If enzyme binding is reversible (as is the case with mesotrione), enzyme activity will recover and plasma tyrosine concentrations will fall once exposure to mesotrione ceases (Brammer, 1995). After a single dose of mesotrione of 2 mg/kg bw administered by gavage to rats, enzyme activity starts to recover after 8 hours (Fig. A-2).

Fig. A-2. The 24-hour plasma tyrosine concentration and HPPD activity in male rats after a 2 mg/kg bw single oral gavage dose of mesotrione



Note 1: dotted line for plasma tyrosine = the mean value in control rats.
Note 2: broken line for HPPD activity = the limit of quantification of the assay used.
Values on each graph represent the mean plus standard deviation, where $n = 5$.

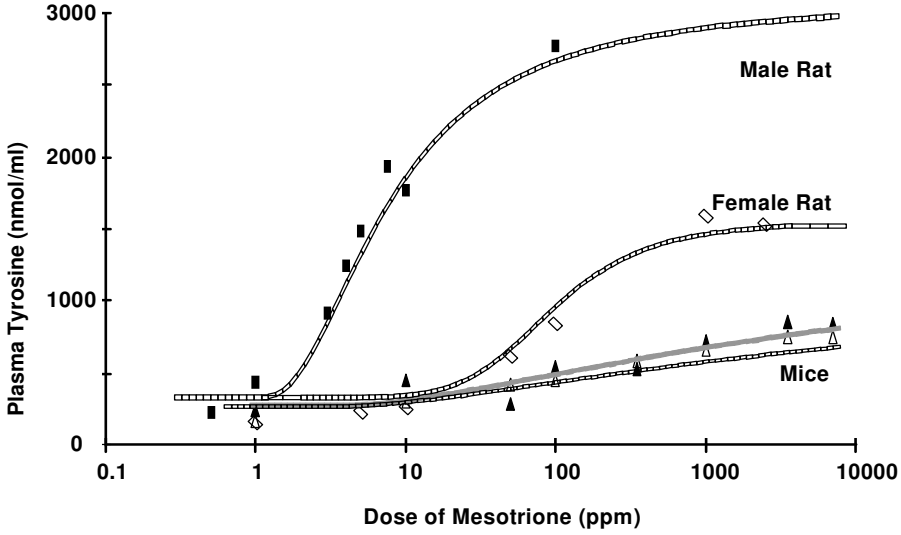
Source: Syngenta (2014)

Clearance of excess tyrosine

Inhibition of HPPD leads to a buildup of its substrate HPPA, which is found in urine (Ellis et al., 1995). The formation of HPPA from tyrosine by TAT is reversible, and a buildup of HPPA results in an elevation of tyrosine levels in the plasma (tyrosinaemia). TAT, the first enzyme in the catabolic pathway, is the limiting and controlling enzyme of tyrosine catabolism. HPPD normally operates at a fraction of its maximum velocity (Lock et al., 1996), and tyrosine concentration is a function of the rate of formation/absorption of tyrosine, the activity of TAT and the efficiency of HPPA elimination by the kidney.

As detailed in Odum (1997), the innate hepatic TAT activity is higher in the mouse, and a sex difference in the rat results in TAT activity being higher in the female rat than in the male rat. The difference in TAT activity results in species and sex differences in tyrosine accumulation (Fig. A-3).

Fig. A-3. Mesotrione-induced tyrosinaemia: dose–response relationship in male and female rats and mice



Source: Syngenta (2014)

Tyrosine-related spectrum of toxicological effects

In standard regulatory studies, a range of toxicities has been seen in rats. Some of these toxicities are seen in mice, but only at very high dose levels (Table A-2).

Table A-2. Toxicities seen in the rat and mouse following mesotrione administration

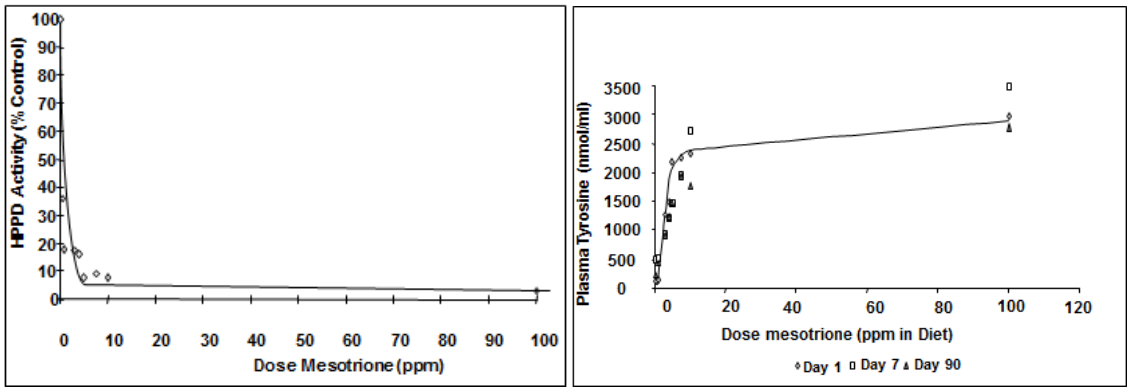
	Presence/absence	
Effect	**Rat**	**Mouse**
Corneal opacity	+	–
Thyroid proliferation	+	–
Sciatic demyelination	+	–
Glomerulonephropathy	+	–
Liver weight increase	+	+/–
Kidney weight increase	+	+/–
Body weight decrease	+	+/–
Reproductive effects		
Litter effects (reduced survival)	+	–
Bilateral hydronephrosis	+	–
Minor modulation in the rate of normal ossification	+	–

Source: Syngenta (2014)

Dose–response relationships

The relationship of dose of mesotrione administered in the diet to the resultant concentration of tyrosine in the plasma is illustrated in Fig. A-3. This correlates with the inhibition of HPPD, as illustrated in Fig. A-4.

Fig. A-4. HPPD inhibition (a) and plasma tyrosine concentration (b) in male rats – 90 day administration



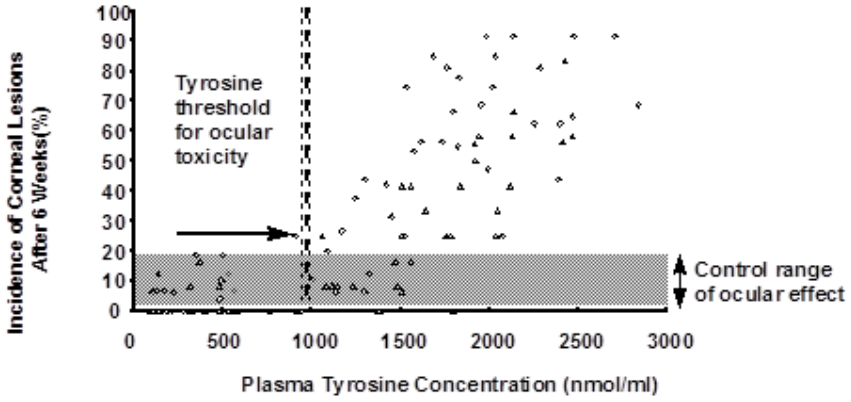
Note: Data taken from studies conducted according to Organisation for Economic Co-operation and Development (OECD) Test Guideline 408

Source: Syngenta (2014)

Tyrosine dose–response relationships: direct association between plasma tyrosine concentration and toxicity

It has been shown (Rich, Beard & Burns, 1973; Burns, Gipson & Murray, 1976; Robinson, 1995) that feeding rats on low-protein/high-tyrosine diets causes corneal opacity identical to that seen in rats administered mesotrione in diets for at least 2 weeks. Data taken from a large series of studies in which groups of rats were dosed for 6 weeks with different triketone HPPD inhibitors showed that there was a linear relationship between plasma tyrosine concentration and ocular toxicity. This study also demonstrated that there was a threshold plasma concentration (approximately 1000 nmol/mL) below which tyrosine-induced corneal lesions do not occur (Fig. A-5).

Fig. A-5. Relationship of plasma tyrosine concentration to corneal lesions in rats



Source: Syngenta (2014)

The dose–response relationship between tyrosine and all adverse effects seen in subchronic and chronic studies in rats has been considered in the non-guideline short-term toxicity studies. In summary, all adverse effects described in the systemic toxicity studies in the rat correlate closely with plasma tyrosine concentration, providing good but indirect evidence that tyrosine, rather than mesotrione itself, is the causal agent of these toxicities.

Strength, consistency and specificity of association

More direct evidence supporting the correlation of tyrosine concentration with toxicological effect is available from exacerbation studies and by examining the species differences in the toxicity of mesotrione.

Tyrosine exacerbation studies

Studies in which HPPD is inhibited by mesotrione and the resulting tyrosinaemia is exacerbated by adding excess tyrosine to the diet have been conducted in pregnant rats to evaluate the effect of tyrosine on pregnancy and in rabbits to investigate the effect of tyrosine on fetal ossification. These studies are discussed in Williams (2000) and Moxon (2000). They show a consistency in the direct role of tyrosine as the cause of developmental and reproductive effects in the rat and rabbit and strengthen the evidence that excessive plasma tyrosine is responsible for the toxicity observed.

Species comparison

The spectrum of toxicities seen in rats following administration of mesotrione is different from that seen in mice administered similar doses and/or doses orders of magnitude higher than those administered to rats. The difference is concluded to be entirely attributable to the significantly higher plasma tyrosine concentrations seen in rats (Table A-3).

Table A-3. Toxicities and plasma tyrosine concentrations seen in the rat and mouse following mesotrione administration

	Rat			Mouse		
Effect	**Presence/ absence**	**Plasma tyrosine[a] (nmol/mL)**	**Mesotrione dose (mg/kg bw per day)**	**Presence/ absence**	**Plasma tyrosine[a] (nmol/mL)**	**Mesotrione dose (mg/kg bw per day)**
Corneal opacity	+	> 1 000	0.16	–	~800	> 1 000
Thyroid proliferation	+	> 1 000	< 0.48	–	~800	> 1 000
Sciatic demyelination	+	> 1 000	< 0.48	–	~800	> 1 000
Glomerulonephropathy	+	> 1 000	< 0.48	–	~800	> 1 000
Liver weight increase	+	~800–1 000	< 0.48	+/–	~800	> 1 000
Kidney weight increase	+	~800–1 000	< 0.48	+/–	~800	> 1 000
Body weight decrease	+	~800–1 000	< 0.48	+/–	~800	> 1 000
Reproductive effects						
Litter effects	+	~800–1 000	1.2	–	~800	> 1 000
Bilateral hydronephrosis	+	~800–1 000	0.3	–	~800	> 1 000
Ossification effects	+	~800–1 000	< 100	–	~800	> 600

[a] Measured or extrapolated from research data.

Source: Syngenta (2014)

Temporal associations and reversibility

The time dependency of the relationship between elevated plasma tyrosine and genesis of an adverse biological event is demonstrated by the data on ocular opacity. A sustained plasma tyrosine elevation in excess of a threshold of approximately 1000 nmol/mL plasma needs to be maintained for 6 weeks to reliably induce ocular change. Most of the pathological findings associated with mesotrione exposure, such as sciatic nerve demyelination, thyroid proliferation and degenerative kidney change, are seen only following exposures longer than 1 year. Furthermore, effects noted in the rat after mesotrione dosing for 90 days are reversible within 4 weeks after cessation of dosing. This reversal coincides with clearance of mesotrione, cessation of HPPD inhibition and return of tyrosine plasma concentrations to control group levels (see Brammer, 1997; Tinston, 1997).

It can be concluded that all early effects occurring with repeated doses of mesotrione are reversible and that all chronic effects are seen only when severe tyrosinaemia is sustained for longer than 1 year (Table A-4).

Life stage sensitivity

Data are available to demonstrate that neonatal rats and mice are not more sensitive than adults to the inhibition of HPPD. Hepatic TAT activity was measured in untreated rats and mice from day 1 postpartum to 6 weeks of age. TAT expression at birth was as high as or higher than adult levels. However, in the case of rats (but not mice) between birth and puberty, the TAT levels of male rats fell, whereas those of the females remained constant (Fig. A-6).

Despite this consistency in TAT expression, the plasma concentration of tyrosine in control mice is higher in pups than in adults (Fig. A-7).

Nevertheless, it has also been shown that elevation of plasma tyrosine, as a consequence of exposure to mesotrione, is lower in mouse pups than in the maternal animal (Fig. A-8).

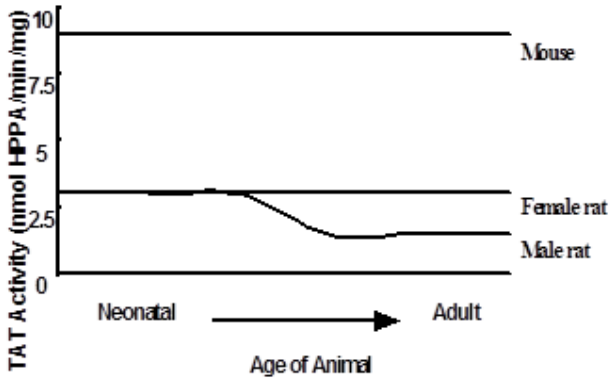
Table A-4. Reversibility of tyrosine-induced toxicity in the rat: values at termination and following a 4-week recovery period in a 90-day study conducted according to OECD Test Guideline 409

	5 ppm		100 ppm		2 500 ppm	
	Week 0	**Week 4**	**Week 0**	**Week 4**	**Week 0**	**Week 4**
HPPD activity (% of control)	11	55	3.5	62	3.8	N/D
Plasma tyrosine concentration (% of control)	828	101	1382	106	1039	374
TAT activity (% of control)	114	104	119	102	166	N/D
Body weight (% difference from control)	−3	0	−2.5	+2	−9	−8
Liver weight/body weight (% of control)	110	101	113	107	120	113
Kidney weight/body weight (% of control)	112	100	112	99	110.5	110
Ocular effects						
NAD/total examined	67/80	15/16	24/80	8/16	28/76	6/16
Opacity	10	0	56	0	46	0
Vascularization	2	0	44	0	41	0
Ghost vessels	0	0	0	0	0	10

HPPD: 4-hydroxyphenylpyruvate dioxygenase; NAD: no abnormality detected; N/D: not determined; OECD: Organisation for Economic Co-operation and Development; TAT: tyrosine aminotransferase

Key: Week 0 = week 13/14 of dosing (90 days)

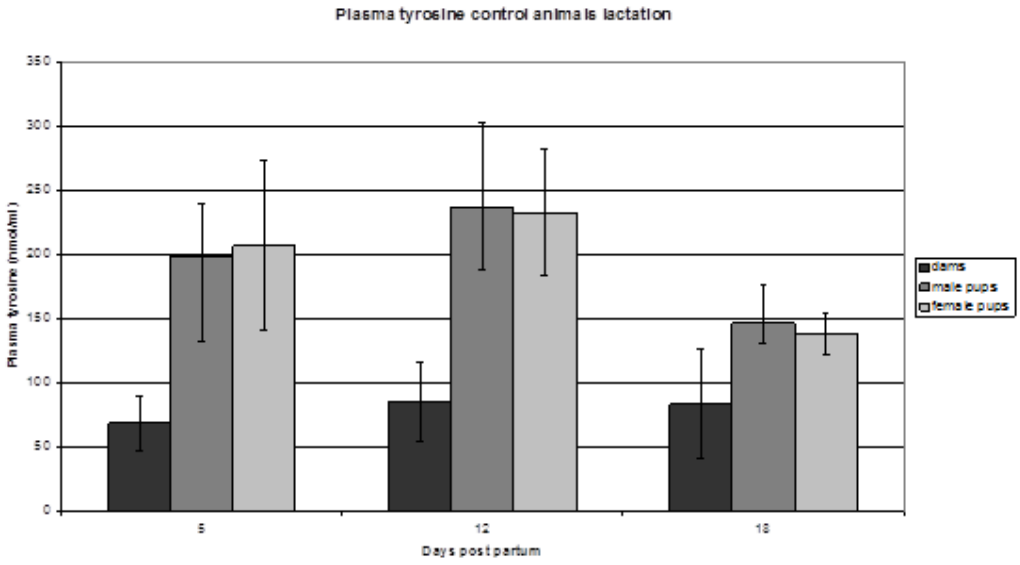
Fig. A-6. Expression of TAT in rats and mice with age



Source: Syngenta (2014)

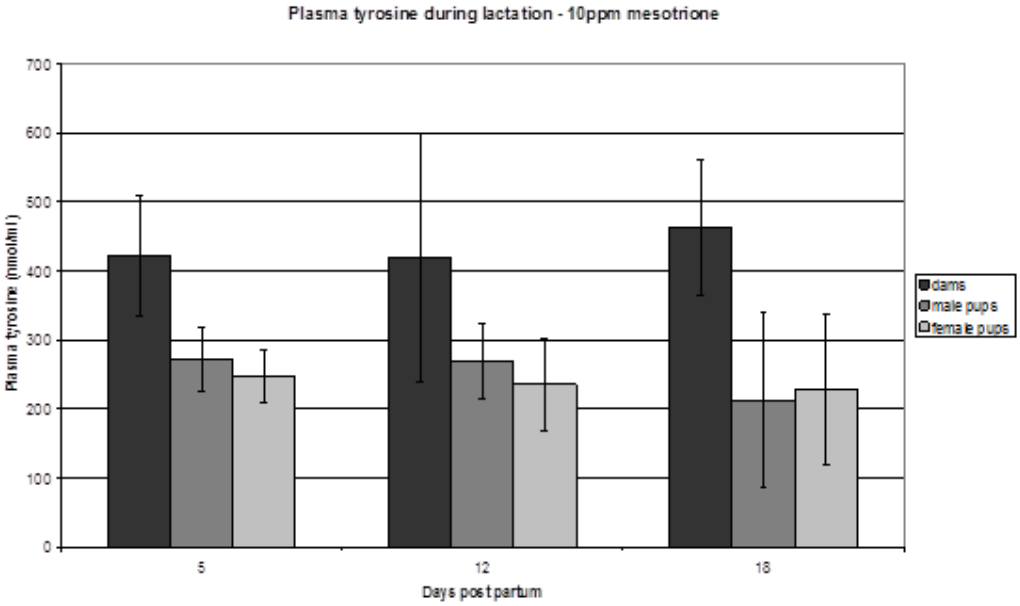
From these data, it can be concluded that plasma tyrosine concentrations in control pups are higher than those in adults. The addition of 10 ppm mesotrione to diet results in a significant elevation of plasma tyrosine in adults, although average levels do not exceed 500 nmol/mL. In contrast, plasma tyrosine levels in pups from the 10 ppm mesotrione group are not significantly elevated above age-matched control levels during lactation and remain less than 500 nmol/mL. These data demonstrate that there is no evidence for increased sensitivity of neonatal animals to the effects of mesotrione.

Fig. A-7. Plasma tyrosine concentration in control animals during lactation



Source: Syngenta (2014)

Fig. A-8. Plasma tyrosine concentration in animals given 10 ppm mesotrione in diet during lactation



Source: Syngenta (2014)

Biological plausibility and coherence of the database

A remarkable consistency is observed in the incidence of plasma tyrosine concentrations in excess of 1000 nmol/mL and the occurrence of adverse effects (ocular change, liver and kidney weight increase, reduced body weight and exacerbation of a range of spontaneous pathologies).

In the assessment of early life stage toxicity in the developmental and reproductive database, the same marked association between the biological end-points of minor changes in ossification and reduced pup survival and elevated plasma tyrosine levels has been demonstrated.

There is also consistency between the systemic and reproductive toxicity databases, where the same elevated liver and kidney weights, reduced body weights and ocular changes are again observed in the presence of elevations in plasma tyrosine levels.

The key events in the animal MOA are summarized in Table A-5.

Table A-5. Key events in the animal MOA

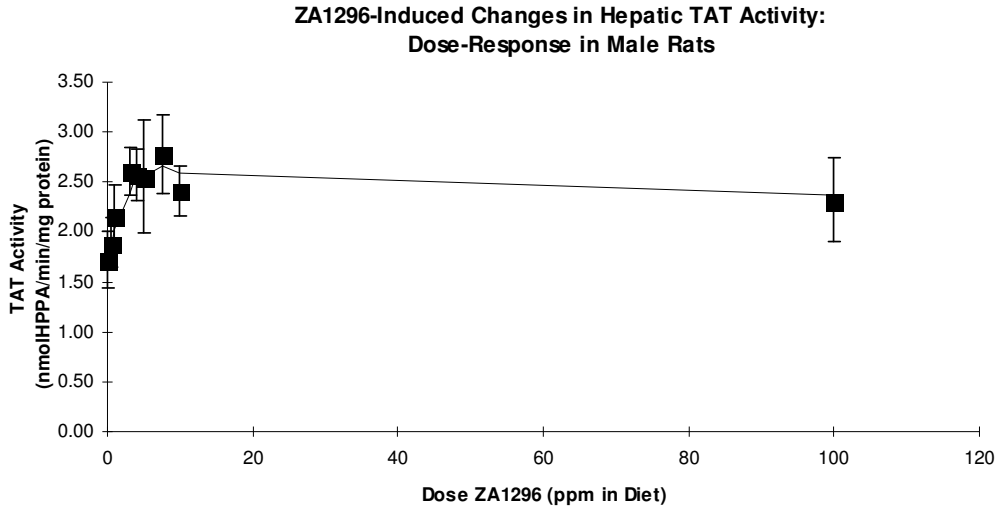
Key event	Evidence	References
Inhibition of HPPD	• YES: This is the established herbicidal MOA in plants • Has been shown to be the MOA for HPDD inhibitors in mammals • HPPD inhibitors are structurally diverse, but have a common substructure, which binds tightly to a single, common active site in both plant and mammalian HPPD • HPPD active site sequence ➢ similar across plants and animals ➢ highly conserved across mammalian species	Lock et al. (1994); Lee et al. (1997)
Increase in systemic tyrosine concentrations	• YES: Increases in plasma tyrosine are mesotrione dose dependent in rats, mice and humans • Degree of tyrosinaemia is species specific and dependent upon the innate activity of TAT, the rate-limiting enzyme in the tyrosine catabolic pathway	Nixon (2001)
Clearance of excess tyrosine via TAT	• YES: TAT is the first enzyme in the catabolic pathway for tyrosine. If the second enzyme (HPPD) is inhibited, excess tyrosine is cleared as phenolic acids in the urine. Rate of clearance depends on inherent activity of TAT, which is species dependent – higher in the mouse than in the rat (mouse > 2× female rat/4× male rat)	Nixon (2001)
Tyrosine-related spectrum of toxicological effects	• YES: Mesotrione administration results in effects on body weight, liver, kidney, eyes, thyroid in subchronic/chronic rat studies and on reproduction/development in the rat. These effects are attributable to severe tyrosinaemia based on: ➢ direct causation of effect by tyrosine alone (ocular opacity) ➢ correlation of effect with tyrosine levels – chronic effect on body weight, liver and kidney, sciatic nerve and thyroid ➢ exacerbation – effect of mesotrione and excess dietary tyrosine on fetal ossification and reproduction	Rich, Beard & Burns (1973); Burns, Gipson & Murray (1976)

HPPD: 4-hydroxyphenylpyruvate dioxygenase; MOA: mode of action; TAT: tyrosine aminotransferase

Alternative MOAs

Given the strength and consistency of the correlation of tyrosine levels to various toxicological outcomes, the obvious species-dependent generation of high plasma tyrosine levels and the clear dose–response relationship for tyrosine rather than mesotrione, it is unlikely that there are alternative MOAs. No other MOAs have been identified for mesotrione that are able to unite the range of biological effects observed. Mesotrione does not inhibit TAT, the first enzyme in the tyrosine catabolic cascade (Fig. A-9).

Fig. A-9. Mesotrione-induced changes in hepatic TAT activity – male rats



Source: Syngenta (2014)

Nuclear magnetic resonance analysis of urine from rats dosed with mesotrione shows excess phenolic acids (Lock et al., 1996), which is consistent with the inhibition of HPPD.

There was no evidence, from urinary analysis, of excess levels of tyrosine itself, tyramine or *N*-acetyltyrosine, which would have indicated TAT inhibition, or of fumarylacetoacetate, maleylacetoacetate or homogentisate, which would have indicated an inhibition of fumarylacetoacetate hydroxylase, maleylacetoacetate isomerase or homogentisic acid oxidase. In addition, mesotrione does not inhibit tyrosine hydroxylase, a key enzyme in the anabolic pathway of tyrosine. Therefore, mesotrione has been shown to inhibit a single enzyme (HPPD) in the catabolic pathway of tyrosine and to not affect the key enzyme in the anabolic pathway.

There are some other MOAs that are known to operate with other HPPD inhibitors, such as hepatic P450 induction. The lack of significant P450 induction by mesotrione was confirmed by the modest increase in liver weight in chronic studies, the lack of pathological changes in the liver and a short-term study (Odum, 1997) showing that the relevant enzymes were not induced.

Conclusions: Assessment of postulated MOA in animals and statement of confidence

Overall, there is a high level of confidence in the postulated MOA that tyrosine elevation leads to a range of biological effects, which are consistent with those seen predominantly in rats following mesotrione exposure. This confidence is based on evidence for direct causation between plasma tyrosine levels and ocular changes, where ocular tyrosine concentrations and effects in the eye are highly correlated. Furthermore, there is a large and convincing database showing a consistent positive correlation between all the biological end-points described following mesotrione treatment and elevations in plasma tyrosine concentration. Additionally, tyrosine exacerbation studies have established a firm link between the severity of developmental and reproductive changes and the degree of elevation of plasma tyrosine concentration.

It is concluded that the weight of evidence is sufficient to establish an MOA in animals.

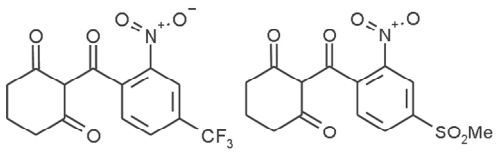
Are the key events in the animal MOA plausible in humans?

HPPD inhibition and increase in tyrosine

Humans have an HPPD that is similar to both the rat and mouse HPPD; in particular, the amino acid sequence homology in the active site is nearly identical, differing by only one amino acid

residue. NTBC (also known as nitisinone and Orfadin®) is an HPPD inhibitor (Lock et al., 1994) that is structurally similar to mesotrione (Fig. A-10) and is used therapeutically to treat humans with tyrosinaemia type 1 (an autosomal recessive fumarylacetoacetate hydrolase deficiency).

Fig. A-10. Chemical structures of NTBC and mesotrione



Nitisinone (NTBC) Mesotrione

Source: Syngenta (2014)

Human data are available from volunteer studies, clinical trials and over 2000 patient years of experience that demonstrate that HPPD inhibitors (NTBC and mesotrione) cause an increase in plasma tyrosine concentration (Lindstedt et al., 1992; Hall et al., 2001). Therefore, based on HPPD enzyme similarities between humans and rodents and based on studies with HPPD inhibitors, it is considered that mesotrione is able to inhibit human HPPD and can increase plasma tyrosine concentrations.

Clearance of tyrosine via TAT

The level of TAT activity in humans is similar to that of male mice, and humans are thus able to efficiently catabolize tyrosine (Table A-6).

Table A-6. Comparison of innate hepatic TAT activity – rats, mice and humans

	Hepatic TAT activity (nmol HPPA/min/mg protein)		
	Rat	**Mouse**	**Humans[a]**
Males	1.7 ± 0.2	7.8 ± 1.5	7.17 ± 1.17
Females	3.3 ± 0.5	10.5 ± 1.9	

HPPA: 4-hydroxyphenylpyruvate; TAT: tyrosine aminotransferase

[a] From Henderson et al. (1981).

As is the case for mice and rats, TAT is fully active 24 hours after birth (Kretchmer, 1959).

Tyrosine-related spectrum of toxicological effects

There are limited reliable data on toxicity in humans occurring as a consequence of elevated plasma tyrosine concentrations. In patients treated with NTBC, it is reported that eye disorders, including conjunctivitis, photophobia, eye pain, keratitis and corneal lesions, have been noted, some of which were transient and/or reappeared (USFDA, 2013), although none occurred in adults treated for alkaptonuria (Sunwannarat et al., 2005). Although alkaptonuria patients were recorded with plasma tyrosine concentrations that periodically reached 800 nmol/mL, none showed treatment-related ocular effects. However, there are no data from patients with plasma tyrosine concentrations that significantly exceed these concentrations for prolonged periods.

Nonetheless, there is no evidence to suggest that humans would react differently from rats to high, sustained levels of tyrosine. For NTBC, it is recognized that humans could exhibit adverse effects should tyrosine levels reach toxicologically relevant concentrations, and the USFDA (2013) therefore recommended that plasma tyrosine levels should be kept below 500 µmol/L in order to

avoid toxic effects (i.e. corneal lesions and hyperkeratotic lesions and neurological symptoms) caused by high plasma tyrosine levels.

In summary, the key events seen predominantly in rats are plausible in humans, although there are significant species differences in the level of plasma tyrosine that can accumulate.

Taking into account kinetic and dynamic factors, is the animal MOA plausible in humans?

The MOA for mesotrione-related adverse effects seen predominantly in rats depends on the sustained elevation of tyrosine. Two factors determine whether or not humans would sustain high plasma tyrosine levels: (1) residence time of the HPPD inhibitor in the body (basic kinetics) and (2) the efficiency of clearance of tyrosine by TAT (Table A-7).

Table A-7. Key events in the animal MOA and human relevance

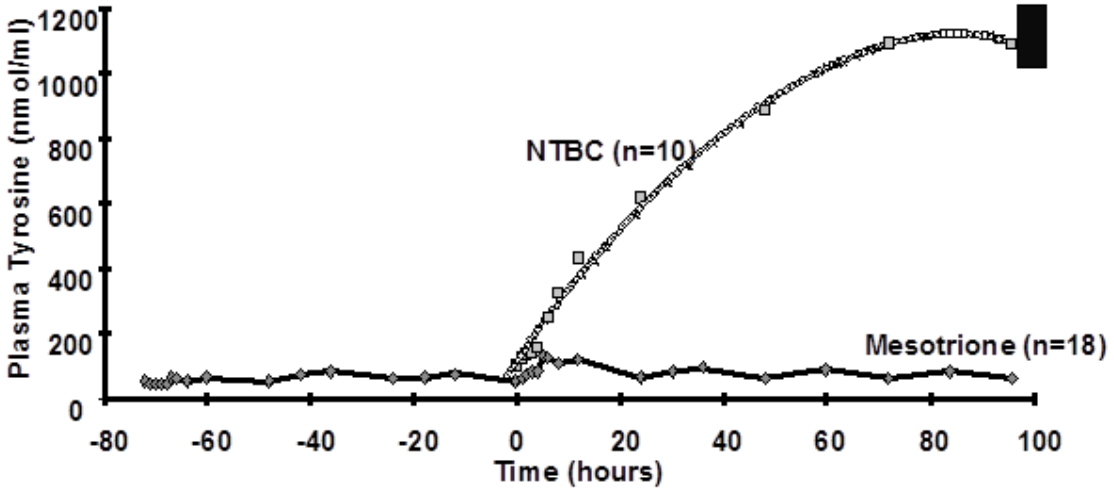
Key event	Evidence in rats	Evidence in mice	Evidence in humans	References
Inhibition of HPPD	• YES: Mesotrione – measured in rats	• YES: Mesotrione – measured in mice	• YES: Indirect data from HT1 patients treated with NTBC	Lindstedt et al. (1992)
Increase in systemic tyrosine concentrations	• YES: Dose-dependent increase in plasma tyrosine concentration	• YES: Dose-dependent increase in plasma tyrosine concentration	• YES: Dose-dependent increase in plasma tyrosine concentration	
Clearance of excess tyrosine via TAT	• YES: Tyrosine cleared slowly • TAT activity (nmol HPPA/min/mg protein) ➢ 1.7 (males) ➢ 3.3 (females) • Maximum plasma tyrosine concentration (nmol/mL) ➢ 2 500–3 000 (males) ➢ 1 500–2 000 (females)	• YES: • TAT activity (nmol HPPA/ min/mg protein) ➢ 7.8 (males) ➢ 10.5 (females) • Maximum plasma tyrosine concentration is 800 nmol/mL	• YES: • TAT activity (nmol HPPA/min/mg protein) ➢ 7.17 • Plasma tyrosine maximum concentration similar to that in mouse when HPPD completely inhibited	Henderson et al. (1981) Hall et al. (2001)
Tyrosine-related spectrum of toxicological effects	• Ocular toxicity evident when plasma tyrosine concentration > 1 000 nmol/mL • Chronic effects and reproductive/ developmental effects at similar plasma tyrosine concentrations	• Minimal effects on liver and kidney weight seen in mouse studies – plasma tyrosine < 1 000 nmol/mL	• No adverse effects seen in human volunteer study with mesotrione or in healthy volunteers with NTBC • NTBC showed minor effects in patients if plasma tyrosine exceeds 800–1 000 nmol/mL • NTBC has no adverse effects in adults treated for alkaptonuria where plasma tyrosine reaches 600–700 nmol/mL	Hall et al. (2001) USFDA (2013) Sunwannarat et al. (2005)

HPPD: 4-hydroxyphenylpyruvate dioxygenase; HT1: tyrosinaemia type 1; MOA: mode of action; NTBC: 2-(2-nitro-4-fluoromethylbenzoyl)-1,3-cyclohexanedione; TAT: tyrosine aminotransferase

Kinetics

Experience with NTBC, a potent HPPD inhibitor used as a drug in children for the treatment of tyrosinaemia type 1 (HT1), shows that elevated plasma tyrosine levels can be achieved with this drug. Limited data for mesotrione indicate that comparable doses do not cause significantly elevated tyrosine levels in human volunteer studies (Fig. A-11), although higher doses (4 mg/kg bw) can produce minor transient elevations in plasma tyrosine (Hall et al., 2001).

Fig. A-11. Plasma tyrosine levels in humans after NTBC or mesotrione dosing



NTBC: 1 mg/kg bw; mesotrione: 0.5 mg/kg bw

Source: Syngenta (2014)

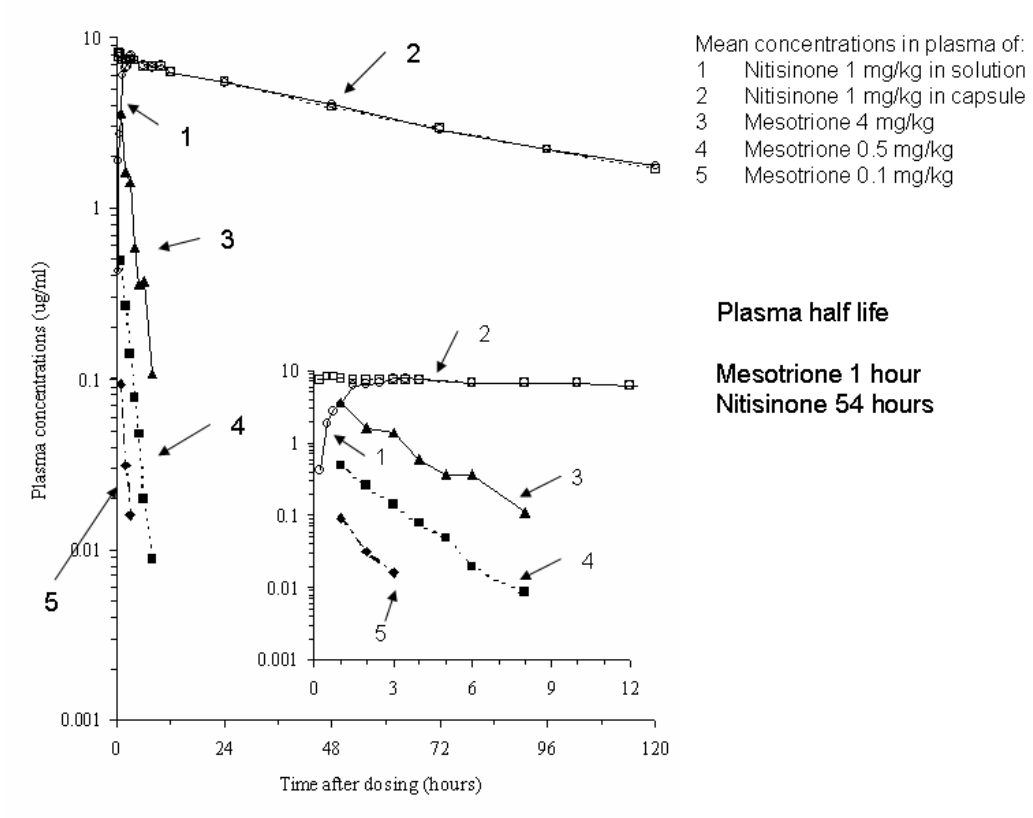
The reason for the difference between NTBC and mesotrione plasma tyrosine concentrations is that NTBC has a plasma half-life of 54 hours compared with mesotrione's half-life of 1 hour (Fig. A-12), resulting in a plasma area under the concentration–time curve that is 400 times greater for NTBC than for mesotrione. The sustained presence of NTBC results in elevated tyrosine levels that plateau at about 1000 nmol/mL.

Therefore, the kinetics of an HPPD inhibitor are important in understanding whether or not the MOA would, in reality, occur in humans. Mesotrione's short half-life would suggest that the MOA would not result in adverse findings in humans unless very high repetitive doses were administered to elevate tyrosine levels and for a sustained period of time.

Clearance of tyrosine

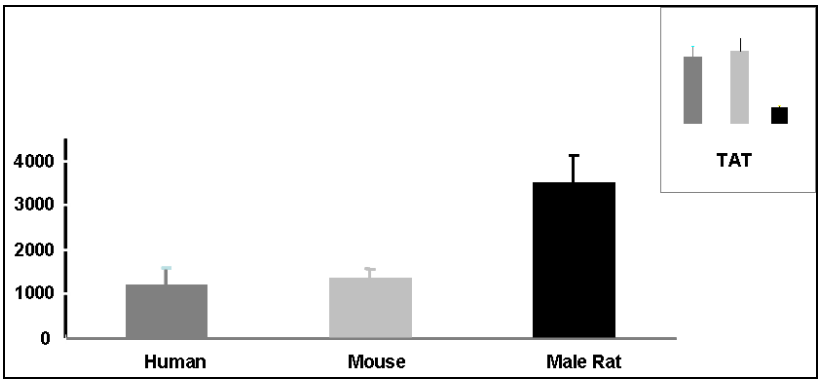
The toxicities attributable to mesotrione have been shown to be significantly different in the two rodent species studied in detail, rats and mice (Table A-2). The metabolic fate of mesotrione has been studied following single and repeated doses in rats and mice, and it has been demonstrated that there are no species differences in the metabolism and excretion of mesotrione that could explain the species differences in toxicity reported (see metabolism studies in sections 1.1 and 1.2 and Gledhill, Jones & Laird, 2001). The differences in toxicity profile are attributable to differences in the steady-state plasma tyrosine concentrations under conditions of complete HPPD inhibition, which in turn have been shown to be dependent upon the animals' innate TAT activity. Human TAT activity is much higher than that of the rat; thus, at equivalent doses of the potent HPPD inhibitor NTBC, plasma tyrosine concentrations in mice and humans are much lower than those seen in the rat (Fig. A-13).

Fig. A-12. Clearance of NTBC or mesotrione from human volunteers



Source: Syngenta (2014)

Fig. A-13. NTBC-induced plasma tyrosine (nmol/mL) elevation in rats, mice and humans



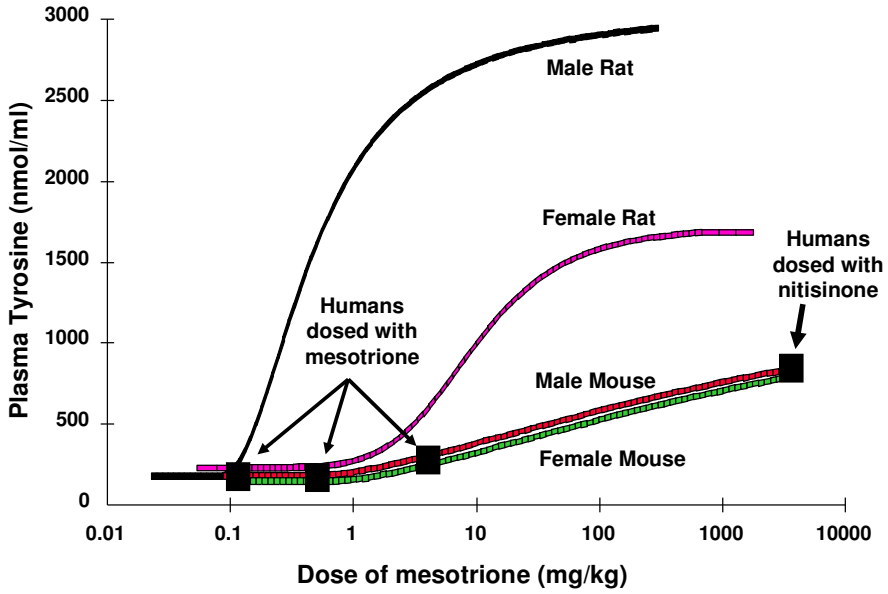
NTBC dose = 1 mg/kg bw
Human data from clinical trial in 10 male volunteers (Hall et al., 2001)
Data for rat from Lock et al. (1996)
Data for mouse from Lock et al. (2000)

Source: Syngenta (2014)

Using values taken from the human volunteer study with mesotrione and the clinical trial with NTBC (Hall et al., 2001) and assuming a 400- to 1000-fold difference in potency of NTBC and mesotrione, it is possible to extrapolate steady-state plasma tyrosine concentrations in humans following mesotrione exposure. These data points have been added to the graph produced for mice and rats and show that humans achieve tyrosine levels similar to those of mice and that these do not

exceed 1000 nmol/mL (the threshold established for induction of adverse effects seen in the rat) (Fig. A-14).

Fig. A-14. Mesotrione-induced tyrosinaemia in mice, rats and humans: relationship between plasma tyrosine concentrations and dose of mesotrione



Source: Syngenta (2014)

On the basis of these data, which clearly indicate that humans will not achieve the high tyrosine levels seen in the rat, the effects of mesotrione in humans would be expected to be the same as those seen in the mouse and to be significantly different from those seen in the rat.

In summary, the kinetic and dynamic characteristics of mesotrione in humans are such that the short half-life (kinetics) and the efficient removal of tyrosine (dynamics) obviate the likelihood of any tyrosine-related effects that are seen in the rat after mesotrione dosing. Therefore, taking into account kinetic and dynamic factors, the animal MOA is plausible in humans, but with the practical certainty that adverse effects will not be observed in humans.

Conclusion and statement of confidence

Based on the available data, the MOA and the key events for mesotrione-related adverse effects have been identified and, on a qualitative basis, are plausible in humans. Given the quantitative factors (kinetics and dynamics) of this MOA (short half-life and the significant differences in TAT activity between humans and rats), humans are unlikely to exhibit the toxicities seen in rats. Nonetheless, mesotrione at some relatively high dose level may raise tyrosine levels in humans, but certainly not to an extent or for a duration that is likely to cause adverse effects.

In addition, the available data lead to the conclusion that for tyrosine-related toxicities, the mouse is the most appropriate model to use for the human risk assessment of mesotrione.

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METRAFENONE

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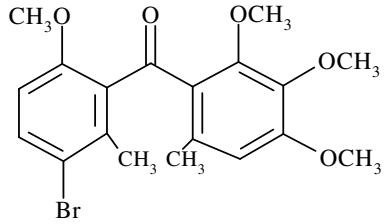
Explanation

Metrafenone (Fig. 1) is the International Organization for Standardization (ISO)–approved common name for (3-bromo-6-methoxy-2-methylphenyl)(2,3,4-trimethoxy-6-methylphenyl)-methanone (International Union of Pure and Applied Chemistry), for which the Chemical Abstracts Service number is 220899-03-6.

Metrafenone is a fungicide initially registered for use on winter and spring varieties of wheat and barley for the control of *Erysiphe graminis* and *Pseudocercosporella herpotrichoides* (eyespot and powdery mildew) and in grapevine for the control of *Uncinaria necator* (powdery mildew). Morphological observations on the mode of action in cereal powdery mildew show that it inhibits growth of the mycelium on the leaf surface, leaf penetration, the formation of haustoria and sporulation. Approvals for expanded use in several fruit and vegetable crops now exist as well and

include use in strawberries for the control of *Sphaerotheca macularis* (powdery mildew), in curcurbits for the control of *Sphaerotheca fuliginea* and *Erysiphe cichoracearum* (powdery mildew) and in tomato and aubergine for the control of *Leveillula taurica* (powdery mildew).

Fig. 1. Structure of metrafenone



Metrafenone 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 (USEPA) test guidelines unless otherwise indicated.

Evaluation for acceptable daily intake

1. Biochemical aspects

1.1 Absorption, distribution and excretion

(a) Oral route

The absorption, distribution and excretion of metrafenone were investigated in Sprague-Dawley rats.

Two label positions of metrafenone, [trimethoxyphenyl-U-^{14}C]metrafenone and [bromophenyl-6-^{14}C]metrafenone, were administered orally at doses of 10 mg/kg body weight (bw) or 1000 mg/kg bw as suspensions in 0.5% carboxymethyl cellulose to groups of male and female rats. An additional group of rats received 14 daily oral doses of non-radiolabelled metrafenone followed by a single oral dose of radiolabelled metrafenone. With the exception of the single low-dose experiments, a single label position (i.e. trimethoxphenyl-U-^{14}C) was used in metabolism experiments because there was evidence that the bond between the bromophenyl ring and trimethoxyphenyl ring remains intact. The experimental design is outlined in Table 1.

In a pilot pharmacokinetic study, groups of two Sprague-Dawley rats of each sex were treated with [trimethoxyphenyl-U-^{14}C]metrafenone orally at a single low dose of 10 mg/kg bw and a single high dose of 1000 mg/kg bw. Blood was collected at 0.5, 1, 2, 4, 6, 12, 24, 48, 72, 96, 120, 144 and 168 hours post-dosing. There were no apparent sex-related differences in blood concentration after administration of a single oral dose of metrafenone. Metrafenone was absorbed quickly into blood and reached a maximum concentration of 1.58 parts per million (ppm) in the low-dose group and 11.6–13.0 ppm in the high-dose group within 6–12 hours. The elimination half-life was approximately 38 hours for males and 32 hours for females in both dose groups. The area under the concentration–time curve from time 0 to time *t* (AUC_{0-t}) from the high-dose group was 10 times greater than that of the low-dose group, indicating a saturation of absorption (Mallipudi, 2002).

In a definitive pharmacokinetic study, groups of four Sprague-Dawley rats of each sex were administered [trimethoxyphenyl-U-^{14}C]metrafenone orally at a single dose of either 10 or 1000 mg/kg

Table 1. Experimental design

Test groups	Radiolabel	No. of rats (M + F)	Target dose (mg/kg bw)	Euthanasia at time post-dosing
Pilot excretion (single oral dose)	[Trimethoxyphenyl-U-^{14}C]	2 + 2	10	At least 72 h to a maximum of 168 h
	[Bromophenyl-6-^{14}C]	2 + 2	10	
Pilot plasma (single oral dose)	[Trimethoxyphenyl-U-^{14}C]	2 + 2	10	At least 72 h to a maximum of 168 h
		2 + 2	1 000	
Single oral low dose	[Trimethoxyphenyl-U-^{14}C]	5 + 5	10	168 h
	[Bromophenyl-6-^{14}C]	5 + 5	10	
Single oral high dose	[Trimethoxyphenyl-U-^{14}C]	5 + 5	1 000	168 h
Multiple oral low dose	[Trimethoxyphenyl-U-^{14}C]	5 + 5	10	168 h
Pharmacokinetic (single oral dose)	[Trimethoxyphenyl-U-^{14}C]	4 + 4	10	168 h
		4 + 4	1 000	
Tissue and organ distribution (single oral dose)	[Trimethoxyphenyl-U-^{14}C]	12 + 12	10	–⅔ T_{max}, T_{max}, $t_{⅓\ elim}$ and $t_{⅔\ elim}$
		12 + 12	1 000	
Biliary excretion (single oral dose)	[Trimethoxyphenyl-U-^{14}C]	4 + 4	10	72 h
		4 + 4	1 000	

bw: body weight; F: female; M: male; $t_{⅓\ elim}$: time for one third elimination (h); $t_{⅔\ elim}$: time for two thirds elimination (h); T_{max}: time to reach peak concentration (h)

Source: Mallipudi (2002)

bw. Blood samples were collected and quantified at 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72, 96, 120, 144 and 168 hours. Carbon-14 residues were observed in blood within 15 minutes and reached maximum concentrations (C_{max}) of 1.22–1.25 µg/g for the low-dose group and 12.74–16.14 µg/g for the high-dose group within 15 hours (8.5–15 hours). A slightly longer time (14–15 hours) was needed to reach a maximum ^{14}C blood level at the high dose compared with the low dose, suggesting prolonged absorption throughout the gastrointestinal tract. The differences between males and females were insignificant. The radioactive residues in the blood declined quickly, with an elimination half-life of approximately 40 hours for the low-dose group and 50 hours for the high-dose group. The AUC from time 0 to infinity ($AUC_{0-\infty}$) for the high dose was only approximately 16-fold higher than that for the low dose, despite there being a 100-fold difference between dose levels. The pharmacokinetic parameters following the administration of a single oral dose of metrafenone are summarized in Table 2 (Mallipudi, 2002).

In a pilot excretion study, groups of two Sprague-Dawley rats of each sex were administered either [trimethoxyphenyl-U-^{14}C]metrafenone or [bromophenyl-6-^{14}C]metrafenone orally at a single low dose of 10 mg/kg bw. Expired carbon dioxide, organic volatiles, urine and faeces were collected at various time intervals. During the 7-day period, total recoveries for the individual dose groups ranged from 95.18% to 104.56% of the administered dose. Total radioactivity of 87.76–95.38% was recovered in faeces and 3.78–14.09% in urine, and negligible amounts of radioactivity (0.05–0.15%) were determined in carbon dioxide and organic volatiles. The results suggested that there was no need for a closed system to conduct the rat metabolism study (Mallipudi, 2002).

A definitive mass balance study was conducted in four groups of male rats and four groups of female rats. Groups of five rats of each sex were orally administered either [trimethoxyphenyl-U-^{14}C]metrafenone or [bromophenyl-6-^{14}C]metrafenone at a single low dose of 10 mg/kg bw; a group of

Table 2. Pharmacokinetic parameters following a single oral dose of [^{14}C]metrafenone

Pharmacokinetic parameter	Low dose (10 mg/kg bw)		High dose (1 000 mg/kg bw)	
	Males	Females	Males	Females
T_{max} (h)	8.5	11	14	15
C_{max} (µg/g)	1.25	1.22	16.14	12.74
$t_{½\ elim}$ (h)	42.66	39.03	54.30	45.32
$AUC_{0-\infty}$ (µg·h/g)	51.16	55.97	930.41	826.76
AUC_{0-t} (µg·h/g)	48.02	52.86	843.57	762.23
MRT (h)	53.22	52.23	62.36	61.49

$AUC_{0-\infty}$: area under the concentration–time curve from time 0 to infinity; AUC_{0-t}: area under the concentration–time curve from time 0 to last measured time; C_{max}: maximum blood concentration; MRT: mean residence time; $t_{½\ elim}$: elimination half-life; T_{max}: time of maximum blood concentration

Source: Mallipudi (2002)

10 rats (five of each sex) was administered a daily oral dose of non-radiolabelled metrafenone at 10 mg/kg bw per day for 14 days followed by a single oral dose of [trimethoxyphenyl-U-^{14}C]metrafenone at 10 mg/kg bw; and a group of 10 rats (five of each sex) was orally administered a single dose of [trimethoxyphenyl-U-^{14}C]metrafenone at 1000 mg/kg bw. Urine, cage rinse and faeces were collected at different time intervals during the 7-day period. Selected tissue samples were collected at the study termination. Urine samples over a time period up to 168 hours were analysed for the single low dose, repeated low dose and single high dose groups.

The percentage of total administered radioactivity excreted in 7 days in urine ranged from 0.69% to 6.60%. Excretion in urine was relatively low, slightly lower in males than in females, and was not affected by repeated dosing. Seven days following the single oral low dose administration, the excretion in urine was 3.83% and 6.6% of the administered dose for males and females, respectively, for [bromophenyl-6-^{14}C]metrafenone and 2.31% and 4.9% of the administered dose for males and females, respectively, for [trimethoxyphenyl-U-^{14}C]metrafenone. The percentage of the administered dose found in urine was 2.0% and 3.65% for males and females, respectively, at 7 days after the multiple low dose and 0.69% and 1.04% for males and females, respectively, at 7 days after the single high dose. The majority of the administered dose was excreted via faeces (84.09–98.83%, depending on the group). Results were similar between single and multiple dose groups. Seven days following the single oral low dose administration, the excretion in faeces was 89.79% and 84.09% of the administered dose for males and females, respectively, for [bromophenyl-6-^{14}C]metrafenone and 91.34% and 90.22% of the administered dose for males and females, respectively, for [trimethoxyphenyl-U-^{14}C]metrafenone. The percentage of the administered dose found in faeces was 96.68% and 94.42% for males and females, respectively, at 7 days after the multiple low dose and 98.83% and 96.25% for males and females, respectively, at 7 days after the single high dose. The majority of the dose was excreted within 48 hours post-dosing, indicating moderately rapid elimination (Mallipudi, 2002).

In a biliary excretion study, groups of four rats of each sex were administered a single oral dose of [trimethoxyphenyl-U-^{14}C]metrafenone at either 10 or 1000 mg/kg bw. Bile, urine and faeces were collected at different time intervals post-dosing, and gastrointestinal tract, gastrointestinal tract contents and residual carcass were collected at termination. In the low-dose group, the majority of the administered dose was eliminated in the bile (90.09% in males and 85.23% in females after 72 hours), with 6.46% and 10.81% (males and females, respectively) recovered in the faeces. In the high-dose group, after 72 hours, the majority of the administered dose was excreted in faeces (81.10% in males and 79.79% in females), and 14.76% and 17.39% (males and females, respectively) were recovered in the bile. Urine and cage rinse contained very small amounts of dose (0.16–3.14%) in both the low- and high-dose groups (Table 3). The average recovery of the administered dose was greater than 96% in 72 hours after dosing. The total radioactive residue (TRR) was also measured in the gastrointestinal

tract, gastrointestinal tract contents and residual carcasses. The radioactivity in urine, bile, gastrointestinal tract (without contents) and residual carcass was added together to determine the extent of total absorption (Table 3). In the low-dose group, the majority of the dose was eliminated in the bile, and 88.72–91.54% of the dose was absorbed, indicating that biliary excretion and metabolism were prominent processes. In the high-dose group, only 14.97–18.34% of the dose was eliminated in the bile and urine, indicating a much lower percentage of absorption and that the absorption process had been saturated (Mallipudi, 2002).

Table 3. Total absorption of metrafenone after 72 hours

	% of administered radioactive dose			
	Low dose (10 mg/kg bw)		High dose (1 000 mg/kg bw)	
Matrix	**Males**	**Females**	**Males**	**Females**
Urine and cage rinses	1.23	3.14	0.16	0.84
Bile	90.09	85.23	14.76	17.39
Gastrointestinal tract	0.01	0.04	0.00	0.00
Carcass	0.21	0.31	0.05	0.11
Total absorption	91.54	88.72	14.97	18.34

bw: body weight

Source: Mallipudi (2002)

In a tissue and organ distribution study, animals received a single oral dose of trimethoxyphenyl-labelled metrafenone at either a low dose of 10 mg/kg bw or a high dose of 1000 mg/kg bw. The TRR in various organs and tissues was determined at four time points, corresponding approximately to ⅔ T_{max}, T_{max}, $t_{½\ elim}$ and $t_{⅔\ elim}$ (4, 8, 24 and 48 hours for the low dose and 4, 14, 36 and 72 hours for the high dose). Data are presented as mean microgram equivalents of radiolabelled metrafenone per gram of tissue (ppm) in Tables 4 and 5.

In females, peak levels in most tissues were recorded at 4 hours at both groups. In males, peak levels in most tissues were observed at 8 hours for the low dose and 14 hours for the high dose. At all time points, the highest proportion of administered dose was always recovered from the gastrointestinal wash.

At the low dose, the highest levels of radioactivity (as ppm metrafenone equivalents) were found in the gastrointestinal tract (gastrointestinal wash excluded), liver, plasma, blood, kidney and fat (in descending order). Over 4–24 hours, levels of radioactivity were significantly higher in the gastrointestinal tract than in any other organ or tissue. By 48 hours, the highest levels were found in the liver.

At the high dose, the highest levels of radioactivity (as ppm metrafenone equivalents) were found in the gastrointestinal tract (gastrointestinal wash excluded), liver, fat, plasma, blood and adrenals (in broadly descending order). The pattern of distribution was therefore similar to that observed at the low dose, although levels were approximately 10-fold higher.

The proportion of the orally administered dose remaining by 48–72 hours had declined to 3.93–4.17% at the low dose and to 0.23–1.20% at the high dose, with levels recorded in organs and tissues other than the gastrointestinal tract substantially lower than these. The high levels throughout in the gastrointestinal tract and gastrointestinal wash were consistent with extensive biliary excretion. The low residues remaining at the last time point indicate no significant potential for bioaccumulation.

Although tissues in females contained more radioactive residues than tissues in males at the last time point, the sex difference was not significant overall, considering the small amount of radioactivity that was contained in the tissues (Mallipudi, 2002).

Table 4. Total radioactive residues in tissues of the low-dose group

	Mean TRR (ppm) at 10 mg/kg bw							
	Male rats (*n* = 3)				Female rats (*n* = 3)			
Matrix	**4 h**	**8 h**	**24 h**	**48 h**	**4 h**	**8 h**	**24 h**	**48 h**
Adrenal	0.41	0.44	0.19	0.09	0.71	0.38	0.19	0.08
Blood	0.93	1.47	0.66	0.34	1.63	1.11	0.69	0.31
Bone	0.19	0.30	0.13	0.07	0.25	0.17	0.09	0.04
Brain	0.06	0.07	0.03	0.01	0.11	0.05	0.02	0.01
Carcass	0.49	0.55	0.20	0.08	0.30	0.64	0.45	0.06
Fat	0.84	0.52	0.14	0.04	0.88	0.54	0.22	0.08
Heart	0.36	0.46	0.24	0.11	0.60	0.37	0.20	0.09
Gastrointestinal tract	21.55	18.10	2.67	0.57	19.45	13.58	5.23	0.58
Kidneys	0.84	1.15	0.54	0.27	1.20	0.65	0.44	0.16
Liver	4.32	4.81	1.10	0.66	6.24	3.33	1.59	0.66
Lungs	0.49	0.79	0.32	0.17	0.84	0.65	0.39	0.18
Muscle	0.10	0.15	0.09	0.05	0.13	0.08	0.06	0.03
Pancreas	0.28	0.38	0.15	0.07	0.54	0.31	0.15	0.06
Pituitary	0.31	0.54	0.22	0.08	0.56	0.33	0.21	0.07
Plasma	1.87	2.90	1.26	0.58	3.30	2.16	1.28	0.53
Red blood cells	0.65	0.94	0.46	0.20	1.05	0.56	0.44	0.18
Skin	0.22	0.32	0.17	0.10	0.34	0.21	0.16	0.08
Testes	0.20	0.33	0.15	0.08	–	–	–	–
Thymus	0.12	0.20	0.11	0.05	0.20	0.13	0.10	0.05
Thyroid	0.34	0.43	0.20	0.10	0.59	0.31	0.21	0.08
Gastrointestinal wash	49.68	32.82	5.30	1.05	32.13	35.33	9.00	1.41
Spleen	0.14	0.25	0.10	0.04	0.27	0.18	0.11	0.06
Bone marrow	0.26	0.33	0.15	0.08	0.49	0.26	0.18	0.07
Uterus	–	–	–	–	0.54	0.38	0.32	0.12
Ovaries	–	–	–	–	0.78	0.51	0.29	0.12

bw: body weight; ppm: parts per million; TRR: total radioactive residue

Source: Mallipudi (2002)

(b) Dermal route

The dermal absorption, distribution and excretion of radioactivity were studied in male rats following a single dermal administration of ^{14}C-labelled metrafenone in the formulation concentration (BAS 560 02 F) and a 1/2000 aqueous dilution thereof. These dose preparations resulted in nominal dose levels of 5.0 and 0.0025 mg/cm^2 (corresponding nominally to about 50 and 0.024 mg/animal and about 180 and 0.1 mg/kg bw). Groups of four animals were treated with metrafenone for 10 hours and then were terminated at 10, 24 and 168 hours. No tape stripping was performed.

Table 5. Total radioactive residues in tissues of the high-dose group

	Mean TRR (ppm) at 1 000 mg/kg bw							
	Male rats (n = 3)				Female rats (n = 3)			
Matrix	**4 h**	**14 h**	**36 h**	**72 h**	**4 h**	**14 h**	**36 h**	**72 h**
Adrenal	10.67	6.07	3.15	0.98	37.25	18.36	2.97	2.09
Blood	9.62	14.96	10.57	2.97	8.34	13.52	9.32	7.62
Bone	1.95	2.84	1.78	0.48	3.75	3.10	1.26	0.97
Brain	1.87	1.30	0.59	0.31	6.96	2.81	0.53	0.50
Carcass	6.16	14.44	3.10	0.67	19.36	20.87	2.79	1.73
Fat	19.00	40.91	3.43	0.56	86.40	264.15	15.96	6.14
Heart	4.73	5.57	3.00	0.88	8.71	5.88	2.77	1.96
Gastrointestinal tract	446.09	128.45	41.71	2.25	368.28	273.73	37.90	13.48
Kidneys	9.51	10.89	9.38	2.49	13.21	10.55	5.28	4.31
Liver	63.23	34.29	21.11	6.20	62.11	51.55	16.81	10.46
Lungs	6.78	8.50	4.66	1.72	11.03	9.32	4.81	3.69
Muscle	1.95	2.06	1.46	0.42	5.22	4.31	4.12	0.82
Pancreas	5.04	4.55	2.18	0.52	14.77	7.18	1.83	1.27
Pituitary	4.66	6.13	3.33	1.57	7.17	5.81	3.93	2.53
Plasma	17.67	26.52	17.35	4.10	14.95	24.07	15.52	11.92
Red blood cells	4.79	5.89	6.22	2.30	2.84	5.75	4.91	4.10
Skin	4.93	4.98	3.65	1.10	23.28	12.67	3.16	2.91
Testes	3.03	3.52	2.23	0.53	–	–	–	–
Thymus	2.31	1.88	1.22	0.35	5.86	3.84	1.15	1.04
Thyroid	6.44	4.82	3.45	1.16	24.95	8.50	2.74	2.32
Gastrointestinal wash	3 280.42	405.58	86.50	3.40	3 718.22	901.96	82.25	30.88
Spleen	2.28	2.71	1.57	0.53	5.25	4.32	1.37	1.26
Bone marrow	3.72	6.48	2.27	0.46	6.99	6.34	2.27	1.94
Ovaries	–	–	–	–	24.14	35.46	5.46	3.19
Uterus	–	–	–	–	10.23	15.59	6.25	3.36

bw: body weight; ppm: parts per million; TRR: total radioactive residue

Source: Mallipudi (2002)

Mean recoveries of radioactivity from all dose groups were in the range of 96.11–108.83% of the total radioactivity administered. The largest proportion of radioactivity was recovered from the skin wash. The amount of radioactivity absorbed (including excreta, cage wash, tissues/organs and carcass) generally increased with increasing termination time but decreased with increasing dose and was 1.15% for the concentrate and 18.72% for the dilution. The amount in skin at and surrounding the application site was 0.48% for the concentrate and 0.40% for the dilution. For the formulation concentration (5.0 mg/cm^2), absolute absorption excluding skin was about 1.15%, and the potential absorption including skin was 1.63%. For the aqueous dilution, absolute absorption excluding skin

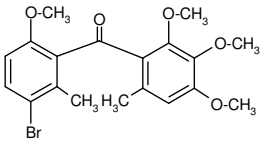
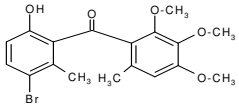
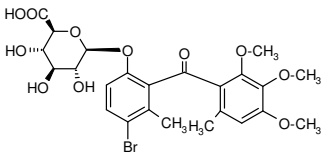
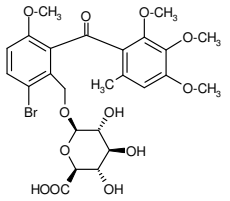
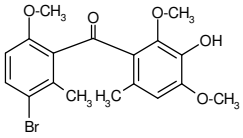
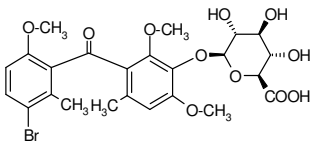
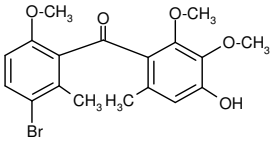
was 18.72%, and the potential absorption including skin was 19.12%. As the amount of absorbed radioactivity (including excreta, cage wash, tissues/organs and carcass) was still increasing after the test substance was washed off, it must be assumed that the test substance in the skin can potentially be absorbed. Dermal absorption at the high dose was considerably lower than that at the low dose, indicating saturation of skin penetration at the high dose.

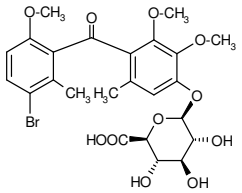
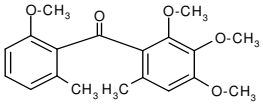
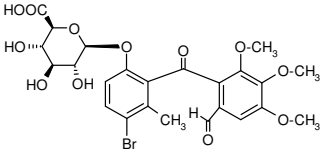
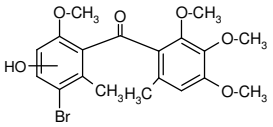
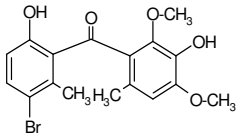
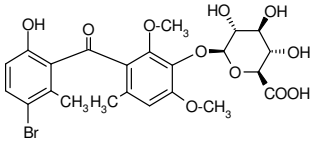
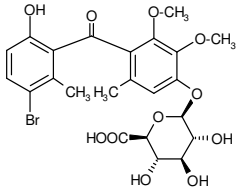
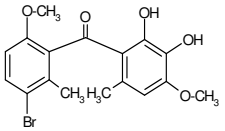
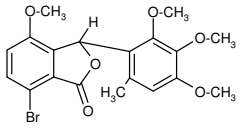
The radioactivity absorbed was excreted mainly via the faeces. The highest tissue concentration of radioactivity was found in the remaining carcass (Leibold & van Ravenzwaay, 2002).

1.2 Biotransformation

The identified rat metabolites of metrafenone are shown in Table 6.

Table 6. Summary of metrafenone-derived metabolites in the rat

Metabolite code	Chemical name	Structure
Metrafenone (AC 375839; CL 375839)	Methanone, (3-bromo-6-methoxy-2-methylphenyl)(2,3,4-trimethoxy-6-methylphenyl)- Benzophenone, 3′-bromo-2,3,4,6′-tetramethoxy-2′,6-methyl-	
CL 376991	Methanone, (3-bromo-6-hydroxy-2-methylphenyl)(2,3,4-trimethoxy-6-methylphenyl)-	
CL 1500701	Methanone, [3-bromo-6-(β-D-glucopyranuronosyloxy)-2-methylphenyl](2,3,4-trimethoxy-6-methylphenyl)-	
CL 1500700	Methanone, [3-bromo-2-[(β-D-glucopyranuronosyloxy)methyl]-6-methoxyphenyl](2,3,4-trimethoxy-6-methylphenyl)-	
CL 377160	Methanone, (3-bromo-6-methoxy-2-methylphenyl)(3-hydroxy-2,4-dimethoxy-6-methylphenyl)-	
CL 1500698	Methanone, (3-bromo-6-methoxy-2-methylphenyl)[3-(β-D-glucopyranuronosyloxy)-2,4-dimethoxy-6-methylphenyl]-	
CL 434223	Methanone, (3-bromo-6-methoxy-2-methylphenyl)(4-hydroxy-2,3-dimethoxy-6-methylphenyl)-	

Metabolite code	Chemical name	Structure
CL 1500699	Methanone, (3-bromo-6-methoxy-2-methylphenyl)[4-(β-D-glucopyranuronosyloxy)-2,3-dimethoxy-6-methylphenyl]-	
CL 375228	Methanone, (6-methoxy-2-methylphenyl)(2,3,4-trimethoxy-6-methylphenyl)-	
CL 1023426	Methanone, [3-bromo-6-(β-D-glucopyranuronosyloxy)-2-methylphenyl](2,3,4-trimethoxy-6-formyl)-	
AC 12071-12B	Methanone, [3-bromo-6-methoxy-(4 or 5)-hydroxy-2-methylphenyl](2,3,4-trimethoxy-6-methylphenyl)-	
CL 1023427	Methanone, [3-bromo-6-hydroxy-2-methylphenyl](3-hydroxy-2,4-dimethoxy-6-methylphenyl)-	
CL 1500702	Methanone, (3-bromo-6-hydroxy-2-methylphenyl)[3-(β-D-glucopyranuronosyloxy)-2,4-dimethoxy-6-methylphenyl]-	
CL 1500697	Methanone, (3-bromo-6-hydroxy-2-methylphenyl)[4-(β-D-glucopyranuronosyloxy)-2,3-dimethoxy-6-methylphenyl]-	
CL 377096	Methanone, (3-bromo-6-methoxy-2-methylphenyl)(2,3-dihydroxy-4-methoxy-6-methylphenyl)-	
CL 3000402	1(3*H*)-isobenzofuranone, 7-bromo-4-methoxy-3-(2,3,4-trimethoxy-6-methylphenyl)-	

Source: Mallipudi (2002)

As more than 83.0% of the administered dose was eliminated in urine and faeces within 72 hours for all the groups, the samples of urine and faeces collected at 0–12 hours, 12–24 hours, 24–

48 hours and 48–72 hours were pooled according to sex and radiolabel for the single low dose, multiple low dose and single high dose and used to analyse and quantify the respective metabolites.

Metabolites in urine were similar regardless of dose level, radiolabel, sex or time interval. The main metabolites in urine (all $\leq 0.41\%$ of the administered dose) were all glucuronic conjugates of either metrafenone or metrafenone with the bromophenyl ring hydroxylated instead of methoxylated. The parent compound was not found in urine.

Unchanged metrafenone was the largest single component identified in faecal samples. The other metabolites identified in the largest amounts in faeces all differed from metrafenone only by the substitution of one or two of the methoxy groups in metrafenone (on either the bromophenyl ring or the trimethoxyphenyl ring) with hydroxyl groups. The other metabolites identified were the same glucuronic acid conjugates also identified in urine. There were no metabolites identified that did not have both the bromophenyl and trimethoxyphenyl rings present and intact. In all groups, there were also numerous other minor radioactive compounds (29–45) that were not identified, accounting in total for 0.24–10.99% of the administered dose.

In addition to urine and faeces, certain organs and tissues (fat, liver and kidney) were also analysed from these groups. The majority (approximately 60%) of the material in these organs and tissues remained unidentified, and the post-extraction solids represented about a further 20%. Given the relatively low levels of radioactivity and the fact that the radioactivity was distributed among a large number of components, the low level of identification is not a concern – all major components of the residue will have been identified, as the radioactivity in the unknowns was spread over so many different components. The unknown components numbered between 11 and 26 individual components across the different groups (total amount of unknowns was < 0.1 ppm at the low dose and < 1 ppm at the high dose). The metabolites that were identified in various organ/tissue components (which represented mostly < 1 ppm) were as follows:

- *Fat*: the largest component identified was metrafenone, with smaller amounts of the same glucuronic acid conjugates as identified in urine.
- *Liver*: broadly equivalent amounts of metrafenone and glucuronic acid conjugates were recorded at low doses, with a slight increase in the level of metrafenone in liver at the high dose.
- *Kidney*: broadly equivalent amounts of metrafenone and glucuronic acid conjugates.
- *Bile*: the only metabolites identified were the glucuronic acid conjugates as identified in urine. Bile samples were hydrolysed by the enzyme β-glucuronidase, and structures were confirmed by liquid chromatography with mass spectrometry proton nuclear magnetic resonance analysis. There were also numerous other unknowns (14–24) in bile, which collectively varied from about 0.5% of the dose to about 20% of the dose. Unchanged metrafenone was not identified in bile.

The proposed metabolic pathway is presented in Fig. 2 (Mallipudi, 2002).

2. Toxicological studies

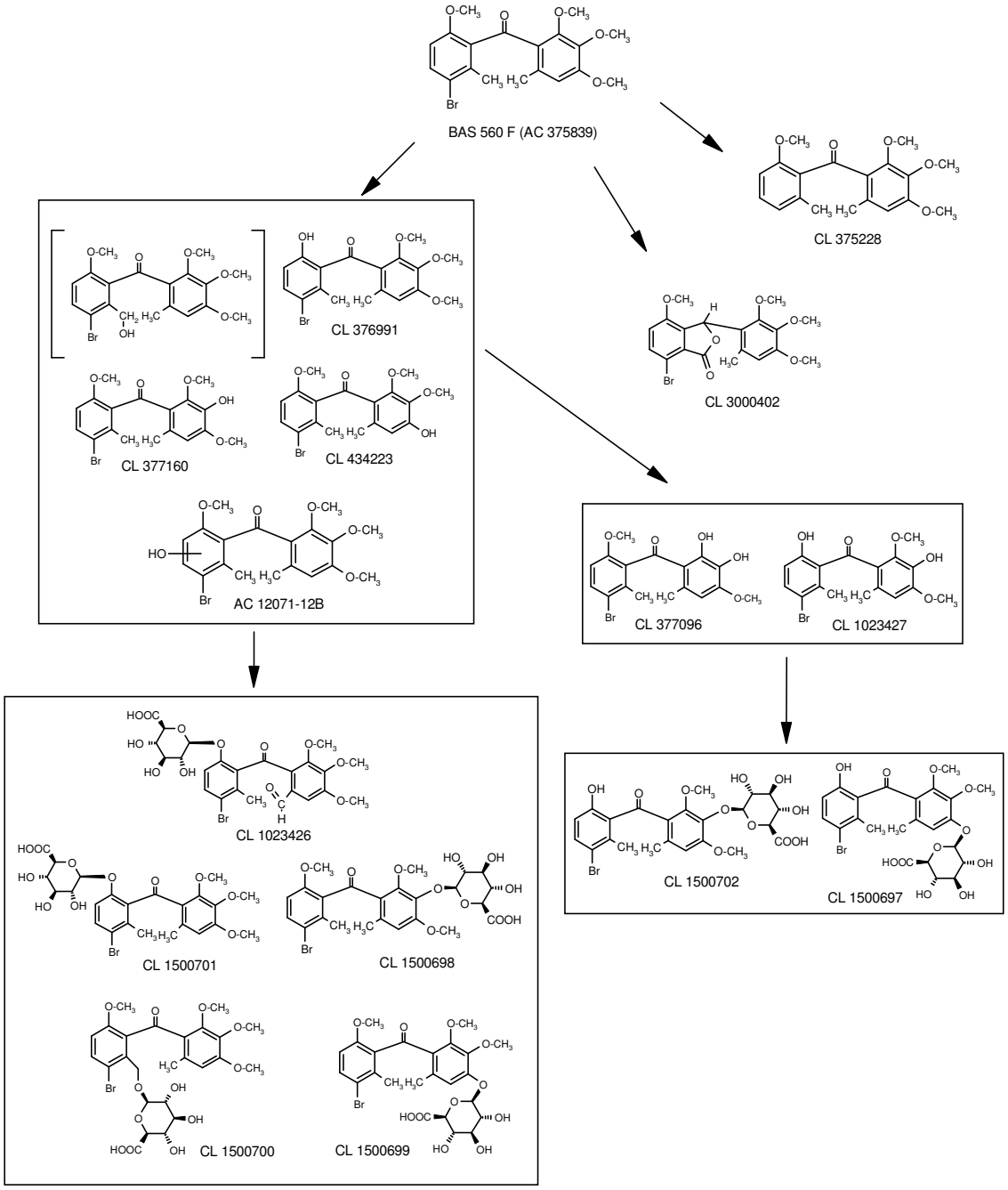
2.1 Acute toxicity

(a) Lethal doses

The results of acute toxicity studies with metrafenone administered orally, dermally or by inhalation are summarized in Table 7. All the studies were certified to comply with GLP and performed according to USEPA and OECD guidelines.

Metrafenone has low acute toxicity when administered orally, dermally and via inhalation to rats.

Fig. 2. Proposed metabolic pathway of metrafenone in the rat



Source: Mallipudi (2002)

In an acute oral toxicity study, groups of five Sprague-Dawley rats of each sex received an oral dose of 5000 mg/kg bw of metrafenone (purity 95.86%). The test material (solid) had been ground to a fine powder and then mixed with 0.5% aqueous carboxymethyl cellulose. There were no deaths or notable clinical signs of toxicity, and body weight gains were normal. There were no gross necropsy findings. The median lethal dose (LD_{50}) following oral exposure was greater than 5000 mg/kg bw (Lowe, 1999).

Table 7. Acute toxicity of metrafenone

Species	Strain	Sex	Route	Purity (%)	Result	Reference
Rat	Sprague-Dawley	Male and female	Oral	95.86	LD_{50} > 5 000 mg/kg bw	Lowe (1999)
Rat	Sprague-Dawley	Male and female	Dermal	95.86	LD_{50} > 5 000 mg/kg bw	Bradley (1999)
Rat	Sprague-Dawley	Male and female	Inhalation	95.86	LC_{50} > 5.0 mg/L (maximal attainable concentration)	Hoffman (2000)

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

In an acute dermal toxicity study, groups of five Sprague-Dawley rats of each sex were treated with metrafenone (purity 95.86%) via dermal exposure at a limit dose of 5000 mg/kg bw. The test material (solid) was ground to a fine powder and administered as a paste in 0.5 mL of tap water to approximately 10% of body surface area under an occlusive dressing for 24 hours. There were no deaths or notable clinical signs of toxicity, and body weight gains were normal. No skin irritation was noted. There were no gross necropsy findings. The LD_{50} following dermal exposure was greater than 5000 mg/kg bw (Bradley, 1999).

In an acute inhalation toxicity study, groups of five Sprague-Dawley rats of each sex were exposed to metrafenone (purity 95.86%) for 4 hours via nose-only inhalation exposure at a test atmosphere concentration of 5.0 mg/L. There were no deaths, and body weight gains were normal, except for a single female that lost weight (3 g) during the second week of the study. Clinical signs recorded during exposure were laboured breathing during the last 3 hours of exposure. Immediately following exposure, signs recorded were red nasal discharge, excessive salivation, chromodacryorrhoea, dried red material on the facial area, laboured breathing and moist rales. Laboured breathing and moist rales continued in some animals until day 3 (48 hours after exposure). Red nasal discharge was recorded sporadically in one or two males and females over the remainder of the 14-day observation period, persisting in a single female at termination (although this female had not shown this sign throughout – only on days 2, 7, 10, 14 and 15). There were no notable necropsy findings at termination. The acute inhalation median lethal concentration (LC_{50}) was greater than 5.0 mg/L. The clinical signs recorded following the completion of the exposure period are considered to be a nonspecific effect related to animals breathing a highly dusty atmosphere (Hoffman, 2000).

(b) Dermal irritation

No dermal irritation study was submitted. No skin irritation was noted in the acute dermal toxicity study.

(c) Ocular irritation

No ocular irritation study was submitted.

(d) Dermal sensitization

No dermal sensitization study was submitted.

2.2 Short-term studies of toxicity

(a) Oral administration

Mice

In a 90-day toxicity study, groups of 10 male and 10 female CD-1 mice received metrafenone (purity 97.1%) in their diet at a concentration of 0, 1000, 3500 or 7000 ppm (mean substance intakes: 0, 163, 622 and 1206 mg/kg bw per day for males and 0, 216, 788 and 1663 mg/kg bw per day for females, respectively) for 13 weeks. Investigations included clinical signs, body weights, feed

consumption, haematology and clinical chemistry at termination, organ weights (adrenals, brain, heart, kidneys, liver, ovaries, spleen, testes and thymus), gross necropsy and histopathological examination (full range of tissues from control and high-dose groups, plus lungs, liver, kidneys and gross lesions from remaining groups).

There were no deaths and no clinical signs of toxicity. There were no effects of treatment on feed consumption. Reduced body weight gain was recorded in males at 7000 ppm from week 5 onwards, such that overall body weight gains in this group over the study period were approximately 20% lower than in controls, although not attaining statistical significance. At the end of the study, body weights in these top-dose males were reduced by 7%. Body weight gains in the other male groups and in all female groups were similar to those of controls.

Treatment had no effect on haematological parameters. Clinical chemistry showed treatment-related changes in hepatic parameters: increases in total bilirubin for both male and female mice at 3500 and 7000 ppm (attaining statistical significance in males only), slight increases in cholesterol values at 7000 ppm (attaining statistical significance in females only) and slight, non-statistically significant increases in triglyceride levels in females at 3500 and 7000 ppm (Table 8).

Table 8. Clinical chemistry results obtained in a 90-day study in mice given metrafenone in the diet

Dietary concentration (ppm)	Cholesterol (mg/dL)		Triglycerides (mg/dL)		Total bilirubin (U/L)	
	Males	Females	Males	Females	Males	Females
0	139.9	77.9	148.1	91.4	0.25	0.20
1 000	134.6	92.5	144.3	91.1	0.27	0.21
3 500	136.2	91.3	139.1	113.2	0.44*	0.33
7 000	151.1	119.1*	136.4	132.5	0.48*	0.36

ppm: parts per million; U: units; *: $P < 0.05$

Source: Fischer (2001a)

The only significant organ weight effects were increased liver weights in males and females treated at 3500 and 7000 ppm: relative liver weights were increased by 18% and 22% in males and by 25% and 26% in females, respectively. There were no treatment-related macroscopic findings. Histopathology revealed treatment-related findings in the liver of animals of both sexes treated at 3500 and 7000 ppm; hepatocellular hypertrophy (principally in the centrilobular area, but frequently blending into the midlobular areas) was noted at both doses. The affected hepatocytes were filled with eosinophilic cytoplasm with a ground glass appearance. No vacuolation of the cytoplasm was evident. The incidence and severity of this finding showed a dose-related pattern. There were no liver findings in animals receiving 1000 ppm and no treatment-related effects in any other organs or tissues.

The no-observed-adverse-effect level (NOAEL) of the study was 1000 ppm (equal to 163 mg/kg bw per day for males and 216 mg/kg bw per day for females), based on liver effects at 3500 ppm (equal to 622 mg/kg bw per day for males and 788 mg/kg bw per day for females). The small increases in cholesterol levels in females at 1000 ppm are not considered to be toxicologically significant in the absence of any supporting findings (Fischer, 2001a).

In a subsequent 13-week study intended to show a clear no-observed-effect level (NOEL) for liver effects, groups of 10 male and 10 female CD-1 mice received metrafenone (purity 97.1%) in their diets at a concentration of 0, 250 or 500 ppm (equal to 0, 42 and 84 mg/kg bw per day for males and 0, 55 and 113 mg/kg bw per day for females, respectively). Investigations were the same as in the previous study.

There were no mortalities, no clinical signs of toxicity and no effects of treatment on body weights or feed consumption in any group. There were no treatment-related haematological effects. Increased cholesterol levels were recorded at 500 ppm in males (125.9 versus 98.5 mg/dL; $P < 0.05$). Triglyceride levels were increased in females at 500 ppm (118.8 versus 90.1 mg/dL; $P < 0.05$). There were no statistically significant or toxicologically relevant organ weight changes and gross necropsy or histopathological findings in any group.

The NOAEL of the study was 500 ppm (equal to 84 mg/kg bw per day for males and 113 mg/kg bw per day for females), the highest dose level tested, because the slight effects on cholesterol levels in males and triglyceride levels in females were not considered to be toxicologically significant in the absence of accompanying findings (Fisher, 2001b).

Rats

In a 4-week range-finding study, groups of five male and five female Sprague-Dawley (Crl CD(SD)BR) rats were exposed to metrafenone (purity > 99.9%) for 28 days via the diet at a concentration of 0, 1000, 5000, 10 000 or 20 000 ppm (mean substance intakes: 0, 106, 528, 1127 and 2245 mg/kg bw per day for males and 0, 118, 586, 1151 and 2294 mg/kg bw per day for females, respectively) as a preliminary study to a 90-day study. Owing to a sexing error, the top-dose group consisted of four males and six females instead of five animals of each sex. Investigations included clinical signs, body weights, feed consumption, haematology and clinical chemistry at termination, organ weights (adrenals, brain, heart, kidneys, liver, ovaries, spleen, testes and thymus), gross necropsy and histopathological examination (full range of tissues from control and high-dose groups, plus lungs, liver, heart, kidneys and gross lesions from remaining groups). Haematology results were considered to be unreliable because of the limited number of data available for the control groups and males in the high-dose group.

There were no deaths, and no clinical signs of toxicity were observed throughout the study. There was no effect of treatment on feed consumption or body weight gain. There were no obvious haematological effects. Clinical chemistry showed increased cholesterol values in groups of female rats, although without a clear dose–response relationship. The increased mean cholesterol value observed in females at 1000 ppm was not considered to be adverse because only 2/5 females showed slightly elevated values, and there was no correlation with any microscopic change. The only significant organ weight effects were recorded in the liver. Absolute and/or relative liver weights were statistically significantly increased in both sexes at and above 5000 ppm, the increases being more marked in females. Liver weights at 1000 ppm were not increased compared with controls (Table 9).

Table 9. Liver effects observed in a 4-week range-finding study in rats given metrafenone in the diet

Dietary concentration (ppm)	Cholesterol (mg/dL)		Total bilirubin (mg/dL)		Absolute liver weight (g) / % of control		Relative liver weight (%) to body weight / % of control	
	M	F	M	F	M	F	M	F
0	80.8	67.2	0.20	0.18	12.62	7.60	3.84	3.81
1 000	70.2	98.2*	0.10*	0.14	11.67 / 93	7.77 / 101	3.73 / 97	3.89 / 102
5 000	67.0	101.0*	0.16	0.16	14.03 / 111	9.14* / 120	4.48* / 117	4.64* / 122
10 000	63.6	118.2*	0.12	0.10	15.65* / 124	9.55* / 126	4.93* / 128	4.99* / 131
20 000	79.0	115.8*	0.08*	0.13	15.37 / 122	11.18* / 147	4.63* / 121	5.67* / 149

F: female; M: male; ppm: parts per million; *: $P < 0.05$

Source: Fischer (2001c)

At necropsy, discoloured liver was noted in 3/5 females at 10 000 ppm and 2/6 females at 20 000 ppm. There were no other notable gross necropsy findings. Significant microscopic findings were recorded in the liver and consisted of periportal cytoplasmic vacuolation of hepatocytes, recorded at a higher incidence in high-dose males and in females at and above 5000 ppm. This finding was described by the pathologist as being consistent with the accumulation of lipid (only haematoxylin and eosin [H&E] staining performed). Severity in females ranged from minimal to moderate, and severity increased in a dose-related pattern. Severity in males was minimal in all cases (Table 10). Scattered cytoplasmic vacuolation (not limited to periportal localization) was also recorded in some animals, but the pattern of incidence did not suggest a treatment-related effect. It was noted by the pathologist that the cytoplasmic vacuolation was not accompanied by necrosis and was therefore considered to be an adaptive rather than a toxic effect. The gross findings of discoloured liver correlated with periportal cytoplasmic vacuolation in each case.

Table 10. Histopathological liver effects observed in a 4-week range-finding study in rats given metrafenone in the diet

Dietary concentration (ppm)	Periportal cytoplasmic vacuolation of hepatocytes									
	No. examined		Total		Minimal		Slight/mild		Moderate	
	M	F	M	F	M	F	M	F	M	F
0	5	5	1	2	1	2	0	0	0	0
1 000	5	5	0	1	0	1	0	0	0	0
5 000	5	5	0	5	0	5	0	0	0	0
10 000	5	5	1	4	1	0	0	3	0	1
20 000	4	6	4	6	4	1	0	3	0	2

F: female; M: male; ppm: parts per million

Source: Fischer (2001c)

The NOAEL was 1000 ppm (equal to 106 mg/kg bw per day for male rats and 118 mg/kg bw per day for female rats), based on clinical chemistry changes, liver weight effects and liver microscopic findings at 5000 ppm (equal to 528 mg/kg bw per day for male rats and 586 mg/kg bw per day for female rats) (Fischer, 2001c).

In a 90-day toxicity study, groups of 10 male and 10 female Sprague-Dawley (Crl:CD (SD)BR) rats were exposed to metrafenone (purity 97.1%) for 13 weeks via the diet at a concentration of 0, 1000, 5000, 10 000 or 20 000 ppm (mean substance intakes: 0, 79, 404, 800 and 1663 mg/kg bw per day for males and 0, 94, 486, 967 and 1938 mg/kg bw per day for females, respectively). Five additional animals of each sex were added to the control and high-dose groups to be maintained for a 4-week recovery period after the exposure period. Investigations included clinical signs, body weights, feed consumption, haematology, clinical chemistry and urine analysis (at termination of exposure and recovery periods), ophthalmoscopy (pre-exposure and day 87), organ weights (adrenals, brain, heart, kidneys, liver, ovaries, spleen, testes, thymus and thyroid gland), gross necropsy and histopathological examination (full range of tissues from control and high-dose groups [treatment and recovery phases], plus lungs, liver, kidneys and gross lesions from remaining groups).

There were two accidental deaths (mechanical or blood sampling), but no treatment-related mortalities. There were no treatment-related clinical signs. Ophthalmoscopy, urine analysis and haematology did not reveal treatment-related findings. There were no toxicologically significant effects on feed consumption. Weekly body weights and body weight gains for treated males and females receiving 1000 ppm were generally comparable with or in excess of those of the untreated

controls at most measurement intervals. Treatment-related decreases in weekly body weights and body weight gains were observed for females at 5000, 10 000 and 20 000 ppm at most measurement intervals. A statistically significant decrease in overall body weight gain (0–13 weeks) was observed in female rats at 5000 (14.6%), 10 000 (14.6%) and 20 000 ppm (13.2%); there was no evidence of a dose–response relationship, but the absorption, distribution, metabolism and excretion (ADME) studies (see section 1.1) indicate a saturation of absorption at high dose levels. Body weights and body weight gains (weekly and overall) for males and females previously treated at 20 000 ppm for 13 weeks were generally comparable with or in excess of those of the untreated controls at most measurement intervals during the recovery phase of the study. Clinical chemistry investigations at termination revealed increases in cholesterol levels in both males and females, statistically significant at 5000 ppm and above and more marked and more clearly dose related in females. Cholesterol levels were also slightly higher in males and females at 1000 ppm, without attaining statistical significance. Total protein was significantly higher in treated animals, with females more markedly affected. Additionally, albumin values for 20 000 ppm females were also slightly but statistically significantly increased. Following the 4-week recovery period, cholesterol, total protein and albumin values for both sexes previously treated at 20 000 ppm returned to control levels, with no statistically significant differences. Statistically significant increases in absolute and relative (to body weight) liver weights were noted for males and females at 5000 ppm and above when compared with controls. Absolute and relative (to body weight) liver weights for males and females at 1000 ppm were only very slightly increased over control liver weights, with the relative weights for 1000 ppm males being statistically significant. As there were no correlating histopathological changes, the liver weight changes at 1000 ppm were not considered to be adverse. At the end of the 28-day recovery phase, liver weights for rats previously treated at 20 000 ppm were generally comparable with control weights (Table 11).

Table 11. Liver effects observed in a 90-day study in rats given metrafenone in the diet and including a 28-day recovery period

Dietary concentration (ppm)	Cholesterol (mg/dL)		Total protein (g/dL)		Albumin (g/dL)		Absolute liver weights (g) / % of control		Relative liver weights (%) to body weight / % of control	
	M	F	M	F	M	F	M	F	M	F
0	53.7	68.1	6.6	7.2	4.6	5.3	13.28	7.68	2.82	2.87
1 000	61.6	85.6	7.0	7.6*	4.6	5.6	15.35 / 116	8.18 / 107	3.07* / 109	3.21 / 112
5 000	72.1*	97.6*	7.3*	8.2*	4.9	5.7	16.19* / 122	9.94* / 129	3.39* / 120	4.11* / 143
10 000	83.2*	108.8*	7.3*	8.2*	4.7	5.6	16.16* / 122	11.06* / 144	3.54* / 126	4.60* / 160
20 000	77.0*	124.0*	7.2*	8.4*	4.8	5.8*	18.97* / 143	11.54* / 150	3.76* / 133	4.89* / 170
Recovery										
0	54.2	70.8	7.1	7.6	3.9	4.9	13.84	7.77	2.87	2.75
20 000	60.0	71.0	7.3	7.8	4.1	4.7	14.77 / 107	8.10 / 104	2.98 / 104	3.15 / 115

F: female; M: male; ppm: parts per million; *: $P < 0.05$

Source: Fischer (2001d)

There were no notable gross necropsy findings in any group. Significant microscopic findings were recorded in the liver. An increased incidence of periportal hepatocellular vacuolation, consistent with the accumulation of lipid, was recorded in males at 20 000 ppm and in females at and above 5000 ppm. The incidence and severity of this finding in females did not show clear dose-related trends. These findings were shown to be reversible in males and largely reversible in females (Table 12).

Table 12. Histopathological liver effects observed in a 90-day study in rats given metrafenone in the diet and including a 28-day recovery period

Dietary concentration (ppm)	Periportal cytoplasmic vacuolation in hepatocytes									
	No. examined		Total		Minimal		Slight/mild		Moderate	
	M	F	M	F	M	F	M	F	M	F
0	10	10	1	1	1	1	0	0	0	0
1 000	10	10	1	0	0	0	1	0	0	0
5 000	10	10	0	7	0	3	0	2	0	2
10 000	10	10	0	8	0	6	0	2	0	0
20 000	10	10	3	5	2	5	1	0	0	0
Recovery										
0	5	5	0	0	0	0	0	0	0	0
20 000	5	5	0	2	0	1	0	1	0	0

F: female; M: male; ppm: parts per million

Source: Fischer (2001d)

The NOAEL of the study was 1000 ppm (equal to 79 mg/kg bw per day for males and 94 mg/kg bw per day for females), based on decreased body weight gain in females treated at 5000 ppm and liver findings (clinical chemistry, organ weight and histopathology) in both sexes at 5000 ppm (equal to 404 mg/kg bw per day for males and 486 mg/kg bw per day for females). Changes recorded at 1000 ppm (liver weight, total protein and cholesterol concentrations) were considered adaptive rather than toxic in nature; they were slight, and recovery at the highest dose level of 20 000 ppm was demonstrated (Fisher, 2001d).

In a subsequent 13-week study to determine a clear NOEL for liver effects, groups of 10 male and 10 female Sprague-Dawley (Crl CD(SD)BR) rats received metrafenone (purity 97.1%) in their diets at 0, 250 or 500 ppm (equal to 0, 21 and 43 mg/kg bw per day for males and 0, 24 and 48 mg/kg bw per day for females, respectively). Investigations included clinical signs, body weights and feed consumption measurements, haematology, clinical chemistry and urine analysis (at the end of the study), organ weights (adrenals, brain, heart, kidneys, liver, ovaries, spleen, testes, thymus and thyroid gland), gross necropsy and histopathological examination (full range of tissues from control and high-dose groups, plus lungs, liver, kidneys and gross lesions from the intermediate-dose group).

There was one accidental death, but there were no treatment-related mortalities or clinical signs of toxicity in any group. There were no effects on feed consumption, body weight gains or haematological parameters in any group. Clinical chemistry investigations at termination did not reveal any treatment-related effects, and no changes were observed in cholesterol levels in any group. There were also no effects on urine analysis parameters. Absolute liver and spleen weights were significantly higher in males treated at 250 ppm; however, relative weights were not significantly higher, and there was no similar effect at the higher dose (500 ppm). Relative heart weights were 12% lower than in controls in 500 ppm females (a statistically significant reduction); however, in the absence of any correlating histopathological findings in the heart (or a similar effect in high-dose males or animals treated at higher dose levels in the previous study), the change was considered to be a spontaneous finding and not toxicologically significant. There were no notable gross necropsy findings in any group, and histopathological examination did not reveal any lesions considered to be treatment related.

The NOAEL of the study was 500 ppm (equal to 43 mg/kg bw per day for males and 48 mg/kg bw per day for females), the highest dose tested (Fisher, 2001e).

Dogs

In a 4-week range-finding study, groups of two male and two female Beagle dogs received metrafenone (purity 97.1%) in their diets at a concentration of 0, 500, 1000, 10 000 or 20 000 ppm. As poor feed consumption with a corresponding decrease in body weight was observed at 10 000 and 20 000 ppm, the dietary administration was terminated after 4 days. All animals were returned to control diet until body weights and feed consumption values were comparable with control levels. The study was reinitiated, and metrafenone was administered to the same dogs via oral capsule (7 days/week) at a dose level of 12.5, 25, 250 or 500 mg/kg bw per day for a period of at least 28 days. Control animals received empty gelatine capsules. The dogs were observed daily for signs of overt toxicity, morbidity and mortality. Ophthalmological examinations were conducted prior to treatment and at termination. Detailed clinical observations were recorded weekly during the 28-day study period. Individual body weights and feed consumption were recorded weekly during the study period. Doses were adjusted weekly to compensate for changes in body weight. Samples for haematological, clinical chemistry and urine analysis examinations were collected from all surviving dogs pretreatment and at termination. At termination, all surviving animals were subjected to a gross necropsy, and selected organs were weighed. Samples of selected tissues from all surviving test animals were processed for histopathological evaluation.

There were no deaths and no notable ophthalmoscopic findings, and no clinical signs of toxicity were observed. Transient body weight loss (ranging from 1% to 10% of body weight) was recorded in both sexes during the first week of treatment at 500 mg/kg bw per day, but body weights recovered subsequently. There were no effects on body weights at lower dose levels or on feed consumption at any dose level. There were no effects of treatment on haematological, clinical chemistry or urine analysis parameters. The only notable organ weight findings were increased liver weights. At 500 mg/kg bw per day, the relative liver weights (mean of two animals) were 30% higher than those of controls for males or 22% higher than those of controls for females. At the lower dose levels, relative liver weights seemed to be increased as well, although to a lesser extent (17%, 8% and 21% in males and 4%, 5% and 11% in females for the 12.5, 25 and 50 mg/kg bw per day dose groups, respectively). Moreover, only two animals of each sex per dose were tested, and the male control values appeared to be somewhat low. There were no notable gross necropsy or microscopic findings in any group.

As the observed body weight effects were transient and minor (< 10%) and the liver weight increases were not accompanied by concomitant effects, the NOAEL was 500 mg/kg bw per day, the highest dose tested (Kelly, 1999).

In a 90-day toxicity study, groups of four male and four female Beagle dogs received metrafenone (purity 95.86%) via oral capsule at 0, 50, 100 or 500 mg/kg bw per day for 13 weeks. Haematology, blood biochemistry evaluations and urine analysis were performed for all animals prior to initiation and at monthly intervals. Gross necropsy and histopathological evaluations were performed for all animals at termination; organ weights were recorded for all animals.

All animals survived to study termination. Clinical observations, body weights, feed consumption, ophthalmoscopic examinations, haematology, clinical chemistry and urine analysis evaluations, and macroscopic and microscopic examinations did not reveal any adverse effects of treatment with metrafenone. Absolute and relative (to body weight and brain weight) liver weights were increased in male and female dogs that received 500 mg/kg bw per day. However, the finding was not associated with any microscopic findings in the liver (Table 13).

The NOAEL was considered to be 500 mg/kg bw per day, the highest dose tested; although increased liver weights were observed, they were not associated with any other histopathological or clinical chemistry liver effects (Kelly, 2001a).

Table 13. Liver weights obtained in a 13-week study in dogs given metrafenone by oral capsule

Dose (mg/kg bw per day)	Terminal body weight (kg)		Liver weights					
			Absolute (g) / % of control		Relative (%) to body weight / % of control		Relative (%) to brain weight / % of control	
	M	F	M	F	M	F	M	F
0	9.28	9.33	253	246	2.7	2.6	344	334
50	9.05	8.10	277 / 109	240 / 98	3.1 / 115	3.0 / 115	351 / 102	350 / 105
100	8.85	8.25	277 / 109	262 / 107	3.1 / 115	3.2* / 123	378/ 110	369 / 111
500	9.25	8.88	331* / 131	293 / 119	3.6* / 133	3.3* / 127	424* / 123	397 / 119

bw: body weight; F: female; M: male; *: $P < 0.05$

Source: Kelly (2001a)

In a 1-year toxicity study, groups of four male and four female Beagle dogs received metrafenone (purity 95.86%) via oral capsule at 0, 50, 150 or 500 mg/kg bw per day. Ophthalmological examinations were performed on all animals pretest and at termination. Haematology, blood biochemistry and urine analysis evaluations were conducted for all animals prior to treatment initiation and at weeks 13, 26 and 52. Gross necropsy and histopathological evaluations were performed for all animals at study termination; organ weights were recorded for all animals at termination.

Administration of metrafenone resulted in mild increases in mean alkaline phosphatase level in males dosed at 500 mg/kg bw per day after 9 and 12 months of dosing. Both absolute and relative liver weights were statistically significantly increased for 500 mg/kg bw per day females, and relative liver weights were statistically significantly increased for 500 mg/kg bw per day males. However, the recorded increases in liver weights did not show a clear dose–response relationship and did not worsen with the longer-term duration, compared with the 90-day study. Moreover, there were no associated macroscopic or microscopic findings. Therefore, the differences in liver weights and alkaline phosphatase levels (males) were not considered to be of toxicological significance (Table 14).

Table 14. Summary of liver results obtained in a 1-year study in dogs given metrafenone via oral capsule

Dose (mg/kg bw per day)	Alkaline phosphatase (U/L)					Liver weight	
	Pretest	3 months	6 months	9 months	12 months	Absolute (g) / % of control	Relative (%) to body weight / % of control
Males							
0	107	95	78	66	63	333	2.8
50	130	109	81	93	72	354 / 106	2.8 / 100
150	141	120	104	103	82	413 / 124	3.3 / 118
500	131	143	160	180*	143*	395 / 119	3.2* / 114
Females							
0	118	92	77	74	74	222	2.6
50	123	146*	120	152	128	324 / 146	3.5* / 135
150	144	147*	124	151	119	306* / 138	3.3 / 127
500	136	153*	134	136	161	335* / 151	3.5* / 135

bw: body weight; F: female; M: male; U: units; *: $P < 0.05$

Source: Kelly (2001b)

The NOAEL of the study was considered to be 500 mg/kg bw per day, the highest dose tested, because the increased alkaline phosphatase levels and absolute and relative liver weights were not accompanied by any histopathological changes in the liver (Kelly, 2001b).

(b) Dermal application

Rats

In a dermal 28-day study, groups of 10 male and 10 female Wistar (CrlGlxBrlHan:WI) rats were treated dermally with metrafenone (purity 94.2%) as a suspension in 0.5% carboxymethyl cellulose, at a dose level of 0, 100, 300 or 1000 mg/kg bw per day for 4 weeks (5 days/week). The volume of application was 4 mL/kg, and the duration of treatment was 6 hours daily. Observations included body weight and feed consumption measurements, daily clinical observations, detailed weekly clinical observations outside the cage in a standard arena, ophthalmological examinations (before treatment and at week 4) and haematology, clinical chemistry and urine analysis at termination. At the end of the study, the animals were terminated and examined for gross pathology. Absolute and relative organ weights were calculated. After necropsy, select organs and tissues of all animals were fixed and preserved; samples from all animals from the control and high-dose groups as well as all gross lesions in any rat were examined microscopically.

There were no systemic or local effects at any dose level. The NOAEL of the study was 1000 mg/kg bw per day, the highest dose tested (Kaspers, 2003a).

(c) Exposure by inhalation

No study was submitted.

2.3 Long-term studies of toxicity and carcinogenicity

The mechanism underlying the formation of liver tumours is considered under section 2.6(c). Clear NOAELs for liver and kidney effects and for increased incidence of liver tumours were established.

Mice

In a chronic toxicity and carcinogenicity study, groups of 65 male and 65 female CD-1 mice received metrafenone (purity 95.86%) in their diets at a concentration of 0, 250, 1000 or 7000 ppm for 18 consecutive months (mean substance intakes: 0, 39, 156 and 1109 mg/kg bw per day for males and 0, 53, 223 and 1492 mg/kg bw per day for females, respectively). The high-dose male group had 66 animals because of an early death and subsequent replacement. Samples were taken from 10 animals of each sex per dose at week 52 and week 78 for haematology. Clinical chemistry parameters were not assessed. At termination, all surviving animals were subjected to a gross necropsy, and selected organs were weighed. Gross necropsies were performed for all animals that died prior to scheduled termination (found dead or euthanized moribund). Samples of selected tissues were processed for histopathological evaluation from all surviving test animals and from any unscheduled deaths that occurred during the study. In the mid- and low-dose groups, histopathology was limited to lungs, liver, spleen, kidneys and gross lesions.

There was no adverse effect of treatment on survival. Survival at week 78 was 69%, 83%, 86% and 82% for males and 74%, 74%, 72% and 82% for females at 0, 250, 1000 and 7000 ppm, respectively (Table 15). Feed consumption values for both male and female mice at all dietary concentrations were generally comparable with or in excess of those of the control mice, and no adverse effects of treatment were evident from the body weight data. Body weights for males and females in all treated groups were generally similar to control values at most measurement intervals during the study. No treatment-related haematological changes were noted in either sex at either 12 months or study termination (18 months). Statistically significant increases in absolute and relative (to body weight) liver weights were noted for males at 7000 ppm (absolute weight: 136%; relative weight: 131%) and for females at 1000 and 7000 ppm (absolute weight: 116% and 154%, respectively; relative weight: 116% and 150%, respectively), when compared with controls. The only

Table 15. Overview of microscopic findings

Finding	Incidence of finding							
	Males				Females			
	0 ppm	250 ppm	1 000 ppm	7 000 ppm	0 ppm	250 ppm	1 000 ppm	7 000 ppm
Mortality								
No. surviving at week 78	45	54	56	54	48	48	47	53
% survival	69	83	86	82	74	74	72	82
No. of animals found dead	15	10	8	11	14	17	15	11
No. of animals euthanized moribund	5	2	2	1	3	0	3	1
Non-neoplastic								
No. examined	65	65	65	66	65	65	65	65
Liver – Centrilobular hepatocyte hypertrophy	0	0	15**	34**	0	0	0	5*
Minimal	0	0	13	8	0	0	0	5
Slight/mild	0	0	2	12	0	0	0	0
Moderate	0	0	0	13	0	0	0	0
Moderately severe	0	0	0	1	0	0	0	0
Liver – Diffuse hepatocellular hypertrophy	0	0	6*	20**	0	0	0	24**
Minimal	0	0	1	0	0	0	0	14
Slight/mild	0	0	5	11	0	0	0	9
Moderate	0	0	0	9	0	0	0	1
Kidney – Chronic nephropathy	42	52*	59**	64**	40	38	41	64**
Minimal	30	38	33	3	28	30	29	30
Slight/mild	8	12	23	39	5	3	5	22
Moderate	1	1	2	20	1	2	3	3
Moderately severe	1	1	1	1	0	2	3	4
Severe/high	2	0	0	1	6	1	1	5
Spleen – Extramedullary haematopoiesis	23	22	17	21	20	21[a]	26	32*
Minimal	6	4	8	7	7	7	9	8
Slight/mild	7	10	5	6	4	6	10	13
Moderate	9	8	3	4	8	6	5	9
Moderately severe	0	0	1	4	1	2	2	2
Severe/high	1	0	0	0	0	0	0	0
Neoplastic								
Liver – no. examined	65	65	65	66	65	65	65	65
Hepatocellular adenoma	4	2	8	14*	2	1	1	2
Hepatocellular carcinoma	2	1	1	5	0	0	0	2

Table 15 (continued)

Finding	Incidence of finding							
	Males				Females			
	0 ppm	250 ppm	1 000 ppm	7 000 ppm	0 ppm	250 ppm	1 000 ppm	7 000 ppm
Total (adenomas + carcinomas)	6	3	9	19**	2	1	1	4

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$

[a] Only 63 females examined instead of 65.

Source: Fischer (2002)

notable treatment-related gross necropsy finding was an increased incidence of grossly visible liver masses in males treated at 7000 ppm.

Non-neoplastic findings were noted in the liver, kidneys and spleen. In the liver, hepatocellular hypertrophy was recorded in males at and above 1000 ppm and in females at 7000 ppm. In males, this finding ranged from centrilobular to diffuse with dose-related severity, but in females, this finding was less severe and was mostly diffuse. Other histopathological findings in liver were limited in number and did not show any treatment-related increases (in particular, there were no increases in the incidences of findings such as necrosis or basophilic, clear cell or eosinophilic foci). In the kidneys, there was an increase in the incidence and severity of chronic nephropathy in males at 1000 ppm and higher and in females at 7000 ppm. Although there was a slight increase in the incidence of chronic nephropathy in males receiving 250 ppm compared with controls, this finding appeared to be due to the increased survival of animals in this group (53 mice) compared with the survival of the controls (45 mice). There was no difference in severity in males in this group compared with controls. In the spleen, there was an increased incidence of extramedullary haematopoiesis in females receiving 1000 and 7000 ppm, compared with the controls, although it reached statistical significance only at 7000 ppm; no increase in extramedullary haematopoiesis was noted in the spleens of treated male mice (Table 15).

A statistically significant increase in the incidence of hepatocellular neoplasms (adenomas and carcinomas) was observed in the livers of male mice receiving 7000 ppm (19/66, 29%), compared with controls (6/65, 9%). In male mice that died prematurely, one control group mouse was found with a hepatocellular carcinoma, and one mouse at each of 1000 and 7000 ppm was found with a hepatocellular adenoma. Thus, there was no indication of accelerated time to tumour formation in male mice. There was also a marginal increase in the incidence of hepatocellular neoplasms in females receiving 7000 ppm (4/65), compared with the control mice (2/65), but the increase was very small and was not statistically significant, and the time to tumour formation was not apparently shortened by treatment (based on the fact that no females dying prematurely had hepatocellular neoplasms) (Table 15).

Although the number of hepatocellular neoplasms in males receiving 1000 ppm was also slightly increased (9/65, 14%) compared with the untreated controls (6/65, 9%), this slight increase was not statistically significant, and it should be considered that survival was higher than that of controls in this group (56/65 survivors at 1000 ppm compared with 45/65) and almost all of these hepatocellular neoplasms were identified in animals at study termination.

Further statistical analyses of the liver tumours in male mice were performed by the sponsor, taking the differences in survival between the groups into account. The survival-adjusted methods used were Tarone's test and the Poly3 test, which are survival-adjusted quantal response procedures that modify the Cochran-Armitage linear trend test to take survival differences into account. When all male dose groups were analysed (total incidence of hepatocellular adenomas and carcinomas), there was a statistically significant difference between the control and the high-dose groups (Fisher's exact test), with significant linear trends by all three (Cochran-Armitage, Tarone's, Poly3) trend tests ($P \leq$

0.001). When the high-dose male group was dropped from the analysis, there were no significant differences and no significant trends across the groups to the 1000 ppm group. Therefore, based on these statistical analyses, 1000 ppm was considered to be the "no statistical significance of trend" dose. No further details on this statistical analysis were submitted.

The sponsor provided two sets of historical control data (HCD): one set from the performing BASF laboratory in Princeton, NJ, USA, and one set from Charles River (Giknis, 2005). Both sets do not meet the JMPR criteria for HCD, as most studies were not performed within 2 years of study initiation, and the performing laboratories or the strains of mice used were unknown. However, both sets of HCD indicate that male (CD-1) mice are more susceptible than female mice to spontaneous liver adenomas and carcinomas.

No clinical chemistry measurements were performed; hence, it cannot be excluded that the (histo)pathological effects observed in liver, kidney and spleen are accompanied by concomitant findings in blood parameters. The haematological measurements did not show an effect. Therefore, the NOAEL for systemic toxicity was 250 ppm (equal to 39 mg/kg bw per day for males and 53 mg/kg bw per day for females), based on increased incidence and severity of hepatocellular hypertrophy and chronic nephropathy in males and extramedullary haematopoiesis in the spleen and increased liver weights in females at 1000 ppm (equal to 156 mg/kg bw per day for males and 223 mg/kg bw per day for females).

Metrafenone showed carcinogenic potential in the liver of male mice treated for 78 consecutive weeks at 7000 ppm (equal to 1109 mg/kg bw per day, above the limit dose). The NOAEL for carcinogenicity was 1000 ppm (equal to 156 mg/kg bw per day for males) (Fischer, 2002).

Rats

In a combined chronic toxicity and carcinogenicity study in rats, groups of 75 male and 75 female Sprague-Dawley (Crl:CD (SD)IGS BR) rats (65 of each sex in main groups, 10 of each sex in satellite groups) received metrafenone (purity 95.86%) in their diets at a concentration of 0, 500, 5000 or 20 000 ppm for 104 weeks. Owing to a marked decrease in body weight gain (> 40%) and microscopic pathology findings in the liver observed at the 12-month interim kill (increased incidences of hepatocellular centrilobular necrosis [7/11 and 5/10 for females at 5000 and 20 000 ppm, respectively, compared with 1/12 in controls] and hepatocellular polyploidy [2/11 and 8/10 for females at 5000 and 20 000 ppm, respectively, compared with 0/12 in controls]), the dose concentration for the high-dose females was reduced from 20 000 to 10 000 ppm beginning with the first day of study week 69. Mean substance intakes were 0, 25, 260 and 1069 mg/kg bw per day for males and 0, 30, 320, 1419 (up to the end of week 68) and 593 mg/kg bw per day (weeks 72–104) for females. Clinical pathology studies (haematology, coagulation, clinical chemistry and urine analysis) were conducted at 3, 6, 12 and 18 months and at study termination. Neurobehavioural assessments (functional observational battery and motor activity measurement) were conducted on 10 animals of each sex per dose pretest and at week 12. After 12 months of treatment, 10 animals of each sex per dose were terminated, and after 24 months of treatment, all surviving animals were killed. Selected organs were weighed, and organ/body weight and organ/brain weight ratios were calculated. Complete macroscopic examinations and histopathological evaluation of selected tissues were conducted on all animals dying during the study and all control and high-dose animals. Histopathology was limited to lungs, liver, kidneys and gross lesions for animals of the low- and mid-dose groups.

Survival at termination was increased in a treatment-related pattern in females (presumably as a result of lower body weights in treated females). Survival at termination in males was slightly lower in treated animals, but there was no indication of a treatment-related effect, and it was considered to be adequate for assessing carcinogenic potential in males, as survival fell below acceptable levels only during the last month of the study. The timings of premature deaths did not indicate any effect of treatment. No treatment-related effects were observed in feed consumption data, clinical observations, ophthalmoscopic examinations, neurobehavioural evaluations (including functional observational battery and motor activity evaluations) and macroscopic examinations. Body weights and body weight

gains were statistically significantly reduced compared with controls at the end of the study in males at 20 000 ppm (90% and 86% of controls) and in females at 5000 ppm (82% and 74% of controls) and at 20 000/10 000 ppm (72% and 58% of controls). Body weight gains were significantly lower in males receiving 20 000 ppm from the start of the study, achieving a decrease of 10% relative to controls after week 64. Body weight gains were significantly lower in females receiving 20 000 ppm from the start of the study and were more than 10% lower than control values from week 2 onwards. A similar but less marked effect on body weight gains was seen in females at 5000 ppm, whose gains were consistently more than 10% lower than control values from approximately week 9. By week 68, body weights of top-dose females were only 70% of control values, and the top-dose level was subsequently reduced by half to 10 000 ppm, following which body weights did not decline any further relative to controls.

Haematology and coagulation revealed transient decreases in mean haemoglobin, haematocrit and/or erythrocyte values of females administered 5000 or 20 000/10 000 ppm, sometimes with corresponding decreases in mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration and increased platelet counts. Some transient prolongation of prothrombin time and of partial thromboplastin time was seen in animals treated at 5000 or 20 000/10 000 ppm. No differences in these parameters compared with controls were apparent at termination of the study.

Clinical chemistry parameters revealed mainly an increase in gamma-glutamyl transpeptidase, total and/or serum cholesterol, total protein and calcium and a decrease in total bilirubin in males and/or females receiving 5000 or 20 000/10 000 ppm. A minimal increase in blood urea nitrogen was seen in females dosed with 20 000 ppm at the end of 12 months of treatment.

Increased urinary protein levels were noted at urine analysis in high-dose females from 6 months onwards. There was no similar effect in males. A dose-related increase in urinary volume in males treated at and above 5000 ppm was also noted at termination.

Effects on serum liver enzymes, cholesterol and/or albumin in male and female animals treated at 5000 or 20 000/10 000 ppm correlated with dose-related increased liver weights (115–194% of controls), dose-related increased incidences of centrilobular hepatocellular hypertrophy and eosinophilic hepatocellular alteration, dose-related increased incidences of non-zonal vacuolation (females only) and basophilic hepatocellular alteration (females only) seen microscopically. The lesions in females were more severe than in males; in addition, the females had increased incidences of centrilobular necrosis and hepatocellular polyploidy. In addition to liver effects, males and females treated at 5000 or 20 000/10 000 ppm had increased kidney weights (108–173% of controls) and microscopically had a dose-related increased incidence of subacute/chronic interstitial inflammation/chronic nephropathy of the kidney. Effects in females treated at 20 000/10 000 ppm had the greatest severity, which correlated with increases in blood urea nitrogen and urinary protein levels (Table 16).

Table 16. Summary of microscopic findings in liver and kidney

	Incidence of finding							
	Males				**Females**			
Organ / histopathological finding	**0 ppm**	**500 ppm**	**5 000 ppm**	**20 000 ppm**	**0 ppm**	**500 ppm**	**5 000 ppm**	**20 000/ 10 000[a] ppm**
Non-neoplastic findings								
After 12 months (interim kill and decedents)								
Liver – number examined	14	12	12	10	12	10	11	10
Centrilobular hypertrophy	0	0	5	10	0	0	11	10

Organ / histopathological finding	Incidence of finding							
	Males				Females			
	0 ppm	500 ppm	5 000 ppm	20 000 ppm	0 ppm	500 ppm	5 000 ppm	20 000/ 10 000[a] ppm
Centrilobular necrosis	0	1	0	0	1	0	7	5
Hepatocellular polyploidy	0	0	0	0	0	0	2	8
Kidneys – number examined	14	12	12	10	12	10	11	10
Subacute/chronic interstitial inflammation/chronic nephropathy	6	5	7	8	2	0	7	10
After 24 months								
Liver – number examined	75	75	75	73	75	75	75	75
Centrilobular hepatocellular hypertrophy	1	2	15*	39*	0	1	42*	51*
Hepatocellular polyploidy	0	1	0	4	0	2	26*	54*
Hepatocellular alteration – basophilic	24	18	14	23	30	20	39	47*
Hepatocellular alteration – eosinophilic	6	8	12	14	3	4	27*	41*
Hepatocellular necrosis (centrilobular)	2	2	1	0	3	2	7	8
Hepatocyte cytoplasm vesicular / vacuolated	14	8	1	6	3	1	10	13
Bile duct(s) epithelial hyperplasia	37	33	29	37	26	28	44*	53*
Bile duct(s) dilated/cyst(s)	2	7	3	3	7	3	9	17*
Biliary fibrosis	16	18	11	12	12	13	13	21
Kidney – number examined	75	75	75	74	75	75	75	75
Subacute/chronic interstitial inflammation/chronic progressive nephropathy/cysts	45	48	59*	71*	18	21	54*	71*
Brown pigment in cortex/medulla tubular epithelium and/or reticuloendothelial cells	9	7	19	15	8	10	28*	41*
Neoplastic findings								
Liver – number examined	75	75	75	73	75	75	75	75
Hepatocellular adenoma	1	0	1	4	1	0	6	12*
Hepatocellular carcinoma	1	3	1	1	0	0	0	1

ppm: parts per million; *: $P < 0.05$

[a] Dose for high-dose females reduced from 20 000 ppm to 10 000 ppm from week 69.

Source: Kelly (2002)

A dose-related increase in the incidence of hepatocellular adenoma was recorded in females at 5000 and 20 000/10 000 ppm. One high-dose female had both a hepatocellular adenoma and a carcinoma (there were no hepatocellular carcinomas in any other female groups in this study or in 10 previous studies from the testing facility [1993–1998] cited as HCD). Of the 12 hepatocellular adenomas identified in the high-dose female group, two were identified at the interim kill after 12 months (Table 16).

There was a slightly, not statistically significantly increased incidence of benign hepatocellular adenoma for males treated at 20 000 ppm (4/73 or 5.5%). The increase was outside the mean and range of relevant HCD of the performing laboratory (mean 0.8%, range 0–1.9%). Based on this and the slight dose–response relationship, a relationship with treatment cannot be ruled out. The liver adenomas in males at 20 000 ppm can be considered an equivocal response.

The sponsor provided two sets of HCD: one set from the performing BASF laboratory and one set from Charles River (Giknis, 2001). Only three studies from the HCD of the performing laboratory meet the JMPR criteria for HCD, as most studies were not performed within 2 years of study initiation. Eleven studies from the Giknis & Clifford (2004) Charles River HCD were performed within 2 years of study initiation/termination; however, the performing laboratories were unknown. Therefore, this set of HCD is of less value. However, the sets of HCD indicate that male and female Sprague-Dawley rats are equally susceptible to spontaneous liver adenomas, but male rats tend to be slightly more susceptible than female rats to spontaneous liver carcinomas (Table 17).

Table 17. Historical control data

	Overall incidence	Range (%)	Range per study
HCD at the testing facility (1997–1998): three studies with Sprague-Dawley rats			
Males			
Hepatocellular adenoma	4/500 / 0.8%	0.0–0.8	0/60–1/52
Hepatocellular carcinoma	14/500 / 2.8%	0.0–1.6	0/15–1/52
Females			
Hepatocellular adenoma	4/489 / 0.8%	0.0–1.9	0/60–1/53
Hepatocellular carcinoma	0/489 / 0.0%	None in 3 studies	
HCD from the supplier (Charles River Laboratories): 11 control groups between 1997 and 2002 (Giknis & Clifford, 2004)			
Males			
Hepatocellular adenoma	15/865 / 1.7%	0.0–6.7	–
Hepatocellular carcinoma	21/865 / 2.4%	0.0–5.0	–
Females			
Hepatocellular adenoma	4/865 / 0.5%	0.0–1.7	–
Hepatocellular carcinoma	1/865 / 0.2%	0.0–1.7	–

HCD: historical control data

Treatment with metrafenone for 24 months resulted in markedly reduced body weight gains in females at 5000 ppm (26%) and 20 000/10 000 ppm (42%) and a lesser reduction in body weight gain in males at 20 000 ppm (14%). Microscopic effects in the liver and kidney were associated with clinical chemistry changes in males and females at and above 5000 ppm. Slight anaemia was observed in females at and above 5000 ppm, which was reversed at 24 months. An increased incidence of

hepatocellular necrosis after 12 months and of benign hepatocellular adenomas was observed in females treated at 5000 ppm and above and in males treated at 20 000 ppm.

The NOAEL for systemic toxicity was 500 ppm (equal to 25 mg/kg bw per day for males and 30 mg/kg bw per day for females), based on effects on body weight, liver and kidney at the lowest-observed-adverse-effect level (LOAEL) of 5000 ppm (equal to 260 mg/kg bw per day for males and 320 mg/kg bw per day for females). The NOAEL for carcinogenicity was 500 ppm (equal to 30 mg/kg bw per day for females), based on an increased incidence of hepatocellular adenomas at the intermediate and high dose levels in females, with an equivocal response in high-dose males (LOAEL of 5000 ppm, equal to 320 mg/kg bw per day for females) (Kelly, 2002).

2.4 Genotoxicity

The results of studies of genotoxicity with metrafenone are summarized in Table 18. All the studies were certified to comply with GLP and performed according to internationally accepted guidelines.

Table 18. Results of studies of genotoxicity with metrafenone

End-point	Test object	Concentration	Purity (%)	Result	Reference
In vitro					
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, T1537; *Escherichia coli* WP2 uvrA	25–5 000 µg/plate (±S9)	95.86	Negative	Wagner & Sly (1999)
Mammalian cell gene mutation	CHO cells, HGPRT locus assay	17–5 000 µg/mL (±S9)	95.86	Negative	Pant (2001)
Chromosome aberration assay	CHO cells	10–100 µg/mL (–S9) and up to 10–250 µg/mL (+S9)	95.86	Negative	Xu (2001a)
In vivo					
Mouse micronucleus	CD-1 mice	0, 500, 1 000 or 2 000 mg/kg bw (gavage)	95.86	Negative	Xu (2001b)
Unscheduled DNA synthesis	Male Wistar rats	0, 1 000 or 2 000 mg/kg bw (gavage)	99.4	Negative	Schulz (2006)

bw: body weight; CHO: Chinese hamster ovary; DNA: deoxyribonucleic acid; HGPRT: hypoxanthine–guanine phosphoribosyltransferase; S9: 9000 × *g* supernatant fraction from Aroclor 1254–pretreated or phenobarbital/β-naphthoflavone–induced rat liver homogenate

(a) In vitro studies

In a bacterial reverse mutation assay (Ames test), metrafenone (purity 95.86%) in dimethyl sulfoxide (DMSO) was tested in *Salmonella typhimurium* (strains TA98, TA100, TA1535 and TA1537) and *Escherichia coli* (WP2 uvrA) at up to 5000 µg/plate with and without S9 from Sprague-Dawley rats pretreated with Aroclor 1254. The confirmatory assay was repeated using the same method as per the first experiment (i.e. plate incorporation method). Precipitate was observed at and above 1000 µg/plate, but no cytotoxicity was observed up to 5000 µg/plate. The positive controls gave acceptable results. There were no increases in mutation frequency induced by the test material either with or without S9 for any strain in either experiment. Metrafenone was concluded to be non-mutagenic in this assay (Wagner & Sly, 1999).

In a mammalian cell gene mutation assay (HGPRT locus), cultured Chinese hamster ovary (CHO) cells were treated with metrafenone (purity 95.86%) in DMSO at concentrations ranging from 0.1 to 5000 µg/mL with and without S9 mix prepared from Aroclor 1254–pretreated Sprague-Dawley rats. Dosages for the mutation experiments (17, 50, 167, 500, 1667 and 5000 µg/mL) were based on the results of a range-finding toxicity screen, which showed relative cloning efficiencies (RCEs) ranging from 100% to 59% in the non-activated system and from 76% to 31% in the activated system.

In the initial mutation assay, RCEs were 105–56% and 96–60% with and without metabolic activation, respectively. The number of mutants in the treated cultures was very similar to the number in the solvent controls, both with and without metabolic activation. Occasional instances of increases over controls were less than 2-fold, which was the test laboratory criterion to consider a possible positive result. The positive controls (ethylmethanesulfonate and dimethylbenzanthracene) gave the expected results (242–335 mutants per 1×10^6 surviving cells). In the confirmatory assay, RCEs were 108–70% and 111–76% with and without metabolic activation, respectively. The number of mutants was again very similar between solvent control and treated cultures. The positive controls gave acceptable results. Metrafenone gave negative results in this assay (Pant, 2001).

Metrafenone (purity 95.86%) in DMSO was tested on CHO cells up to a concentration of 100 µg/mL in the absence of metabolic activation and of 250 µg/mL in the presence of metabolic activation to investigate clastogenic potential. Tested dose levels were based on a range-finding test (3 hours of exposure, harvest after 18 hours) in which reductions in mitotic index were recorded at 25–250 µg/mL without S9 and at 250 µg/mL with S9. Treatment with metrafenone was associated with a reduction in relative mitotic index of greater than 50% at and above 25 µg/mL without S9 and at and above 125 µg/mL with S9 in the initial assay and at and above 25 µg/mL in the confirmatory assay. There was no increase in the percentage of cells with aberrations at any of the concentrations tested, either with or without metabolic activation, in either assay. No more than one or two aberrations were recorded in any culture (treated or control); most contained no aberrations except for the occasional chromatid gap. The percentages of endoreduplicated and polyploid cells were also recorded separately, but the results were not significant. The positive controls gave the expected results. Metrafenone gave negative results in this assay (Xu, 2001a).

(b) In vivo studies

Groups of CD-1 mice (five of each sex per group) received a single oral dose of metrafenone (purity 95.86%) in DMSO at a dose level of 0, 500, 1000 or 2000 mg/kg bw (dosing volume 4 mL/kg bw) in order to assess the potential to induce micronuclei in bone marrow cells. The test animals were killed at approximately 24, 48 and 72 hours after dosing. The positive control group received a single oral gavage dose of cyclophosphamide at 80 mg/kg bw. Positive controls were included only in the 24-hour harvest. Metrafenone did not induce any increase in the incidence of micronuclei in male or female mice at any sampling time (Xu, 2001b). The results of the ADME study (see section 1.1) showed distribution of orally administered material to the bone marrow (see Tables 4 and 5; Mallipudi, 2002).

In an in vivo study, metrafenone was tested for its ability to induce deoxyribonucleic acid (DNA) repair synthesis (unscheduled DNA synthesis) in rat hepatocytes. Groups of three male Wistar rats received a single oral dose of metrafenone (purity 99.4%) in corn oil at 1000 or 2000 mg/kg bw. Negative control rats received corn oil only (10 mL/kg bw), and the positive control rats received 2-acetylaminofluorene at 50 mg/kg bw. Hepatocytes were harvested 3 and 14 hours after administration. Metrafenone did not lead to an increase in the mean number of net nuclear grain counts. Recorded values at both doses and time points were comparable with vehicle control values and within the historical control range. The induction of DNA repair by the positive control confirmed the sensitivity of the test method and procedures (Schulz, 2006).

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

In a dietary dose range–finding study for a two-generation study, groups of 10 male and 10 female Sprague-Dawley (Crl:CD® (SD) IGS BR) rats received metrafenone (purity 95.86%) in their diets at a concentration of 0, 500, 1000 or 10 000 ppm for a 10-week pre-pairing period and a mating period of up to 14 days, and then females were allowed to deliver and nurse litters over a 21-day lactation period. On day 4 of lactation, litters were standardized to eight pups (four of each sex where possible), and, following weaning, 10 pups of each sex per group were selected to continue on study for a 1-week period (F_1 generation). Investigations included clinical examinations, body weights, feed consumption, estrous cyclicity, reproductive parameters and litter parameters. At termination, a blood sample for haematological investigation was taken, followed by a gross necropsy, weighing of organs and microscopic examination of reproductive tissues, livers, spleens and gross lesions for the control and high-dose animals and liver and gross lesions only for the low- and mid-dose animals. Brain, liver, spleen and thymus weights were recorded for all F_1 pups (postnatal day [PND] 21 and PND 29), and livers from all 10 control and high-dose F_1 pups (10 of each sex per group) and spleens from five selected F_1 pups of each sex per group were examined microscopically at PND 29.

There were no effects on any reproductive parameters (i.e. estrous cycles, number of animals mating, males siring females or females becoming pregnant, mean number of days prior to mating, gestation length, number of pups per litter and live birth index, sex ratio or pup survival) up to 10 000 ppm (equal to 582–790 mg/kg bw per day for males and 882 mg/kg bw per day for females – pre-pairing period only).

Effects following treatment at 1000 ppm (equal to 55–76 mg/kg bw per day for males and 91 mg/kg bw per day for females – pre-pairing period only) were limited to a slight increase in absolute and/or relative (to body weight) liver weights in parental males and F_1 male and female pups terminated at PND 21 or 29. Microscopically, 3/10 parental males exhibited hepatocellular hypertrophy, whereas the livers of F_1 male and female pups terminated at PND 21 or 29 were unremarkable.

At 10 000 ppm, parental females had lower body weights and gained less weight (11% decrease) during the premating period, compared with controls. During gestation, body weights were statistically significantly lower than control values on days 0, 7 and 14 of gestation, but gains over the entire gestation period were comparable with control values. At termination, slight anaemia (decreases in haemoglobin, haematocrit and red blood cell counts) was observed in the parental females. Absolute and relative liver weights were increased in both male and female parental animals exposed to 10 000 ppm. Microscopic examination of the livers from male and female parental animals exposed to 10 000 ppm revealed centrilobular hepatocellular hypertrophy.

At 10 000 ppm, F_1 pup body weights were decreased at birth and throughout lactation. At weaning, pup body weights were decreased by 21% compared with controls. At PND 29, haematological effects, consisting of slight decreases in haemoglobin and haematocrit (females only) and red blood cell counts (both sexes), were observed in the F_1 pups. Liver weights (absolute and/or relative to body or brain weight) were increased in the F_1 pups at both PND 21 and PND 29. Microscopic examination of the livers from animals showed generalized to diffuse hepatocellular hypertrophy.

The NOAEL for systemic toxicity was 1000 ppm (equal to 55–76 mg/kg bw per day for males and 91 mg/kg bw per day for females during the premating period). The NOAEL for reproductive toxicity and fertility was 10 000 ppm (equal to 582–790 mg/kg bw per day for males and 882 mg/kg bw per day for females during the premating period), the highest dose tested (Schroeder, 2001).

In a two-generation study on reproductive toxicity, groups of 30 male and 30 female Sprague-Dawley (Crl:CD® (SD) IGS BR) rats received metrafenone (purity 95.86%) in their diets at a

concentration of 0, 500, 1000 or 10 000 ppm over the course of two generations (P and F_1). P generation animals were treated for a 10-week premating period prior to pairing. Animals were paired one male to one female for a mating period of a maximum of 14 days. Females delivered and nursed litters to weaning (litter size was standardized at four of each sex where possible on day 4 of lactation). At weaning of the F_1 generation, 30 animals of each sex per group were selected to form the F_1 parental generation. The treatment scheme was the same as that applied to the P generation. F_2 pups were reared to weaning. In-life investigations included clinical signs, body weights, feed consumption, estrous cyclicity, reproductive parameters and litter parameters. Developmental landmarks (vaginal opening and preputial separation) were recorded in F_1 animals, and anogenital distance was recorded for F_2 pups at birth. Haematology was conducted in all parental (P and F_1) animals and in one F_2 pup of each sex per litter at euthanasia. Reproductive tissues, adrenals, liver, spleen, pituitary and gross lesions were examined microscopically for all P and F_1 parental animals from the control and high-dose groups; examination of the liver was extended to all F_1 parental animals of the low- and mid-dose groups. Sperm evaluations were conducted in all parental males, and detailed ovarian examination and primordial follicle count were conducted in females of the control and high-dose groups for the P generation and in all F_1 females. Metrafenone intakes, as average combined premating values for P and F_1 males and females, were 0, 39, 79 and 811 mg/kg bw per day at 0, 500, 1000 and 10 000 ppm, respectively.

In the P generation, there was a single mortality (one male treated at 500 ppm), unrelated to treatment. There were no deaths among P females and no notable clinical signs of toxicity in either sex. Body weights of females at 10 000 ppm were up to 8% lower than those of controls over the second half of the premating period, with feed consumption also reduced. Body weight gains over the entire premating period were 18% lower in 10 000 ppm females than in controls. By the end of the lactation period, the body weights in the 10 000 ppm females were nearly 13% less than those of controls. There was no effect of treatment on estrous cyclicity, mating, fertility or fecundity indices or the time to mating in any group. The number of females delivering litters was 28, 26, 29 and 30 at 0, 500, 1000 and 10 000 ppm, respectively, with all animals identified as pregnant delivering live litters. There was no increase in the number of stillborn pups, and litter size both at birth and throughout lactation was comparable with that of controls in all treated groups. Haematology performed at termination of P animals revealed reduced erythrocyte counts, reduced haemoglobin and reduced haematocrit in animals at 10 000 ppm (both sexes, but more marked in females). There were no notable gross necropsy findings in P generation animals, but liver weights were found to be significantly increased in both sexes at 10 000 ppm (both sexes – relative weights 122–168% of controls). Other organ weight changes at 10 000 ppm included increased kidney weights (both sexes – relative weights 112–118% of controls), lower thymus weights (both sexes – relative weights 79–85% of controls) (Table 19), increased adrenal weights (females only – relative weights 125% of controls) and increased spleen weights (males only – relative weights 113% of controls). The changes in adrenal and spleen weights were not accompanied by any histopathological findings and were not considered to be toxicologically significant. The kidneys and thymus were not evaluated microscopically, and the toxicological significance of the weight change in these organs is unclear. At microscopic examination of P generation animals, minimal hepatocellular hypertrophy was identified in males (17/30 animals) and females (26/30 animals) at 10 000 ppm. There were no notable microscopic findings in other tissues (including reproductive tissues) in any group.

F_1 pup weights at 10 000 ppm were statistically significantly lower than those of controls both at birth and throughout lactation. At birth, the F_1 pups in this group weighed about 8% less than controls, and at weaning they were about 31% lower in weight. There was no effect of treatment on pup survival or sex ratio. Body weights of pups at 1000 ppm were statistically significantly lower than those of controls at weaning, but the differences from control were less than 10%. Body weights of females at 1000 ppm during the post-weaning period were also lower than those of controls, but by less than 10% and not statistically significantly. Among F_1 pups terminated at weaning, there were no treatment-related macroscopic or microscopic findings (only gross lesions were examined microscopically). Increased relative liver weights were recorded at 10 000 ppm (both sexes – relative 137–138% of controls) (Table 19). There were no other notable organ weight findings in F_1 weanlings. Sexual maturation, as indicated by vaginal opening, was delayed in females exposed to

Table 19. Organ weights in a two-generation reproductive toxicity study in rats

	0 ppm		500 ppm		1 000 ppm		10 000 ppm	
	M	**F**	**M**	**F**	**M**	**F**	**M**	**F**
P animals								
Liver weight								
Absolute (g)	21.29	11.97	21.96	12.76	22.35	12.89	26.16**	17.92**
Relative (%)	3.55	3.77	3.58	4.08**	3.63	4.17**	4.33**	6.35
Kidney weight								
Absolute (g)	4.43	2.50	4.43	2.55	4.52	2.54	4.98**	2.62
Relative (%)	0.74	0.79	0.73	0.82	0.74	0.82	0.83**	0.93**
Thymus weight								
Absolute (g)	0.47	0.37	0.46	0.30**	0.44	0.31**	0.37**	0.28**
F_1 generation, parental animals								
Liver weight								
Absolute (g)	24.18	12.75	25.11	13.76	23.76	13.95*	24.24	17.11
Relative (%)	3.63	3.76	3.87*	3.94	3.91**	4.08*	4.31**	6.18**
Kidney weight								
Absolute (g)	4.82	2.52	4.83	2.66	4.52	2.62	4.76	2.31
Relative (%)	0.73	0.74	0.75	0.76	0.75	0.77	0.85	0.94**
F_1 generation, pups at weaning								
Liver weight								
Absolute (g)	2.52	2.53	2.50	2.49	2.48	2.56	2.33	2.45
Relative (%)	4.73	5.00	4.89	5.22	5.00	5.37*	6.53**	6.83**
F_2 generation								
Liver weight								
Absolute (g)	2.56	2.51	2.45	2.36	2.60	2.54	2.33	2.23*
Relative (%)	4.50	4.68	4.75	4.87	4.82*	4.96	6.11**	6.24**

F: female; F_1: first filial generation; F_2: second filial generation; M: male; P: parental generation; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$

[a] Only 63 females examined instead of 65.

Source: Schroeder (2002)

10 000 ppm, likely as a secondary effect to the reduced body weights recorded in these animals rather than a specific effect.

In F_1 parental animals, the death of one male treated at 10 000 ppm on day 18 was attributed to treatment (microscopic examination identified severe atrophy of hepatocytes and hydronephrosis). One female at 500 ppm was killed in extremis and had an adenocarcinoma of the mammary tissue; in the absence of similar findings or mortality in females at higher dose levels, this finding was considered incidental. Body weights and body weight gains of males and females at 10 000 ppm were lower throughout the study period. Body weight gains of males (but not females) at 1000 ppm were also significantly lower than those of controls (statistically significant from week 6 onwards), and

overall (premating and post-mating) body weight gains were approximately 12% lower than in controls. There was no effect of treatment on estrous cyclicity, mating, fertility or fecundity indices or the time to mating in any group. The number of females delivering litters was 24, 26, 23 and 24 at 0, 500, 1000 and 10 000 ppm, respectively, and gestation length was comparable among groups. The number of live pups born per litter was slightly (but not statistically significantly) lower in the high-dose group, but litter size post-cull remained similar to control values. There was no effect of treatment on sex ratio or anogenital distance at birth.

Haematological investigations performed at termination revealed mild but statistically significant reductions in erythrocyte counts, haemoglobin and haematocrit in females at 10 000 ppm. Haematocrit only was slightly but statistically significantly lower in males at 10 000 ppm. Other haematological findings recorded at the high dose level were lower total leukocyte counts (females), lower neutrophil, monocyte and eosinophil (but not total leukocyte) counts (males) and increased platelet counts (females). There were no effects on haematological parameters at 1000 or 500 ppm. There were no notable macroscopic findings in F_1 parental animals, but relative liver weights were increased in animals receiving 10 000 ppm (both sexes – 119–164% of controls). Moreover, relative kidney weights were also increased in this top-dose group (both sexes – 116–127% of controls) (Table 19). At microscopic examination of F_1 parental animals, minimal hepatocellular hypertrophy was identified in males (17/29) and females (25/30) at 10 000 ppm (similar to the finding identified in the P generation). There were no notable microscopic findings in other tissues (including reproductive tissues) in any group.

At 10 000 ppm, a lower proportion of progressively motile sperm and an increase in the proportion of abnormal sperm were observed in F_1 males. The predominant sperm abnormality seen in the 10 000 ppm group was changes in the appearance of the sperm hook (either absence of hook or excessive hook). All other sperm parameters of the high-dose F_1 group were comparable with control values. A relationship with treatment is equivocal, as there were no corresponding effects in P males or accompanying effects on reproductive performance or histopathological findings in the testes and epididymides.

F_2 pup body weights at 10 000 ppm were lower than those of controls at birth and throughout lactation (approximately 10% lower at birth, increasing to approximately 32% lower at weaning). Haematological findings in randomly selected F_2 pups were restricted to longer activated partial thromboplastin times (males and females) and longer prothrombin time values (females only) at 10 000 ppm. These changes were not considered to be treatment related in the absence of similar findings in P and F_1 parental animals. Among F_2 weanlings, there were no treatment-related macroscopic or microscopic findings (only gross lesions were examined microscopically). Increased relative liver weights at 10 000 ppm were attributed to treatment (both sexes – 133–136% of controls). Spleen and thymus weights, absolute and relative to either body weight or brain weight, for the F_2 pups (males and females) at 10 000 ppm were statistically significantly lower than those of controls; this finding was considered to be related to the smaller size of these animals compared with controls. There were no other notable organ weight findings in F_2 weanlings.

The NOAEL for parental systemic toxicity was 500 ppm (equal to 39 mg/kg bw per day), based on effects on body weight gain in F_1 parental males at 1000 ppm (equal to 79 mg/kg bw per day).

The NOAEL for effects on reproductive parameters was 1000 ppm (equal to 79 mg/kg bw per day), based on an increased proportion of abnormal sperm in F_1 males at 10 000 ppm (811 mg/kg bw per day). There were no effects on reproductive performance at any dose level.

The NOAEL for effects on pups was 1000 ppm (equal to 79 mg/kg bw per day), based on adverse effects on pup weights and increased liver weights at 10 000 ppm (equal to 811 mg/kg bw per day) (Schroeder, 2002).

(b) Developmental toxicity

Rats

In a study of prenatal developmental toxicity performed in line with OECD Test Guideline 414 (adopted in 2001), groups of 25 presumed pregnant Sprague-Dawley (Crl:CD® (SD)IGS BR VAF/Plus®) rats were administered metrafenone (purity 95.86%) at a dose of 0, 50, 500 or 1000 mg/kg bw per day in 0.5% carboxymethyl cellulose via oral gavage on gestation day (GD) 6 to GD 20. In-life observations were clinical signs, body weights and feed consumption. All animals were killed on GD 21, and a blood sample was taken for haematological analyses. A caesarean section and gross necropsy were performed, with liver and gravid uterine weights recorded. Livers from control and high-dose animals were fixed and examined microscopically. The following reproductive parameters were recorded: number and distribution of corpora lutea, pregnancy status, number and distribution of implantations, live and dead fetuses, and early and late resorptions. Fetuses were weighed and examined for gross external alterations and sex. Approximately half of the fetuses in each litter were examined for soft tissue alterations by microdissection techniques. The heads of these fetuses were fixed and subsequently examined by free-hand sectioning. The remaining fetuses were examined for skeletal alterations after staining with Alizarin Red S. Dose levels were set following a pilot study (same dose levels, eight rats per group, external examination of fetuses only) in which no effects of treatment were identified at doses up to 1000 mg/kg bw per day.

There were no deaths and no notable clinical signs of toxicity related to the test substance administration. Body weight gains and feed consumption were similar between treated and control groups. Relative liver weights were higher than control values at 500 and 1000 mg/kg bw per day, attaining statistical significance at 1000 mg/kg bw per day only, but the increases were lower than 10% and were not considered to be toxicologically significant (relative liver weights were 99%, 103% and 106% of control values at 50, 500 and 1000 mg/kg bw per day, respectively). There were no effects of treatment on any haematological parameters. Histopathological examination of livers from control and high-dose animals did not reveal any notable findings. At termination, the number of rats that were pregnant in each group was 21, 23, 22 and 24 at 0, 50, 500 and 1000 mg/kg bw per day, respectively; all pregnant animals had viable fetuses, and there were no dead fetuses. The numbers of corpora lutea, implantation sites, live fetuses, early and late resorptions, percentage of resorbed conceptuses, percentage of male fetuses per litter and fetal weights were all similar between treated animals and controls. The number of fetuses with alterations was similar between groups, and there were no fetuses with alterations visible at gross external examination. The number and pattern of soft tissue alterations, skeletal alterations and ossification sites were very similar between all treated groups and the controls.

The NOAEL for both maternal toxicity and embryo/fetal toxicity was 1000 mg/kg bw per day, the highest dose tested. Metrafenone did not show teratogenic potential (Barnett, 2001a).

Rabbits

In a study of prenatal developmental toxicity performed in line with OECD Test Guideline 414 (adopted in 2001), groups of 25 presumed pregnant New Zealand White (Hra:(NZW)SPF) rabbits were dosed at 0, 50, 350 or 700 mg/kg bw per day with metrafenone (purity 95.86%) in 0.5% carboxymethyl cellulose via oral gavage on GD 6 to GD 28. In-life observations were clinical signs, body weights and feed consumption. All animals were killed on GD 29, and a blood sample was taken for haematological analyses. A caesarean section and gross necropsy were performed, with liver and gravid uterine weights recorded. Livers from all animals were fixed and examined microscopically. The following reproductive parameters were recorded: number and distribution of corpora lutea, pregnancy status, number and distribution of implantations, live and dead fetuses, and early and late resorptions. Fetuses were weighed, sexed and examined for gross external alterations. All fetuses were dissected to detect soft tissue alterations. The heads of half of the fetuses were processed for evaluation of soft tissue alterations by serial sectioning methods. The heads of the remaining fetuses were sectioned by a single mid-coronal incision for evaluation of the internal structure of the brain and then processed for evaluation of skeletal alterations. All fetuses were examined for skeletal

alterations following staining with Alizarin Red S. Dose levels were set following a pilot study (0, 50, 500 and 1000 mg/kg bw per day, five rabbits per group, external examination of fetuses only) in which abortions, clinical signs and reduced body weight gains at 1000 mg/kg bw per day were recorded.

A number of animals died or were killed prior to scheduled termination: three in the control group (one found dead on GD 15, 12 minutes after dosing, and thus considered likely related to an intubation error; one killed moribund on GD 21 because of an intubation error; and one aborting on GD 26), one treated at 350 mg/kg bw per day (which delivered on GD 29) and four treated at 700 mg/kg bw per day (one found dead on GD 10 because of an intubation error; one aborting on GD 28; and two delivering on GD 29). The deaths, moribund kills and abortions were considered unrelated to treatment, as they occurred in the vehicle control, were not dose related or were attributed to intubation errors. The early deliveries cannot be ruled out as effects of the test substance, because the incidences were dose dependent; however, all three does delivered at GD 29, on the day of scheduled termination. Other than clinical signs associated with abortions or intubation errors, the only clinical sign considered treatment related was scant faeces in animals at 700 mg/kg bw per day. Apart from findings associated with the intubation errors, there were no abnormalities detected at necropsy.

Lower maternal body weight gains were recorded at 350 and 700 mg/kg bw per day, with differences from control starting to become apparent around GD 11 and with some slight body weight losses recorded at 700 mg/kg bw per day in the last week of the study. Feed consumption was reduced at 350 and 700 mg/kg bw per day over the course of the study (reaching statistical significance only at 700 mg/kg bw per day at nearly all time points). Gravid uterine weights were also lower at 350 and 700 mg/kg bw per day (93% and 91% of control values, respectively) (Table 20).

Absolute and relative liver weights were significantly increased in a dose-related pattern at 350 and 700 mg/kg bw per day. The liver weight increases were accompanied by increased incidence and severity of periportal hepatocellular hypertrophy and diffuse hepatocellular cytoplasmic vacuolation at 350 and 700 mg/kg bw per day (dose-related patterns). Haematological investigations at termination revealed significantly increased prothrombin and activated partial thromboplastin times at 700 mg/kg bw per day.

The numbers of animals with litters at termination in each group were 20, 24, 23 and 19 at 0, 50, 350 and 700 mg/kg bw per day, respectively. There were no animals that had totally resorbed their litters, and there were no dead fetuses in the treated groups. Data from the animals that had delivered prematurely on day 29 were excluded from the litter observation data. The number of corpora lutea, implantation sites, live fetuses, early and late resorptions, percentage of resorbed conceptuses and percentage of male fetuses per litter were all similar between treated animals and controls. Fetal weights were slightly, but statistically significantly, reduced at 700 mg/kg bw per day. The number of fetuses with alterations was similar between groups. Gross external alterations were limited to one fetus in the controls (umbilical hernia) and one fetus at 700 mg/kg bw per day (short tail with fused caudal vertebrae and only 14 caudal vertebrae present identified at skeletal examination). The other malformations identified (absent kidneys in a single fetus at 50 mg/kg bw per day and malformations in the thoracic vertebrae of single control fetus) did not indicate a treatment-related teratogenic effect. The pattern of soft tissue and skeletal variations did not suggest any effect of treatment, as the fetal and litter incidences of findings were generally similar in treated and control groups (most findings occurred in only one or two fetuses or litters).

The average numbers of ossification sites per fetus were similar between treated and control groups; the only statistically significant increase was in forelimb phalange ossification sites (13.96, 13.99*, 14.00** and 14.00** at 0, 50, 350 and 700 mg/kg bw per day, respectively; *: $P < 0.05$; **: $P < 0.01$), but the increases were minimal and within the historical control range for the testing facility (average [1998–2000] 13.93; range 13.68–14.00) and therefore not considered to be toxicologically significant.

Table 20. Body weight effects in a developmental toxicity study in rabbits

Interval or parameter	Control	50 mg/kg bw per day	350 mg/kg bw per day	700 mg/kg bw per day
Maternal body weight (kg)				
GD 0	3.58	3.60	3.59	3.61
GD 6	3.65	3.68	3.66	3.69
GD 7	3.67	3.68	3.68	3.69
GD 8	3.67	3.69	3.69	3.69
Maternal body weight gain (kg)				
Days 6–9	0.03	0.02	0.04	0.00
Days 9–12	0.05	0.06	−0.01	0.03
Days 12–15	0.10	0.08	0.11	0.06
Days 15–18	0.06	0.04	0.03	0.03
Days 18–21	0.06	0.06	0.03	0.04
Days 21–24	0.10	0.07	0.07	−0.03**
Days 24–27	0.05	0.04	0.02	−0.01
Days 27–29	0.04	0.02	0.00	−0.01
Days 6–29	0.47	0.39	0.30**	0.15*
Days 0–29	0.54	0.47	0.37*	0.23*
Gravid uterine weight (g)	543.83	551.52	505.13	496.92
Corrected maternal body weight gain (kg)				
Days 6–29	−0.07	−0.16	−0.18	−0.24
Days 0–29	−0.00	−0.08	−0.11	−0.16
Live fetal weight (g)				
Fetal weight/litter	41.95	43.02	40.19	38.84*
Male fetuses	42.29	43.14	40.16	39.08
Female fetuses	41.34	42.90	40.10	38.87
Maternal feed consumption (g/day)				
Days 6–9	172.2	173.1	173.9	150.4
Days 9–12	171.0	172.0	152.6	148.8
Days 12–15	168.5	163.2	147.3	131.2**
Days 15–18	173.6	169.0	149.6	142.3*
Days 18–21	171.5	169.6	152.2	143.3*
Days 21–24	164.9	156.6	143.9	101.4**
Days 24–27	136.0	128.9	114.2	77.4**
Days 27–29	123.1	106.4	98.7	81.0
Days 6–29	160.8	157.5	143.4*	128.5**

bw: body weight; GD: gestation day; *: $P < 0.05$; **: $P < 0.01$

Source: Barnett (2001b)

The NOAEL for maternal toxicity was 50 mg/kg bw per day, based on lower body weight gains and feed consumption, increased liver weights and histopathological effects in the liver at 350 and 700 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 350 mg/kg bw per day, based on lower fetal weights at 700 mg/kg bw per day (Barnett, 2001b).

2.6 Special studies

(a) Neurotoxicity

In an acute neurotoxicity study, groups of five male and five female Wistar (CrlGlxBrlHan:WI) rats received a single oral dose of metrafenone (purity 94.2%) as a suspension in either doubly distilled water or 0.5% carboxymethyl cellulose at 0, 125, 500 or 2000 mg/kg bw. After unexpectedly low concentrations of metrafenone were measured in the doubly distilled water, the vehicle was changed, and doses were administered to further groups of five animals of each sex, resulting in groups consisting of 10 animals of each sex per dose. Doses were chosen on the basis of a peak range-finding study, in which no effects were observed at 2000 mg/kg bw. The animals were observed for up to 14 days after treatment. General observations were performed daily; body weight determinations, functional observational batteries and motor activity assessments were carried out on days −7, 1 (12.5 hours after treatment for functional observational batteries and 16.5 hours after treatment for motor activity assessment, based on toxicokinetic data indicating that C_{max} is attained at 14 and 15 hours for males and females, respectively), 7 and 14. Five animals of each sex per dose (those treated using 0.5% carboxymethyl cellulose as the vehicle) were fixed by in situ perfusion and subjected to neuropathological examinations. The remaining animals were euthanized under carbon dioxide anaesthesia without any further examination.

There was no mortality, and there were no clinical signs, treatment-related effects on body weight or treatment-related effects on functional observational battery or motor activity assessments. Neuropathological investigations did not show any treatment-related findings. The NOAEL for acute neurotoxicity was 2000 mg/kg bw, the highest dose tested (Kaspers, 2003b).

In a repeated-dose neurotoxicity study, groups of 10 male and 10 female Wistar (CrlGlxBrlHan:WI) rats received metrafenone (purity 94.2%) in their diets at 0, 1500, 5000 or 15 000 ppm (equal to 0, 143, 459 and 1371 mg/kg bw per day for males and 0, 152, 493 and 1371 mg/kg bw per day for females, respectively) for 4 weeks. The study was performed in accordance with OECD Test Guideline 424. Feed consumption was determined once a week. Body weights were recorded weekly and on the days when functional observational batteries and motor activity assessments were carried out (days −1 and 27). Detailed clinical examinations in an open field were conducted prior to the start of dosing and weekly thereafter, with the exception of the last week. At the end of the study, the first five animals of each sex per dose were killed by perfusion fixation and subjected to neuropathological examinations. The remaining animals were euthanized under carbon dioxide anaesthesia without any further examination.

All rats survived to scheduled termination. No specific signs of systemic toxicity or neurotoxicity were noted. Piloerection was observed in two males and one female treated at 5000 ppm (by day 21) and in three males treated at 15 000 ppm (by day 16, and also noted at functional observational battery performed on day 27). In addition, red discoloured urine was noted in one female treated at 5000 ppm (by day 15) and in five females treated at 15 000 ppm (by day 16). Body weight, body weight gain and feed consumption were affected by treatment at 15 000 ppm. Mean body weights of males were significantly reduced at days 7 and 14 (up to −8.5%); body weight gains were significantly reduced in males at days 7, 14 and 21 and in females at days 14 and 28, and feed consumption was significantly reduced in males at days 7 and 14 and in females throughout the entire study.

Several deviations from “zero value” were noted in the functional observational batteries; however, as most of these were equally distributed between the treated and control groups, were without a dose–response relationship, were observed in single animals or were observed before the

start of treatment, they were considered to be incidental and not related to treatment. The same applied to motor activity assessments, which showed no effect on the overall activity, but significantly decreased values at intervals 10 and 11 in females treated at 1500 ppm and at intervals 1 and 3 in females treated at 15 000 ppm, without any dose–response relationship. Neuropathological investigations showed no differences between the treated and control groups. Axonal degeneration (grade 1) was observed in the peripheral nerves of one control male and in one male and one female of the high-dose group and was considered unrelated to treatment.

The NOAEL for systemic toxicity was 1500 ppm (equal to 143 mg/kg bw per day for males and 152 mg/kg bw per day for females), based on clinical signs (piloerection and red discoloured urine) at 5000 ppm (equal to 459 mg/kg bw per day for males and 493 mg/kg bw per day for females). The NOAEL for neurotoxicity was 15 000 ppm (equal to 1371 mg/kg bw per day for males and females), the highest dose tested (Kaspers, 2003c).

(b) Immunotoxicity

The immunotoxic potential of metrafenone was investigated in female Wistar (Crl:WI(Han)) rats. The study was reported to comply with GLP and performed according to internationally accepted guidelines (USEPA Test Guideline OPPTS 870.7800). Groups of eight female Wistar rats received metrafenone (purity 94.2%) in their diets at 0, 1000, 4000 or 12 000 ppm (equal to 0, 80, 315 and 1086 mg/kg bw per day, respectively) for 4 weeks. A similar sized positive control group was treated with cyclophosphamide monohydrate at 4.5 mg/kg bw per day by oral gavage (drinking-water as vehicle). All animals were immunized 6 days before blood sampling and necropsy using 0.5 mL of sheep red blood cells (4×10^8 sRBCs/mL) administered by intraperitoneal injection. Feed consumption was determined once a week, and body weight was determined twice 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 treatment and weekly thereafter. Enzyme-linked immunosorbent assay was used to measure sRBC immunoglobulin M (IgM) antibody titres at study termination. In addition, animals were subjected to a gross examination, with liver, spleen and thymus weights recorded.

No mortality and no signs of toxicity were noted. Treatment with metrafenone at concentrations up to and including 12 000 ppm had no impact on body weight or feed consumption, whereas that with the positive control (cyclophosphamide) caused a consistently reduced feed intake (attaining statistical significance on day 28), significantly impaired body weights at days 14 (−5.18%) and 28 (−8.62%) and significantly reduced body weight gains (maximum −23.59% at day 28). No changes in the sRBC IgM titres were found in female rats treated with up to 12 000 ppm of metrafenone, whereas the sRBC titres were lower in rats of the positive control group. At necropsy, liver changes were observed only in females treated at 12 000 ppm. Organ weight data showed increased absolute and relative liver weights in animals treated at 4000 and 12 000 ppm and significantly decreased absolute and relative spleen and thymus weights in the positive control animals.

Metrafenone did not reveal any signs of immunotoxicity when administered via the diet over a period of 4 weeks to female Wistar rats. Thus, the NOAEL for immunotoxicity was 12 000 ppm (equal to 1086 mg/kg bw per day), the highest dose tested. The NOAEL for systemic toxicity was 1000 ppm (equal to 80 mg/kg bw per day), based on significantly increased absolute and relative liver weights observed at 4000 ppm (equal to 315 mg/kg bw per day). The sensitivity of the assay was confirmed by results obtained in animals treated with the positive control cyclophosphamide (Buesen, 2010).

(c) Mechanistic studies

In a study aimed at determining whether metrafenone can induce liver enzyme activities or peroxisome proliferation, groups of five male and five female Sprague-Dawley (Crl:CD) rats received metrafenone (purity 95.86%) in their diet at 0 or 20 000 ppm (equal to 0 and 1526 mg/kg bw per day for males and 0 and 1654 mg/kg bw per day for females, respectively) for 4 weeks. Investigations

included clinical signs, body weights and feed and water consumption. At the end of the study, animals were killed, and livers were perfused with 0.9% sodium chloride solution via the portal vein. The following parameters indicative of peroxisome proliferation or liver enzyme induction were analysed:

- cyanide-insensitive palmitoyl-coenzyme A oxidation in liver homogenate;
- glutathione (GSH) concentration in liver homogenate;
- ethoxyresorufin *O*-deethylase (EROD) activity in S9 fraction; and
- pentoxyresorufin *O*-dependentylase (PROD) activity in S9 fraction.

There were no deaths and no clinical signs of toxicity. Water consumption was significantly increased in males at day 21 and was also increased, but to a lesser extent (and not significantly), at day 28 (prior to day 21, water bottles had been observed visually only; as a result of obviously increased water consumption, weighing of water bottles was commenced on day 21). Feed consumption was significantly lower in treated females on days 7, 21 and 28. Decreased body weight gains were recorded in treated animals, such that after 4 weeks, body weight gains were 10% or 19% lower than control values in males and females, respectively.

PROD was significantly increased in females, but the increase was less than 2-fold. EROD was significantly increased in males (17-fold increase over controls) and females (10-fold increase over controls). There was no effect of treatment on palmitoyl-coenzyme A or GSH.

It can be concluded that metrafenone induces liver enzymes of the cytochrome P450 family in Sprague-Dawley rats following treatment at a high dose (> 1000 mg/kg bw per day) via the diet for 4 weeks (Mellert, 2002a).

To provide positive control data for the previous study (Mellert, 2002a), groups of five male and five female Sprague-Dawley (Crl:CD) rats received phenobarbital in their diet at 0 or 500 ppm (equal to 0 and 38.9 mg/kg bw per day for males and 0 and 42.6 mg/kg bw per day for females, respectively) for 4 weeks. Investigations included clinical signs, body weights and feed and water consumption. At the end of the study, animals were killed, and livers were perfused with 0.9% sodium chloride solution via the portal vein. The following parameters were analysed:

- cyanide-insensitive palmitoyl-coenzyme A oxidation in liver homogenate;
- GSH concentration in liver homogenate;
- cytochrome P450 microsomal content;
- EROD activity in S9 fraction; and
- PROD activity in S9 fraction.

There were no deaths and no clinical signs. Feed consumption was slightly, but statistically significantly, higher in males over the first 2 weeks of the study. Body weight gains of males and females were significantly higher in the first week and remained higher (but not significantly) in males over the remainder of the study.

There were marked increases in cytochrome P450 content (89% in males and 66% in females) in the liver. PROD activity was statistically significantly increased in both sexes (18-fold increase in males and 25-fold increase in females). Additionally, EROD activity was slightly, but statistically significantly, increased (2.8-fold) in males, with corresponding females showing a slight, non-statistically significant increase (1.4-fold), which was considered to be biologically significant. No effects on palmitoyl-coenzyme A oxidation or GSH concentration were observed in either sex treated with phenobarbital.

Administration of phenobarbital in the diet at 500 ppm for 4 weeks was associated with increases in cytochrome P450 content and EROD and PROD activities. The methodology used in the previous enzyme induction assay with metrafenone is therefore validated (Mellert, 2002b).

The proliferation of liver cells following treatment with metrafenone was evaluated in rats. Groups of eight male and eight female Sprague-Dawley (Crl:CD (SD)IGS BR) rats received metrafenone (purity 95.86%) in their diets at 0, 500 or 20 000 ppm (approximately equal to 0, 30 and 1000 mg/kg bw per day, respectively) over a period of either 1 or 4 weeks. A further group received a dose of 20 000 ppm in the diet for 4 weeks and was then maintained on control diet for a 2-week recovery period. In-life investigations included clinical signs, body weights and feed and water consumption. Seven days prior to termination, osmotic minipumps containing 5-bromo-2′-deoxyuridine (BrdU) were implanted subcutaneously. At termination, a gross necropsy was performed, and livers were weighed and prepared for both histopathological examination (H&E staining) and immunohistochemistry (BrdU staining for cell proliferation [DNA synthesis] and terminal deoxynucleotidyl transferase dUTP nick end labelling [TUNEL] stain for apoptotic cells).

There were no deaths considered to be treatment related and no clinical signs of toxicity. Significantly increased water consumption and significantly decreased feed consumption were recorded in animals treated at 20 000 ppm. Significantly lower body weight gains were also recorded in these animals, mainly in week 1. Females in the 20 000 ppm dose group even lost weight after 1 week. There were no notable gross necropsy findings. Liver weights were significantly increased in both sexes at 20 000 ppm. Relative (to body weight) liver weights were 17% higher (after 1 week of treatment) or 15% higher (after 4 weeks of treatment) than those of controls in males and 48% higher (after 1 week) or 65% higher (after 4 weeks) than those of controls in females. After 1 week of treatment, centrilobular hypertrophy of hepatocytes (minimal to slight) was identified in all males and in 5/7 females at 20 000 ppm. After 4 weeks of treatment, centrilobular hypertrophy of hepatocytes (minimal to moderate) was identified in all males and in 7/8 females at 20 000 ppm. Two high-dose females also showed minimally or moderately increased numbers of mitotic figures in the periportal region after 4 weeks of treatment. No hypertrophy or increased mitosis was identified in animals treated at 20 000 ppm following the 2-week recovery period. There were no effects on liver weight or liver histopathology at 500 ppm.

After 1 week of treatment at 20 000 ppm, there were marked significant increases in cell proliferation (as measured by BrdU index) in both males and females. Females were affected to a greater extent, and most of the increased activity was located in the periportal zone. After 4 weeks of treatment, there was no clear increase in BrdU index at either dose level in males. In females, there were significant increases at 20 000 ppm (again, mostly in the periportal zone), but the increases were of much smaller magnitude than after 1 week of treatment (Table 21).

In both males and females after the 2-week recovery period following the 4-week treatment at 20 000 ppm, there was a significant decrease in BrdU index relative to the control activity recorded at 4 weeks. The decrease was more marked in females, and in both sexes the largest decrease occurred in the periportal zone (which had previously shown the highest increases) (Table 21).

The apparent significant increase in BrdU index relative to controls in females treated at 500 ppm after 1 week was considered to be due to an unusually low control value in zone 1. Available HCD from 12 studies in 20 groups of female Wistar rats aged between 11 and 19 weeks for BrdU index in zone 1 ranged between 0.47 and 12.44, with a mean of 3.94 (standard deviation ± 2.48) and a median of 3.65. Although no specific data for female Sprague-Dawley rats were available, the control value in the current study is lower than the historical control values at week 1, whereas it was within the historical control range at week 4. For this reason, the statistically significant increase was not considered to be of biological relevance; the slight but significant decrease recorded in females treated at 500 ppm at week 4 was also not considered biologically relevant (Table 21).

Table 21. S-phase liver response (absolute and relative) following treatment with metrafenone in the diet for 4 weeks

		Zone 1		Zone 2		Zone 3		All zones	
Treatment period	**Test group**	**%**	**LI**	**%**	**LI**	**%**	**LI**	**%**	**LI**
Males									
1 week	Control	100	1.77	100	1.47	100	0.95	100	1.40
	500 ppm	108	1.92	97	1.43	61	0.58	94	1.31
	20 000 ppm	645**	11.41	214	3.14	62	0.59	361**	5.05
4 weeks	Control	100	0.55	100	1.87	100	0.10	100	0.84
	500 ppm	93	0.51	128	2.40	160	0.16	122	1.02
	20 000 ppm	120	0.66	80	1.50	60	0.06	88	0.74
4 weeks with 2 weeks' recovery	20 000 ppm	33**[a]	0.18	111	2.07	40	0.04	91*	0.76
Females									
1 week	Control	100	0.17	100	2.71	100	0.63	100	1.17
	500 ppm	394**	0.67	100	2.70	81	0.51	111	1.29
	20 000 ppm	9 559**	16.25	241**	6.52	22**	0.14	653**	7.64
4 weeks	Control	100	2.70	100	1.69	100	1.54	100	1.98
	500 ppm	69*	1.87	71	1.20	47*	0.73	64	1.27
	20 000 ppm	418**	11.29	111	1.88	117	1.80	252**	4.99
4 weeks with 2 weeks' recovery	20 000 ppm	7**[a]	0.19	8**	0.13	20**	0.31	11**	0.21

LI: 5-bromo-2′-deoxyuridine labelling index; ppm: parts per million; Zone 1: periportal zone; Zone 2: intermediate zone between zones 1 and 3; Zone 3: centrilobular zone; * P: < 0.05; ** P: < 0.01

[a] Compared with the 4-week controls.

Source: Mellert (2002c)

Very low numbers of positively labelled hepatocytes with TUNEL staining were recorded in control males after 1 week. By comparison with the control values after 4 weeks, it can be concluded that there was no clear effect of treatment on the number of apoptotic cells in any male group. Comparison with the 4-week control values was considered acceptable, as the values in control males after 4 weeks were similar to the control values in females, and the female values did not show any differences between 1 and 4 weeks. Therefore, the 1-week male control values appeared to be atypical. Among the female groups, there was a clear, significant increase after 4 weeks at 20 000 ppm (mostly in zones 1 and 2), and a smaller increase was still apparent in zone 1 after the 2-week recovery period.

Treatment with metrafenone at 20 000 ppm (approximately 1000 mg/kg bw per day) was associated with the following effects on the liver:

- Liver weights were increased after both 1 and 4 weeks of treatment. Females were affected to a greater extent than males, and in females only the increase after 4 weeks was larger than after 1 week. Liver weight increases were fully reversible after 2 weeks of recovery.
- Centrilobular hypertrophy was noted after both 1 and 4 weeks of treatment. Males were affected to a greater extent than females in terms of incidence, but not severity (all males affected after 1 week, in contrast to females, for which incidence increased with increasing duration). This effect was also reversible following 2 weeks of recovery. In addition to

centrilobular hypertrophy, minimally or moderately increased numbers of mitotic figures were identified in the periportal region of two females after 4 weeks of treatment. No such finding was identified after 2 weeks of recovery.

- Cell proliferation was significantly increased in both males and females after 1 week of treatment, but only in females after 4 weeks, and the magnitude of the increase was smaller. Females were affected to a greater extent than males in terms of magnitude of effect after 1 week, and increased cell proliferation was identified mostly in the periportal region. After the 2-week recovery period, there was a significant decrease recorded in cell proliferation.
- The number of apoptotic liver cells was increased after 4 weeks (but not after 1 week) in females (but not males), mostly in the periportal and intermediate zones (Mellert, 2002c).

To evaluate the initiation potential of metrafenone, groups of 12 male and 12 female partially hepatectomized (14 hours earlier) Sprague-Dawley (Crl:CD (SD)IGS BR) rats received a single oral dose of metrafenone (purity 95.86%) in double-distilled water by gavage at 5000 mg/kg bw. The positive control for initiation, nitrosomorpholine (NNM), was administered as a single dose by oral gavage at 50 mg/kg bw in double-distilled water. After a 2-week recovery period, phenobarbitone (PB) was given as a known promoter at a dietary concentration of 500 ppm for 8 weeks. Negative control groups were included and consisted of untreated animals or animals treated with PB only. At termination, a gross necropsy was performed, and liver sections were prepared for histopathology with H&E staining and for staining for glutathione *S*-transferase placental form (GST-P). GST-P positive foci, considered as preneoplastic lesions, were evaluated quantitatively (foci/cm^2 of live tissue).

Several animals from different groups died during or shortly after partial hepatectomy (no more than one or two from each group). There were no treatment-related deaths, no clinical signs and no effects of metrafenone treatment on body weights or feed consumption (NNM adversely affected body weight gains and feed consumption).

In the positive control groups (NNM with and without subsequent PB treatment), a significantly increased number of GST-P positive foci was observed when compared with the corresponding control groups, confirming the known initiating capacity of NNM.

After treatment with metrafenone, the number of GST-P positive foci per square centimetre was very low, and there were no significant differences from the corresponding controls. In addition, the ratio of the foci area to the total liver area did not show significant differences between treatment groups and the corresponding control groups.

The results of this study indicate that a very large single dose of metrafenone does not initiate the formation of foci of cellular alteration in rat liver. This includes both foci that could be identified by histopathology following H&E staining and GST-P positive foci identified by immunohistochemistry. No increase in foci was recorded in metrafenone-treated animals compared with appropriate controls (Mellert, 2002d).

(d) Impurity

A technical impurity of metrafenone, Reg. No. 4087263 (purity 97.7%), was tested in an Ames test. The study consisted of a standard plate test and preincubation test, both with and without metabolic activation (Aroclor-induced rat liver S9 mix). *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* strain WP2 uvrA were exposed to the impurity dissolved in DMSO at a dose of 0, 20, 100, 500, 2500 or 5000 µg/plate (standard plate test) or a dose of 0, 4, 20, 100, 500 or 2500 µg/plate (preincubation test).

Precipitation of test material was observed at and above 500 µg/plate. A slight bacteriotoxic effect was recorded at and above 2500 µg/plate. The positive controls gave acceptable results. There were no increases in mutation frequency induced by the test material either with or without S9 for any

strain in either experiment. The impurity Reg. No. 4087263 was non-mutagenic in this assay (Engelhardt, 2002).

3. Observations in humans

As indicated by the sponsor, medical data on metrafenone 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. As there are no specific parameters available for monitoring the effects of metrafenone, the medical monitoring programme is designed as a general health checkup, with special interest in the primary target organs presumed to be relevant by analogy from animal experiments. The surveillance programme includes a general physical examination, including neurological status, red and white blood cell counts and serum liver enzyme levels. Adverse health effects suspected to be related to metrafenone exposure have not been observed, and no associations of adverse health effects in the course of production, transportation, formulation or packaging of metrafenone have been reported or documented in the BASF internal medical files.

No data on exposure of the general public or epidemiological studies are available to BASF SE, nor is BASF SE aware of any epidemiological studies performed by third parties. There is no evidence or data available to support any findings in relation to poisoning with metrafenone. According to the Proposed Registration Decision of the Pest Management Regulatory Agency of Canada dated 26 April 2013, there were no incident reports from Canada or the USA as of 30 January 2013 for products containing metrafenone.

Comments

Biochemical aspects

Following gavage dosing in rats, absorption of metrafenone was rapid and complete (> 88%) at the low dose of 10 mg/kg bw but was limited to 15–20% at the high dose of 1000 mg/kg bw, suggesting saturation of the absorption processes. Metrafenone was widely distributed in the body, with highest residue levels found mainly in the gastrointestinal tract, liver and fat. There was no evidence of accumulation. The labelled material was relatively rapidly excreted into the gastrointestinal tract via the bile (85–90%), resulting in extensive excretion via faeces. Excretion via urine was relatively low at 10 mg/kg bw (1–3%), and even lower at the high dose of 1000 mg/kg bw (< 1%). Metrafenone was extensively metabolized, with most of the labelled products excreted as glucuronic acid conjugates in bile and urine. Five possible sites of conjugation with glucuronic acid were identified, following *O*-demethylation of the molecule. Residues in faeces consisted primarily of parent compound and the aglycones of bile and urine conjugates. The transformation steps included:

- *O*-demethylation of the aromatic methoxy group(s) followed by mono-*O*-glycosidation;
- hydroxylation of the bromophenyl ring; and
- hydroxylation of the methyl substituent to hydroxymethyl followed by *O*-glycosidation or further oxidation to aldehyde or lactone.

The bond between the bromophenyl ring and trimethoxyphenyl ring remained intact.

Toxicological data

Metrafenone has low acute toxicity when administered orally and dermally (LD_{50}s > 5000 mg/kg bw) and via inhalation (LC_{50} > 5 mg/L) to rats. No studies on skin and eye irritation or skin sensitization were available.

The major target organ for toxicity was the liver in short-term and long-term studies in mice and rats. Increased liver weight was the most common finding. Hepatocyte vacuolation was observed in rats, and hepatocyte hypertrophy was observed in mice. In the long-term studies, the kidneys were

also a target organ in rodents, as chronic nephropathy was observed (with or without increased kidney weights). Chronic nephropathy observed only in male rats would not normally be considered relevant for a human risk assessment; however, this finding was observed in mice and both sexes of rats and therefore was considered relevant for the risk assessment.

In two 90-day studies of toxicity, mice were treated with metrafenone either at a dietary concentration of 0, 1000, 3500 or 7000 ppm (equal to 0, 163, 622 and 1206 mg/kg bw per day for males and 0, 216, 788 and 1663 mg/kg bw per day for females, respectively) or at a dietary concentration of 0, 250 or 500 ppm (equal to 0, 42 and 84 mg/kg bw per day for males and 0, 55 and 113 mg/kg bw per day for females, respectively). The overall NOAEL was 1000 ppm (equal to 163 mg/kg bw per day), on the basis of liver effects (increased total bilirubin, increased liver weight and centrilobular hepatocellular hypertrophy) observed at 3500 ppm (equal to 622 mg/kg bw per day).

In two 90-day studies of toxicity, rats were treated with metrafenone either at a dietary concentration of 0, 1000, 5000, 10 000 or 20 000 ppm (equal to 0, 79, 404, 800 and 1663 mg/kg bw per day for males and 0, 94, 486, 967 and 1938 mg/kg bw per day for females, respectively) or at a dietary concentration of 0, 250 or 500 ppm (equal to 0, 21 and 43 mg/kg bw per day for males and 0, 24 and 48 mg/kg bw per day for females, respectively). The overall NOAEL was 1000 ppm (equal to 79 mg/kg bw per day), on the basis of effects on liver (increased cholesterol and total protein, increased liver weight and periportal hepatocellular vacuolation) noted at 5000 ppm (equal to 404 mg/kg bw per day). This overall NOAEL was supported by findings in a 28-day dose range–finding study.

In a 90-day study, dogs received metrafenone via oral capsule at 0, 50, 100 or 500 mg/kg bw per day. In a 1-year study, dogs received metrafenone via oral capsule at 0, 50, 150 or 500 mg/kg bw per day. The overall NOAEL was 500 mg/kg bw per day, the highest dose tested, because minor changes in liver weight and/or clinical chemistry parameters were not accompanied by any microscopic abnormality and were therefore not considered adverse. This overall NOAEL was supported by findings in a 28-day dose range–finding study.

In an 18-month dietary toxicity and carcinogenicity study, mice were treated with metrafenone at 0, 250, 1000 or 7000 ppm (equal to 0, 39, 156 and 1109 mg/kg bw per day for males and 0, 53, 223 and 1492 mg/kg bw per day for females, respectively). Significant treatment-related effects were observed in the liver, kidneys and spleen at 1000 ppm (equal to 156 mg/kg bw per day) and above. Increased incidence and severity of extramedullary haematopoiesis in the spleen of female mice were recorded. Increased incidence and severity of chronic nephropathy in the kidneys (more severe in males) were recorded. Liver effects included increased weights (more severe in females) and increased incidence and severity of centrilobular and diffuse hepatocellular hypertrophy (more severe in males). The NOAEL for chronic toxicity was 250 ppm (equal to 39 mg/kg bw per day). The NOAEL for carcinogenicity was 1000 ppm (equal to 156 mg/kg bw per day), based on a treatment-related increase in hepatocellular adenomas and carcinomas in high-dose male mice at 7000 ppm (equal to 1109 mg/kg bw per day).

In a 2-year dietary study of toxicity and carcinogenicity, rats were treated with metrafenone at 0, 500, 5000 or 20 000 ppm; the dietary concentration for the high-dose females was reduced from 20 000 to 10 000 ppm beginning with study week 69 (doses equal to 0, 25, 260 and 1069 mg/kg bw per day for males and 0, 30, 320 and 1419 mg/kg bw per day [up to the end of week 68] or 593 mg/kg bw per day [weeks 72–104] for females, respectively). Body weight gains were reduced, and the liver and kidney were the target organs. Kidney effects included increased weights and increased incidence and severity of chronic nephropathy in both sexes. Effects on the liver were generally more marked in females and included increased weights, histopathological effects consistent with liver enlargement, such as centrilobular hypertrophy, and polyploidy and necrosis. The NOAEL for chronic toxicity was 500 ppm (equal to 25 mg/kg bw per day), based on effects on body weight, liver and kidney at 5000 ppm (equal to 260 mg/kg bw per day). The NOAEL for carcinogenicity was 500 ppm (equal to 30 mg/kg bw per day), based on an increased incidence of hepatocellular adenoma at the intermediate and high dose levels in females, with an equivocal response in high-dose males (LOAEL of 5000 ppm, equal to 320 mg/kg bw per day).

The Meeting concluded that metrafenone is carcinogenic in mice and rats.

Mechanistic studies in rats did not reveal tumour initiating potential in rat liver and showed that the treatment-related liver tumours identified in mice and rats were induced by a mechanism that is non-genotoxic and is expected to have a threshold. The postulated mechanism is that metrafenone induces an increased rate of hepatocyte proliferation (cytochrome P450 enzyme induction as an associated marker); continuing exposure to metrafenone leads to chronic stimulation of proliferation, which is a known mechanism that can give rise to tumours. Levels of exposure that are insufficient to give rise to induction of liver enzymes and liver cell proliferation would not be expected to cause liver tumours following chronic exposure.

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

The Meeting concluded that metrafenone is unlikely to be genotoxic.

In view of the lack of genotoxicity and the fact that the observed carcinogenicity in mice and rats is expected to have a threshold, the Meeting concluded that metrafenone is unlikely to pose a carcinogenic risk to humans from the diet.

In a two-generation study of reproductive toxicity, rats were given a diet containing metrafenone at a concentration of 0, 500, 1000 or 10 000 ppm (equal to 0, 39, 79 and 811 mg/kg bw per day based on the average combined premating values for P and F_1 males and females). The NOAEL for effects on reproductive parameters was 1000 ppm (equal to 79 mg/kg bw per day), based on an increased proportion of abnormal sperm in F_1 males at 10 000 ppm (equal to 811 mg/kg bw per day), although there were no clear effects on reproductive performance at any dose. The NOAEL for parental toxicity was 500 ppm (equal to 39 mg/kg bw per day), based on effects on body weight gain in F_1 parental males at 1000 ppm (equal to 79 mg/kg bw per day). The NOAEL for effects on pups was 1000 ppm (equal to 79 mg/kg bw per day), based on adverse effects on pup weights and increased liver weights at 10 000 ppm (equal to 811 mg/kg bw per day).

In a study of prenatal developmental toxicity, rats received metrafenone via gavage at 0, 50, 500 or 1000 mg/kg bw per day. There was no evidence of either maternal or embryo/fetal toxicity. The NOAEL for both maternal and embryo/fetal toxicity was 1000 mg/kg bw per day, the highest dose tested.

In a study of prenatal developmental toxicity, rabbits received metrafenone via gavage at 0, 50, 350 or 700 mg/kg bw per day. The NOAEL for maternal toxicity was 50 mg/kg bw per day, based on lower body weight gains and feed consumption, increased liver weights and histopathological effects in the liver at 350 and 700 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 350 mg/kg bw per day, based on decreased fetal weights at 700 mg/kg bw per day.

The Meeting concluded that metrafenone is not teratogenic.

In an acute neurotoxicity study, rats received metrafenone via gavage at 0, 125, 500 or 2000 mg/kg bw per day. The NOAEL was 2000 mg/kg bw, the highest dose tested.

In a 28-day neurotoxicity study, rats were given a diet containing metrafenone at a concentration of 0, 1500, 5000 or 15 000 ppm (equal to 0, 143, 459 and 1371 mg/kg bw per day for males and 0, 152, 493 and 1371 mg/kg bw per day for females, respectively). The NOAEL for systemic toxicity was 1500 ppm (equal to 143 mg/kg bw per day), based on clinical signs (piloerection and red discoloured urine) at 5000 ppm (equal to 459 mg/kg bw per day). The NOAEL for neurotoxicity was 15 000 ppm (equal to 1371 mg/kg bw per day), the highest dose tested.

The Meeting concluded that metrafenone is not neurotoxic.

In a 28-day immunotoxicity study, female rats were given a diet containing metrafenone at a concentration of 0, 1000, 4000 or 12 000 ppm (equal to 0, 80, 315 and 1086 mg/kg bw per day, respectively). The NOAEL for immunotoxicity was 12 000 ppm (equal to 1086 mg/kg bw per day), the highest dose tested. The NOAEL for systemic toxicity was 1000 ppm (equal to 80 mg/kg bw per

day), based on significantly increased absolute and relative liver weights observed at 4000 ppm (equal to 315 mg/kg bw per day).

The Meeting concluded that metrafenone is not immunotoxic.

Toxicological data on metabolites and/or degradates

No metabolites of concern were identified.

Human data

In reports on manufacturing plant personnel, no adverse health effects were noted.

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

Toxicological evaluation

An acceptable daily intake (ADI) of 0–0.3 mg/kg bw was established, on the basis of the NOAEL of 25 mg/kg bw per day for liver and kidney effects in the 2-year dietary study in rats, using a safety factor of 100. The upper bound of the ADI provides a margin of exposure of at least 1000 relative to the LOAEL for the induction of liver tumours in rats and mice.

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

Levels relevant to risk assessment of metrafenone

Species	Study	Effect	NOAEL	LOAEL
Mouse	Eighteen-month study of toxicity and carcinogenicity[a]	Toxicity	250 ppm, equal to 39 mg/kg bw per day	1 000 ppm, equal to 156 mg/kg bw per day
		Carcinogenicity	1 000 ppm, equal to 156 mg/kg bw per day	7 000 ppm, equal to 1 109 mg/kg bw per day
Rat	Ninety-day studies of toxicity[a,b]	Toxicity	1 000 ppm, equal to 79 mg/kg bw per day	5 000 ppm, equal to 404 mg/kg bw per day
	Two-year study of toxicity and carcinogenicity[a]	Toxicity	500 ppm, equal to 25 mg/kg bw per day	5 000 ppm, equal to 260 mg/kg bw per day
		Carcinogenicity	500 ppm, equal to 30 mg/kg bw per day	5 000 ppm, equal to 320 mg/kg bw per day
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	1 000 ppm, equal to 79 mg/kg bw per day	10 000 ppm, equal to 811 mg/kg bw per day
		Parental toxicity	500 ppm, equal to 39 mg/kg bw per day	1 000 ppm, equal to 79 mg/kg bw per day
		Offspring toxicity	1 000 ppm, equal to 79 mg/kg bw per day	10 000 ppm, equal to 811 mg/kg bw per day
	Developmental toxicity study[c]	Maternal toxicity	1 000 mg/kg bw per day[d]	–
		Embryo and fetal toxicity	1 000 mg/kg bw per day[d]	–

Species	Study	Effect	NOAEL	LOAEL
Rabbit	Developmental toxicity study[c]	Maternal toxicity	50 mg/kg bw per day	350 mg/kg bw per day
		Embryo and fetal toxicity	350 mg/kg bw per day	700 mg/kg bw per day
Dog	Ninety-day and 1-year studies of toxicity[b,c]	Toxicity	500 mg/kg bw per day[d]	–

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

Estimate of acceptable daily intake (ADI)

0–0.3 mg/kg bw

Estimate of acute reference dose (ARfD)

Unnecessary

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

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

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

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	> 88% within 72 hours at 10 mg/kg bw; 15–20% within 72 hours at 1 000 mg/kg bw
Dermal absorption	1.6% for the concentrate and 19% for a 1/2 000 aqueous dilution
Distribution	Widely distributed; highest concentrations in gastrointestinal tract and liver
Potential for accumulation	No evidence of accumulation
Rate and extent of excretion	> 80% (mainly in faeces via bile) within 72 hours at 10 mg/kg bw
Metabolism in animals	Extensive; mostly *O*-demethylation and subsequent conjugation with glucuronic acid
Toxicologically significant compounds in animals and plants	Parent compound
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 mg/L
Rabbit, dermal irritation	No data
Rabbit, ocular irritation	No data

Dermal sensitization	No data
Short-term studies of toxicity	
Target/critical effect	Liver / increased weights and associated histopathological findings
Lowest relevant oral NOAEL	79 mg/kg bw per day (90-day study in rats)
Lowest relevant dermal NOAEL	1 000 mg/kg bw per day (28-day study in rats)
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Liver / hepatocyte hypertrophy and hepatocellular adenomas
Lowest relevant NOAEL	25 mg/kg bw per day (2-year study in rats)
Carcinogenicity	Hepatocellular adenomas and carcinomas in male mice (at 1 109 mg/kg bw per day) and hepatocellular adenomas in female rats (at 320 mg/kg bw per day) Non-genotoxic mechanism proposed (chronic induction of cell proliferation/enzyme induction) Unlikely to pose a carcinogenic risk to humans from the diet
Genotoxicity	
	Unlikely to be genotoxic
Reproductive toxicity	
Target/critical effect	Reduced pup weights (F_1 and F_2) and increased proportion of abnormal sperm (F_1 only) with no adverse effects on reproductive performance in the presence of parental toxicity
Lowest relevant parental NOAEL	39 mg/kg bw per day
Lowest relevant offspring NOAEL	79 mg/kg bw per day
Lowest relevant reproductive NOAEL	79 mg/kg bw per day
Developmental toxicity	
Target/critical effect	Lower fetal weight at maternally toxic doses (rabbit)
Lowest relevant maternal NOAEL	50 mg/kg bw per day (rabbit)
Lowest relevant embryo/fetal NOAEL	350 mg/kg bw per day (rabbit)
Neurotoxicity	
Acute neurotoxicity NOAEL	2 000 mg/kg bw, highest dose tested
Subchronic neurotoxicity NOAEL	1 371 mg/kg bw per day, highest dose tested
Developmental neurotoxicity NOAEL	No data
Other toxicological studies	
Immunotoxicity NOAEL	1 086 mg/kg bw per day, highest dose tested
Studies on toxicologically relevant metabolites	No metabolites of concern were identified
Mechanistic studies	Reversible induction of P450 enzymes and cell proliferation in rat liver; no tumour initiating potential in rat liver
Medical data	
	No evidence of adverse effects in personnel exposed to metrafenone; no incident reports

Summary

	Value	Study	Safety factor
ADI	0–0.3 mg/kg bw	Two-year toxicity and carcinogenicity study (rat)	100
ARfD	Unnecessary	–	–

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MYCLOBUTANIL

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Explanation

Myclobutanil is the International Organization for Standardization (ISO)–approved common name for (*R*,*S*)-2-(4-chlorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)hexanenitrile (International Union of Pure and Applied Chemistry), with Chemical Abstracts Service number 88671-89-0. It is a broad-spectrum fungicide of the substituted triazole chemical class of compounds. It acts by inhibiting sterol biosynthesis in fungi.

Myclobutanil was evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 1992, when an acceptable daily intake (ADI) of 0–0.03 mg/kg body weight (bw) was established, on the basis of a no-observed-adverse-effect level (NOAEL) of 2.5 mg/kg bw per day in a 2-year study in rats. No acute reference dose (ARfD) was established, because the establishment of ARfDs by JMPR was not common practice in 1992. Myclobutanil was reviewed by the present Meeting as part of the periodic review programme of the Codex Committee on Pesticide Residues.

New studies on acute toxicity, skin and eye irritation, skin sensitization and carcinogenicity in rats and mice as well as a reanalysis of a developmental toxicity study in rats were submitted. New studies on the acute toxicity, short-term toxicity or genotoxicity of metabolites, degradates or

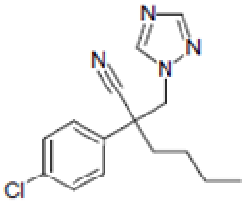
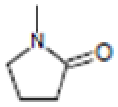
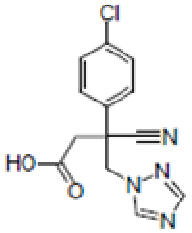
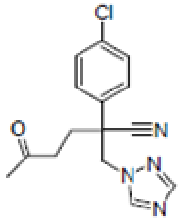
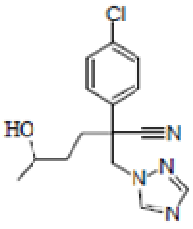
impurities were also submitted. All critical studies contained statements of compliance with good laboratory practice (GLP). Overall, the Meeting considered that the database was adequate for the risk assessment.

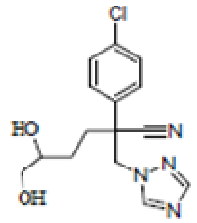
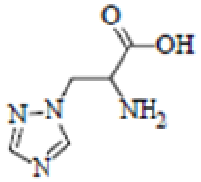
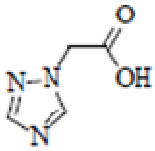
Evaluation for acceptable daily intake

1. Biochemical aspects

Structures of parent compounds, metabolites and degradation products in various crops and animals are shown in Table 1.

Table 1. Structures of parent compounds, metabolites and degradates in various crops and animals

Code/common name	Chemical name	Structural formula
Myclobutanil	(*RS*)-2-(4-Chlorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)hexanenitrile	
1-Methylpyrrolidin-2-one	1-Methylpyrrolidin-2-one	
Myclobutanil butyric acid	(3*RS*)-3-(4-Chlorophenyl)-3-cyano-4-(1*H*-1,2,4-triazol-1-yl)butanoic acid	
RH-9089 (#3)[a]	(2*RS*)-2-(4-Chlorophenyl)-5-oxo-2-(1*H*-1,2,4-triazol-1-ylmethyl)hexanenitrile	
RH-9090 (#4)	(2*RS*,5*RS*)-2-(4-Chlorophenyl)-5-hydroxy-2-(1*H*-1,2,4-triazol-1-ylmethyl)hexanenitrile	

Code/common name	Chemical name	Structural formula
RH-0294 (#6)	(2*RS*,5*RS*)-2-(4-Chlorophenyl)-5,6-dihydroxy-2-(1*H*-1,2,4-triazol-1-ylmethyl)hexanenitrile	
Triazolyl alanine	(2*RS*)-2-Amino-3-(1*H*-1,2,4-triazol-1-yl)propanoic acid	
Triazolyl acetic acid	1*H*-1,2,4-Triazol-1-ylacetic acid	
1,2,4-Triazole	1*H*-1,2,4-Triazole	

[a] Numbers in parentheses refer to the metabolites in Table 2 and Fig. 1 below.

1.1 Absorption, distribution and excretion

(a) [^{14}C]Myclobutanil labelled in the chlorophenyl ring

Mice

The disposition and metabolism (see section 1.2) of [^{14}C]myclobutanil labelled in the chlorophenyl ring were investigated in a study with male and female CD-1 mice, following the administration of non-radiolabelled myclobutanil (TD83-086; lot no. LSPL0016E; purity 81.1%) for 2 weeks in the diet at 0, 10, 100 or 1000 parts per million (ppm) (equal to 0, 2.1, 22.3 and 218 mg/kg bw per day, respectively). The [^{14}C]myclobutanil (TD83-184; lot no. 424.0107; specific activity 380.4 MBq/g; radiochemical purity 99.7%) was administered as a single oral dose by gavage at 0, 2, 20 or 200 mg/kg bw, respectively, in 0.5% weight per volume (w/v) methyl cellulose. Whole blood, urine and faeces were collected and analysed. This study was conducted in compliance with GLP, but not in accordance with test guidelines.

Absorption. Radiolabel was rapidly absorbed by mice, with a half-life for absorption ranging from 0.04 hour (at 10 ppm in females) to 0.31 hour (at 1000 ppm in males), but with no clear difference between the sexes. Maximum whole blood concentrations were reached within 0.25–1 hour and were proportional to dose in both sexes. Elimination of radiolabel from blood of males at 1000 ppm was monophasic (half-life = 6.2 hours). Elimination of radiolabel from blood in both sexes of all other groups was biphasic: a rapid phase lasting 4–6 hours, with half-lives ranging from 0.63 hour (1000 ppm in females) to 0.88 hour (10 ppm in females), was followed by a more variable slow phase, with half-lives ranging from 6.0 hours (1000 ppm in females) to 30.1 hours (10 ppm in males). The area under the whole blood concentration–time curve (AUC) was proportional to dose and was similar in males and females at each dose. At 24 hours, only 0.07–0.15% of the administered radioactivity remained in the blood.

Distribution. At 1 hour after dosing, plasma concentrations of radiolabel were similar to whole blood levels at each dose, for both sexes (1.18–2.03% of the dose). Liver concentrations were also similar for both sexes at each dose (4.79–10.53% of the dose), with levels 3.9- to 11.1-fold higher than in whole blood. The liver/blood ratio of radiolabel decreased with increasing dose (6.8% or 8.4% at 2 mg/kg bw for males and females, respectively, to 4.0% or 2.9% at 200 mg/kg bw for males and females, respectively). These data suggest that the liver has a greater affinity for radiolabel, and the ability to extract or bind the radiolabel diminished with increasing dose, although this did not cause a disproportionate increase in radiolabel in the whole blood.

Excretion. Most of the administered radioactivity was recovered in the urine and faeces within 24–48 hours after dosing. By 96 hours, the proportion excreted was 61–67% of the dose for males and 67–87.2% for females. An additional significant amount of radiolabel (14.1–24.0%) was recovered in the cage washes at the end of 96 hours.

In conclusion, following its repeated oral dosing to mice, [^{14}C]myclobutanil was completely and rapidly absorbed and essentially completely excreted by 96 hours. Maximum plasma and tissue ^{14}C concentrations were achieved within 1 hour after oral administration and were dose proportional. Plasma elimination was biphasic in all but high-dose males. No significant tissue accumulation was observed 96 hours after dosing, although liver concentrations were higher than whole blood levels at 1 hour after dosing (Steigerwalt, Udinsky & Longacre, 1986b).

Rats

The disposition and metabolism (see section 1.2) of [^{14}C]myclobutanil (radiolabelled at all carbons in the chlorophenyl ring), suspended in 0.5% w/v methyl cellulose, were investigated in male and female Sprague-Dawley rats (Steigerwalt, Udinsky & Longacre, 1986a). [^{14}C]Myclobutanil was administered to rats according to three protocols: as single oral doses (10 mL/kg bw) of 1 mg/kg bw (TD83-116; lot no. 424.0102; specific activity 380.4 MBq/g; radiochemical purity 97.3%) or 100 mg/kg bw (TD83-115; lot no. 424.0103; specific activity 59.9 MBq/g; radiochemical purity 96.9%) or 100 mg/kg bw (TD83-115; lot no. 424.0103; specific activity 59.9 MBq/g; radiochemical purity 96.9%) following 2 weeks of dietary administration of 1000 ppm non-radiolabelled myclobutanil (TD83-086; lot no. LSPL0016E; purity 81.1%). An additional group of rats received a single intravenous dose (1 mL/kg bw) of [^{14}C]myclobutanil (TD83-115; lot no. 424.010 3; specific activity 59.9 MBq/g; radiochemical purity 96.9%) at 1 mg/kg bw in 20% w/v 2,5-dimethylisosorbide. Radioactivity in whole blood, plasma, various organs and tissues, urine and faeces was analysed by liquid scintillation counting after combustion.

Absorption. Practically all (89.2–114.6%) of the [^{14}C]myclobutanil was absorbed by the rats, based on the relative percentage of radiolabel excreted in urine following oral and intravenous administration. Plasma concentrations following administration of a single dose or repeated doses reached maximum levels within 1 hour after the treatment. The maximum plasma concentrations were 19.6 and 23.8 ppm in the single-dose and repeated-dose groups, respectively.

Plasma elimination of radiolabel was biphasic, with a rapid phase lasting 12–24 hours. The rapid elimination phase was faster in whole blood for the 100 mg/kg bw myclobutanil group rats. The slower phase was even slower and the AUC was slightly greater in whole blood for both single and repeated orally dosed rats. Kinetics data are shown in Table 2.

Distribution. Radiolabel rapidly appeared in all tissues of male rats, reaching maximum concentrations within 1 hour, ranging from 6.29 ppm (brain) to 56.65 ppm (liver) for single orally dosed males and from 15.01 ppm (brain) to 153.7 ppm (liver) for repeatedly dosed males. Radiolabel was rapidly eliminated from the tissues in a biphasic manner, as observed in plasma, with no evidence of significant accumulation. Less than 0.7% of the dose remained in tissues of single or repeatedly dosed rats at 96 hours.

Excretion. Following an intravenous dose of 1 mg/kg bw, 54.86–64.36% was recovered in urine (including funnel wash) and faeces within 24 hours of dosing. Total ^{14}C excretion by 96 hours post-dosing was 72.6% and 78.72% for males and females (representing 94% and 96% of the

Table 2. Kinetics data in rats

Parameter	Single dose (100 mg/kg bw)		Repeated dose	
	Serum	**Whole blood**	**Serum**	**Whole blood**
C_{max} (ppm)	19.6	26.2	23.8	19.9
$t_{½}$ (h)				
Rapid phase	5.25	1.61	1.97	2.04
Slow phase	25.7	38.5	31.5	49.5
AUC (h·µg/g)	246	276	226	289

AUC: area under the concentration–time curve; bw: body weight; C_{max}: maximum concentration; ppm: parts per million; $t_{½}$: half-life

Source: Steigerwalt, Udinsky & Longacre (1986a)

recovered radiolabel), respectively, with an equivalent distribution between both urine and faeces. In oral treatment with 1 or 100 mg/kg bw (both single and repeated dosing), 40.8–81.11% of the dose was recovered in the urine and faeces within 24–48 hours post-dosing. By 96 hours, total excretion ranged from 73.91% (1 mg/kg bw in females) to 88.12% (10 mg/kg bw in females) of the dose, representing 89–98% of recovered radiolabel, again with an equivalent distribution between both urine and faeces. Between 75% and 94% of myclobutanil was excreted within 48 hours following both intravenous and oral treatment.

In conclusion, in the single-dose oral study in rats, [^{14}C]myclobutanil was completely and rapidly absorbed (> 89%) and rapidly and essentially completely excreted. Maximum plasma and tissue ^{14}C levels were achieved within 1 hour after oral administration, and plasma elimination was biphasic. Most myclobutanil was excreted within 48 hours. No significant tissue accumulation was observed 96 hours after dosing.

This study was GLP compliant and conducted in accordance with test guidelines (Steigerwalt, Udinsky & Longacre, 1986a).

(b) [^{14}C]Myclobutanil labelled at carbons 3 and 5 of the triazole ring

Rats

A single-dose oral study in rats was conducted to define the path and rate of excretion of myclobutanil labelled with ^{14}C in the triazole ring, to identify in which organs of rats potential accumulation might occur and to characterize the excreted material for parent and metabolite compounds (see section 1.2). Four male and four female Sprague-Dawley rats were given a single oral gavage dose of 2000 ppm (equal to 150 mg/kg bw) [^{14}C]myclobutanil (lot no. 417.01; specific activity 406.3 MBq/g) radiolabelled at carbons 3 and 5 of the triazole ring. This study was not conducted in compliance with GLP (Streelman, 1984).

Excretion. Most of the radioactivity was excreted in the urine and faeces, with only a small portion appearing in the expired air as carbon dioxide. Excluding the cage washes, an average of 99.3% of the recovered radioactivity was contained in the urine (16–54%) and faeces. The distribution between urine and faeces varied considerably between rats. The excretion pattern for both males and females fit a first-order kinetic model.

Distribution. The highest concentrations of radioactivity were found in the liver, kidney, and small and large intestines. Residue levels were reduced with time and lower in the females than in the males.

In conclusion, in this study, myclobutanil was rapidly excreted from rats. There was no accumulation in any organ or tissue (Streelman, 1984).

1.2 Biotransformation

Mice

In a 2-week repeated-dose oral study in mice (see section 1.1), myclobutanil was extensively metabolized to a number of more polar metabolites, with parent compound representing only 1–7% of the administered dose. The metabolite profiles were comparable between males and females at all three doses. There were 3–4 major fractions (i.e. constituting 10% of the total) and six minor fractions detected in the excreta (Steigerwalt, Udinsky & Longacre, 1986b).

Rats

In the first single-dose study in rats (see section 1.1), myclobutanil was extensively metabolized to more polar metabolites, the parent compound representing only 1.0–3.6% of the excreted dose. In orally dosed males, five major fractions were eliminated in the excreta. The excreta of orally dosed females contained one dominant fraction that accounted for 53.0–61.1% of the excreted radiolabel. Metabolites were qualitatively similar in males and females, although quantitatively different in the amounts of certain fractions excreted (Steigerwalt, Udinsky & Longacre, 1986a).

In the second single-dose study in rats (see section 1.1), thin-layer chromatography showed a range of metabolites, all of which were more polar than the parent. The overall distribution of metabolites in urine and faeces of male and female rats is shown in Table 3. The same metabolites (metabolite fractions #2, #3, #4, #5, #6 and #7) were found in urine and faeces of both males and females, although relative distributions were different. In females, metabolite fraction #7 (sulfate of RH-9090) was a major metabolite in both urine and faeces. The major metabolic processes involved oxidation of the butyl group. Among the metabolites formed were RH-9090 and RH-9089 (metabolite fractions #4 and #3, respectively, in Table 1), the major unconjugated phenethyl triazole-containing metabolites found in plants. The distributions of RH-9090 and RH-9089 were approximately 10% or lower in the overall distribution of metabolites in rats (Streelman, 1984).

In conclusion, myclobutanil was extensively metabolized to more polar compounds (Streelman, 1984; Steigerwalt, Udinsky & Longacre, 1986a).

Table 3. Overall distribution of metabolites in the rat

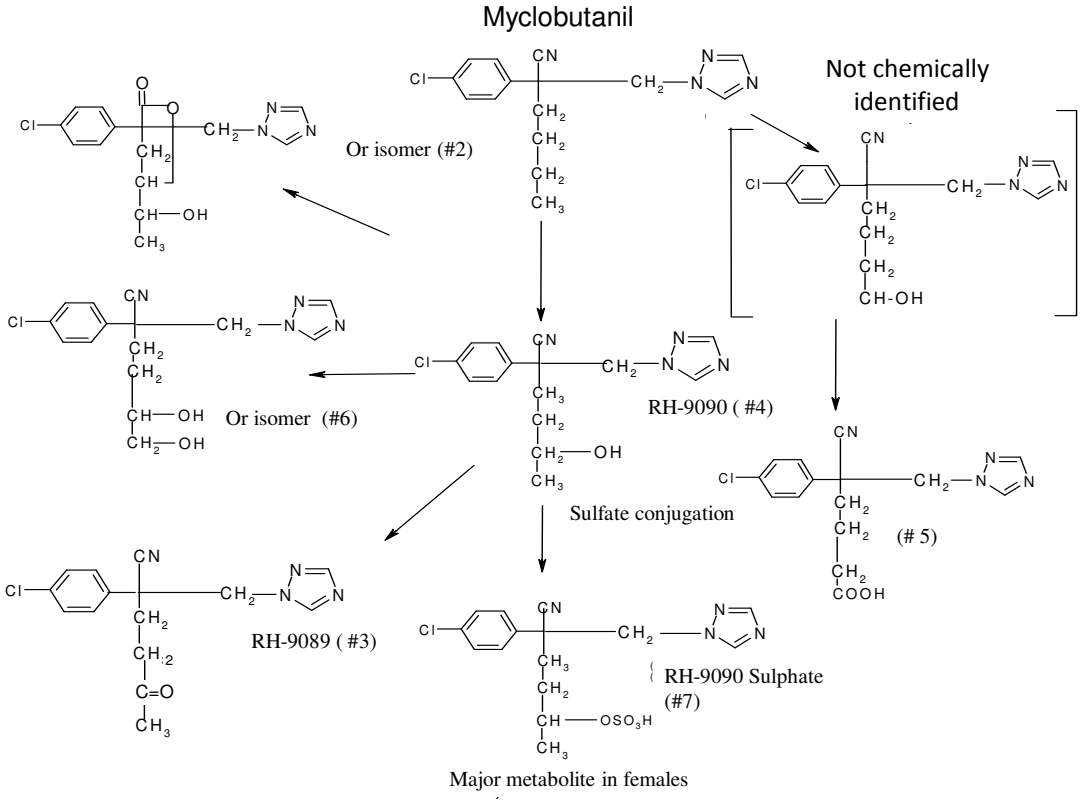
	Distribution of metabolites (%)						
	Urine		**Faeces**		**Total**		
Parent/metabolites	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Rf value**
Myclobutanil	0.6	1.7	4.5	2.2	2.8	2.0	0.66
#2[a]	8.8	2.1	14.3	0.4	11.9	1.0	0.58
#3 RH-9089	8.7	2.5	10.4	0.8	9.7	1.4	0.54
#4 RH-9090	6.4	4.7	14.3	3.5	10.8	3.9	0.50
#5	10.6	1.5	6.8	0.4	8.5	0.8	0.45
#6	26.1	18.1	12.2	5.4	18.3	9.9	0.39
#7 RH-9090 sulfate	11.9	62.3	17.8	81.5	15.2	74.7	0.20
Unknown 1	8.8	1.5	2.4	0.2	5.2	0.7	0.35
Unknown 2	3.3	1.3	3.4	0.0	3.4	0.5	0.14
Origin	9.7	2.0	9.8	0.5	9.8	1.0	–
Remainder	5.1	2.6	4.3	5.3	4.7	4.3	–

Rf: retardation factor

[a] Numbers in parentheses refer to structures given in Table 1 and Fig. 1.
Source: Streelman (1984)

A proposed metabolic scheme in rats is presented in Fig. 1.

Fig. 1. Proposed metabolic pathway in the rat



Source: Streelman (1984)

2. Toxicological studies

2.1 Acute toxicity

Studies on acute toxicity, skin or eye irritation, and skin sensitization are summarized in Table 4.

(a) Lethal doses

Mice

Three acute toxicity studies were performed. In the first study, median lethal doses (LD_{50}s) in male and female ICR mice were 1910 and 1840 mg/kg bw, respectively, and the lowest lethal dose was 1300 mg/kg bw in both sexes (Morrison et al., 1986). The LD_{50} in female ICR mice was 1360 mg/kg bw in the second study (Morrison, Murphy & Chan, 1984). In the third study, LD_{50}s in male and female ICR mice were 2270 and 2440 mg/kg bw, respectively; the lowest lethal dose in the study was 1870 mg/kg bw in both sexes (Shimizu, Tokiwa & Nakayoshi, 1987).

Rats

Two acute toxicity studies were conducted with male and female rats.

Table 4. The acute toxicities, skin or eye irritation, and skin sensitization of myclobutanil

Route (method)	Species/ strain (sex)	Purity (%)	Result	Reference
Oral, gavage	Rat/CD (M + F)	84.5	LD_{50} = 1 750 mg/kg bw (M) LD_{50} = 1 800 mg/kg bw (F)	Krzywicki (1983)
Oral, gavage	Rat/CD (M + F)	91.9	LD_{50} = 1 600 mg/kg bw (M) LD_{50} = 2 290 mg/kg bw (F)	Krzywicki & Morrison (1984)
Oral, gavage ("up and down" method)[a]	Rat/F344 (F)	95.1	LD_{50} = 3 129 mg/kg bw per day	Moore (2005b)
Oral	Mouse/ICR (M + F)	91.9	LD_{50} = 1.91 g/kg bw (M) LD_{50} = 1.84 g/kg bw (F)	Morrison et al. (1986)
Oral	Mouse/ICR (F)	91.9	LD_{50} = 1.36 g/kg bw	Morrison, Murphy & Chan (1984)
Oral	Mouse/ICR (M + F)	91.4	LD_{50} = 2.27 g/kg bw (M) LD_{50} = 2.44 g/kg bw (F)	Shimizu, Tokiwa & Nakayoshi (1987)
Dermal[a]	Rat/F344 (M + F)	95.1	LD_{50} > 5 000 mg/kg bw	Moore (2005a)
Dermal	Rabbit/NZW (M + F)	84.5	LD_{50} > 5 000 mg/kg bw	Krzywicki (1983)
Dermal	Rabbit/NZW (M + F)	91.9	LD_{50} > 5 000 mg/kg bw	Krzywicki & Bonin (1984)
Inhalation, nose only[b]	Rat/CD (M + F)	91.4	LC_{50} > 5.1 mg/L	Fisher, Emmons & Hagan (1987)
Dermal	Rabbit/NZW (M)	84.5	Not irritating	Krzywicki (1983)
Dermal	Rabbit/NZW (M)	91.9	Not irritating	Krzywicki & Bonin (1984)
Dermal[a]	Rabbit/NZW (M)	95.1	Very slightly irritating	Moore (2005c)
Eye	Rabbit/NZW (M)	84.5	Slightly irritating	Krzywicki (1983)
Eye	Rabbit/NZW (M)	91.9	Slightly to moderately irritating	Krzywicki & Bonin (1984)
Eye[a]	Rabbit/NZW (M)	95.1	Slightly irritating	Merkel (2005)
Dermal (Buehler method)[b]	Guinea-pig/ Hartley albino (M + F)	91.4	Minimally sensitizing	Bonin & Hazelton (1987)
Dermal (maximization test)	Guinea-pig/ Hartley albino (M + F)	91.4	Not sensitizing	Kreuzmann (1989)
Dermal (local lymph node assay)[a]	Mouse/BALB/c (F)	95.1	Not sensitizing	Woolhiser, Wiescinski & Anderson (2005)

bw: body weight; F: female; F344: Fischer 344; LC_{50}: median lethal concentration; LD_{50}: median lethal dose; M: male; NZW: New Zealand White

[a] Conducted in accordance with the guidelines of the Organisation for Economic Co-operation and Development, the United States Environmental Protection Agency, the European Commission and the Japanese Ministry of Agriculture, Forestry, and Fisheries.

[b] Conducted in accordance with the guidelines of the Organisation for Economic Co-operation and Development and the United States Environmental Protection Agency.

In the first oral acute toxicity study with CD rats, the LD_{50}s in males and females were 1600 and 2290 mg/kg bw, respectively (Krzywicki & Morrison, 1984). Mortality occurred at doses of 1100 mg/kg bw and above. Clinical signs of toxicity observed in the treated rats occurred from the day of dosing, at greater severity with increasing dose, and included passiveness, ataxia, prostration, emaciation, salivation, abdominal breathing, lacrimation, alopecia and scant droppings. Staining of the urogenital/anogenital area, muzzle and eyes was also observed. There were no treatment-related clinical observations in control rats, which showed some staining of anogenital areas and muzzle only (Krzywicki, 1983).

In the second study with F344 rats and using the "up and down" procedure, no treatment-related changes were detected at 175 mg/kg per bw. At 1750 mg/kg per bw, all rats survived, but clinical signs of toxicity were observed in two rats, including anogenital staining and/or hypoactivity. However, the rats recovered by day 3. At 5000 mg/kg bw, all four females died within 1 day of test substance administration. Prior to death, all rats were hypoactive and/or exhibited abnormal posture, anogenital staining, piloerection and diarrhoea (Moore, 2005b).

Based on these two studies, LD_{50} values for myclobutanil in rats were 1600–3129 mg/kg bw.

(b) Dermal irritation

In two of three skin irritation studies using male New Zealand White rabbits, no irritation was detected (Krzywicki, 1983; Krzywicki & Bonin, 1984). In one study, very slight erythema was observed at 30–60 minutes after dosing. Recovery occurred in the three rabbits by 24, 48 and 72 hours, respectively (Moore, 2005c).

Based on the three studies, myclobutanil showed no irritation to rabbit skin.

(c) Ocular irritation

Three New Zealand White rabbit eye irritation studies of myclobutanil have been made available, using unformulated material.

In the first of these, the test substance stained the skin and fur around the treated eyes. Hair loss was also observed in these areas. Signs of eye irritation in the conjunctiva (report does not state whether there was redness or chemosis) were observed only in two rabbits with unwashed eyes at 24 hours post-dosing. These effects were reversible by 72 hours, and no other effects were recorded (Krzywicki, 1983).

In the second study, slight to moderate irritation was observed in the cornea (5/6), iris (3/6) and conjunctiva (6/6) at 24 hours post-dosing for rabbits with unwashed eyes. An uneven, pitted area was observed at the centre of the cornea of one rabbit with corneal and conjunctival effects. Myclobutanil deposits were noted to be present in the eyes of 3/6 rabbits and around the eyes of the remaining three rabbits. Irritation of reduced severity was present at 48 and 72 hours in the cornea (4/6 and 3/6, respectively) and conjunctiva (6/6 at both time points). The test substance was noted to be present around the eyes of the same three rabbits as before at 48 hours and in one rabbit at 72 hours. At 7 days, irritation of the conjunctiva was still present in two rabbits. No irritation was observed at 14 and 21 days. However, a few blood vessels had extended into the cornea of one rabbit from day 7 (one of the three rabbits with test material observed in the treated eye at 24 hours) and, after dosing, a 3 mm distinct hazy yellow stained area was seen on the cornea of another rabbit (one of the three rabbits with test material observed around the treated eye at 24, 48 and 72 hours). For rabbits with washed eyes, irritation was observed in all three animals in the conjunctiva at 24 hours and only in one animal at 72 hours (Krzywicki & Bonin, 1984).

In the third eye irritation study, 1 hour after test substance instillation, all three treated eyes exhibited iritis and conjunctivitis. Corneal opacity was evident in two rabbits by 24 hours. The overall incidence and severity of irritation decreased thereafter. All animals were free of ocular irritation by 72 hours (Merkel, 2005).

On the basis of these three studies, myclobutanil was slightly to moderately irritating to the eyes of rabbits.

(d) Dermal sensitization

In a modified Buehler delayed contact hypersensitivity study in guinea-pigs (six animals of each sex per group), minimal to no erythema was observed in the naive control group at 24 and 48 hours following challenge with either myclobutanil (50% weight per weight [w/w]) or a positive control material, 1-chloro-2,4-dinitrobenzene (DNCB), at 800 ppm. In the positive control DNCB group, 7/12 and 6/12 guinea-pigs responded with erythema at 24 and 48 hours, respectively. In the myclobutanil (50% w/w) group, erythema was observed in 3/12 and 1/12 guinea-pigs at 24 and 48 hours, respectively. The decreasing erythema response to myclobutanil over time suggests either a local irritation effect or a weak sensitization effect, and hence a rechallenge phase was considered necessary. In the rechallenge study, no erythema was observed in the naive control group at 24 or 48 hours following challenge with either myclobutanil or DNCB. In the DNCB group, 6/12 and 5/12 guinea-pigs responded with erythema at 24 and 48 hours, respectively. In the myclobutanil group, 1/12 and 0/12 animals exhibited erythema at 24 and 48 hours, respectively. The same one animal responded at challenge at 24 hours, at challenge at 48 hours and at rechallenge at 24 hours, but showed no reaction at rechallenge at 48 hours. The authors concluded that the potential of myclobutanil to produce delayed contact hypersensitivity in guinea-pigs had not been determined (Bonin & Hazelton, 1987). As the Buehler method does not have the extreme sensitivity of the Magnusson-Kligman protocol, this result suggested that myclobutanil was a mild sensitizer to rabbit eyes.

In a maximization test using guinea-pigs (10 animals of each sex per group), 2/20 animals showed a minimal response to myclobutanil. Therefore, myclobutanil was considered to be not sensitizing (Kreuzmann, 1989).

In a local lymph node assay using BALB/cAnNCrl mice (six females per group), erythema was not observed in the mice treated with 5% or 20% myclobutanil, whereas 5/6 mice treated with 80% myclobutanil showed slight erythema on day 6. Topical application of 5%, 20% or 80% myclobutanil elicited proliferative responses/stimulation indices that were, respectively, 1.1-, 1.5- and 1.6-fold greater than in vehicle controls. Myclobutanil did not demonstrate dermal sensitization potential in the mouse local lymph node assay, as the lymph nodes draining the area of topical application did not demonstrate a 3-fold proliferation (stimulation index) when compared with vehicle-treated mice (Woolhiser, Wiescinski & Anderson, 2005).

Overall, in three studies, myclobutanil was equivocal for sensitization due to a lack of sensitization using the guinea-pig maximization test and the mouse local lymph node assay and mild sensitization using the Buehler method.

2.2 Short-term studies of toxicity

(a) Oral administration

Mice

Myclobutanil (TD 83-076; lot no. LSPL0016/E; purity 81.1%) was administered in the diet to nine groups of Crl:CD-1(IcCR)BR mice (10 of each sex per group) for 3 months at 0, 3, 10, 30, 100, 300, 1000, 3000 or 10 000 ppm (equal to 0, 0.40, 1.54, 4.79, 14.1, 42.7, 132, 542 and 2035 mg/kg bw per day for males and 0, 0.62, 2.11, 6.94, 22.9, 65.5, 232, 710 and 2027 mg/kg bw per day for females, respectively). This study was not conducted in accordance with test guidelines, but all the examinations required by test guidelines were conducted in this study with the exception of an ophthalmological test. Liver tissues from four randomly selected mice of each sex per group at 30 and 10 000 ppm myclobutanil were taken for liver mixed-function oxidase (MFO) assay.

At 1000 ppm, one male died at week 3, the cause being considered unrelated to treatment, because no deaths occurred at any of the higher dose levels. Nevertheless, the cause of death was not identified. No treatment-related clinical signs were observed throughout the study. There was a

statistically significant decrease in body weight gain throughout the treatment period at 10 000 ppm for both sexes; body weights for males and females were reduced by 19% and 7%, respectively, compared with controls, at 13 weeks. Male body weight gains were also statistically significantly decreased at 3000 ppm, with body weights being 7% of control values at 13 weeks. Feed consumption of males at 10 000 ppm was statistically significantly reduced during the first week of dosing (26% of controls) only and was greater than control values during weeks 9–13. For females at this dose level, feed consumption was statistically significantly decreased during week 1 (39% of controls). It was lower than the intake of controls throughout the treatment period, but not statistically significantly. The reduced feed consumption at the beginning of treatment was considered to be due to low palatability, rather than an adverse effect, as no similar change in feed consumption was observed in gavage studies (developmental toxicity studies in rats and rabbits) of myclobutanil, and no effects on the gastrointestinal tract were observed. In haematology, statistically significant decreases in the haematocrit and mean corpuscular volume (MCV) values were seen in males and females at 10 000 ppm, but not at 3000 ppm. Also at 10 000 ppm, males had significant decreases in white blood cells and the number of lymphocytes and an increase in the number of segmented neutrophils, whereas females had decreased haemoglobin and increased platelet values. There were no other treatment-related changes in haematological parameters.

At 10 000 ppm, statistically significantly increased levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT) and urea nitrogen were observed in male and female mice; ALT was also increased in males at 3000 ppm. Glucose and cholesterol levels were statistically significantly reduced in both sexes at 10 000 ppm, glucose was reduced in females at 3000 ppm and cholesterol was reduced in both sexes at 3000 ppm and in males at 1000 ppm. Blood chemistry data are summarized in Table 5. There were no other treatment-related changes in blood chemistry parameters.

Table 5. Summary of blood chemistry in the 90-day oral toxicity study in mice

	0 ppm		**1 000 ppm**		**3 000 ppm**		**10 000 ppm**	
	Males	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
AST (U/L)	78.3	125.0	86.0	111.2	115.2	131.1	410.3*	281.9*
ALT (U/L)	27.6	24.8	46.4	33.1	101.8*	72.7	539.4*	371.7*
ALP (U/L)	40.7	41.1	30.1	38.5	52.1	52.3	100.1*	85.6*
BUN (mg/dL)	22.88	21.06	30.14*	20.82	27.09	23.68	39.22*	28.63*
GLUC (mg/dL)	82.3	90.4	71.2	74.1	76.9	64.0*	51.7*	65.0*
GGT (U/L)	0.2	0	0.3	0	0	0	1.4*	2.3*
CHOL (mg/dL)	99.4	61.9	65.3*	52.9	37.2*	36.9*	19.1*	35.0*

ALP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; BUN: blood urea nitrogen; CHOL: cholesterol; GGT: gamma-glutamyltransferase; GLUC: glucose; ppm: parts per million; U: units; *: $P < 0.05$

Source: Goldman, Harris & Lampe (1986)

Statistically significant, modest treatment-related increases in hepatic MFO activity were noted at 3 months; increases in cytochrome P450 per milligram of microsomal protein at 3000 ppm and 10 000 ppm were 85% and 73%, respectively, in males and 86% and 99%, respectively, in females, compared with controls.

Liver weights (absolute and relative to body weight) were statistically significantly increased in both sexes at 3000 ppm (69% and 48% in males and females, respectively, for relative liver weights) and 10 000 ppm (152% and 141% in males and females, respectively, for relative liver weights), and relative liver weights were statistically significantly increased in both sexes at 1000

ppm (35% and 18% in males and females, respectively). Some changes in relative weights for brain, adrenal and gonads were observed as a result of decreased terminal body weights at 10 000 ppm.

At necropsy, there were enlarged livers with or without accentuated lobular architecture at 1000 ppm and above. Treatment-related microscopic changes observed are summarized in Table 6. The liver was the primary target. Microscopically, hepatocellular hypertrophy was increased in males at 1000 ppm and above and in females at 3000 ppm. The hypertrophied area increased in size with increasing dose. Swollen vacuolated centrilobular hepatocytes and single large hepatocytic vacuoles (both histopathological findings were considered fatty changes) were observed at the same doses as hepatocellular hypertrophy in both sexes. Single-cell necrosis of hepatocytes detected in the centrilobular area in males at 1000 and 3000 ppm and in females at 3000 ppm were considered to progress to necrotic hepatitis or coagulative necrosis at 10 000 ppm in both sexes. Pigmented Kupffer cells were observed in males at 3000 ppm and above and in females at 10 000 ppm, and bile duct proliferation was detected in both sexes at 10 000 ppm. Other treatment-related histopathological changes included cytoplasmic eosinophilia and/or hypertrophy of the zona fasciculata cells of the adrenal glands in males at 1000 ppm and in both sexes at 3000 ppm and above. These adrenal changes might reflect the severe stress state of the animals. Pigment was seen in the macrophages in the spleen at 3000 ppm and above. Lymphoid necrosis in the spleen was seen in one or both sexes at 3000 ppm and above. An increased myeloid:erythroid ratio, primarily involving granulocytes in the bone marrow in some females at 10 000 ppm, might be related to inflammatory or necrotic changes in the liver or lymphoid tissues. Atrophy of the uterus and absence of corpora lutea in the ovaries, suggesting no ovulation, were observed at 10 000 ppm. An increased mononuclear cell infiltration in the skin in both sexes was observed at 10 000 ppm.

The NOAEL for 90-day oral toxicity in mice was 300 ppm (equal to 42.7 mg/kg bw per day), based on hepatotoxicity, including hepatocellular hypertrophy, fatty changes and hepatocellular necrosis, in males at 1000 ppm (equal to 132 mg/kg bw per day) (Goldman, Harris & Lampe, 1986).

Rats

Myclobutanil (TD 83-076; lot no. LSPL0016/E; purity 81.1%) was administered in the diet to nine groups of CD (SD)BR rats (10 of each sex per group) for 3 months at a dietary concentration of 0, 5, 15, 50, 150, 500, 1500, 5000 or 15 000 ppm for weeks 1 and 2; 0, 7, 21, 70, 210, 700, 2100, 7000 or 21 000 ppm, respectively, for weeks 3 and 4; and 0, 10, 30, 100, 300, 1000, 3000, 10 000 or 30 000 ppm, respectively, for the remainder of the study. These dietary concentrations were equal to doses of 0, 0.52, 1.60, 5.22, 15.3, 51.5, 158, 585 and 1730 mg/kg bw per day for males and 0, 0.67, 2.03, 6.85, 19.7, 65.8, 195.2, 665 and 1811 mg/kg bw per day for females, respectively. This study was not conducted in accordance with test guidelines, but almost all examinations required by test guidelines were conducted in this study. Haematological and clinical chemistry examinations were conducted on day 32 and day 95. Samples of hepatic tissue were taken from three rats of each sex per group at 100, 300, 1000, 3000 and 10 000 ppm myclobutanil for liver MFO assay.

All rats treated with 15 000/21 000/30 000 ppm myclobutanil died during the treatment period, males during days 17–63 and females during days 18–49. Clinical signs observed prior to death included a brown-stained anogenital area, red- or brown-stained muzzle, scant faecal droppings and emaciation. One or two rats of each sex were also lethargic or ataxic prior to death. One male at 5000/7000/10 000 ppm was found dead on day 83 because of an accident. Body weights of males and females were significantly decreased at 5000/7000/10 000 ppm and above throughout the study. At 1500/2100/3000 ppm, statistically significant decreases in body weight were noted during weeks 6–12 of the study in males (8% at week 13), but only during week 9 in females. Feed consumption was significantly decreased in males and females at 15 000/21 000/30 000 ppm and in males at 5000/7000/10 000 ppm throughout the study. Feed consumption was statistically significantly decreased in females at 5000/7000/10 000 ppm for the first 7 weeks and in week 9 of the study. These constant decreases in feed consumption were considered adverse. At 1500/2100/3000 ppm, transient but statistically significant decreases in feed consumption were not considered treatment related in either sex.

Table 6. Summary of histopathological changes in the 90-day oral toxicity study in mice

	Incidence of histopathological changes									
	0 ppm		**300 ppm**		**1 000 ppm**		**3 000 ppm**		**10 000 ppm**	
	M	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
No. of mice examined	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*
Liver										
Centrilobular hepatocellular hypertrophy	6	0	5	0	10	0	10	10	0	0
Centrilobular and midzonal hepatocellular hypertrophy	0	0	0	0	0	0	0	0	10	10
Swollen vacuolated centrilobular hepatocytes	0	0	0	0	3	0	10	5	10	10
Single large hepatocytic vacuoles	0	0	0	0	3	0	6	8	5	4
Centrilobular single-cell hepatocytic necrosis	0	0	0	0	3	0	8	2	0	0
Coagulative necrosis	1	2	0	3	2	1	6	3	10	10
Centrilobular necrotic hepatitis	0	0	0	0	0	0	0	0	10	10
Pigmented Kupffer cells	0	0	0	0	0	0	3	1	10	7
Bile duct proliferation	0	0	0	0	0	0	0	0	5	7
Adrenal										
Cytoplasmic eosinophilia of the zona fasciculata cells	0	0	0	0	4	0	7	8	10	10
Hypertrophy of the zona fasciculata cells	0	0	0	0	0	0	5	2	8	6
Spleen										
Pigment in the macrophages	3	3	6	6	3	6	8	10	10	9
Lymphoid necrosis	0	0	0	0	2	0	0	2	3	3
Bone marrow										
Increased myeloid:erythroid ratio	2	0	0	0	0	0	1	0	1	5
Female reproductive tract										
Absence of corpora lutea	–	2	–	0	–	3	–	1	–	10
Atrophy of uterus	–	1	–	0	–	0	–	1	–	4

F: females; M: males; ppm: parts per million

Source: Goldman, Harris & Lampe (1986)

No treatment-related changes in ophthalmology were detected.

In haematology, haematocrit, haemoglobin or MCV was decreased at 5000/7000/10 000 ppm in both sexes at one or three time points. Increased GGT, total cholesterol and blood urea nitrogen were observed as treatment-related abnormalities in blood chemistry in both sexes at 5000/7000/10 000 ppm. ALT was slightly increased in males at 5000/7000/10 000 ppm. Many haematological and clinical chemistry abnormalities seen at 15 000/21 000/30 000 ppm in both sexes

were considered the result of the maximum tolerated dose (MTD) being exceeded, because all rats died at this dose level. No treatment-related changes were observed in the urine analysis parameters.

Statistically significant increases in hepatic MFO activity were noted at 3 months, with increases in males at 150/210/300 ppm (1.7-fold) and higher (6.5-fold at 5000/7000/10 000 ppm) and in females at 500/700/1000 ppm (2-fold) and higher (8-fold at 5000/7000/10 000 ppm). Female MFO activity was 1.4- to 1.5-fold higher than the control value at 150/210/300 ppm, but this was not a statistically significant change.

Treatment-related changes in the liver and kidneys were seen in males and females at 500/700/1000, 1500/2100/3000 and 5000/7000/10 000 ppm, as shown in Table 7. These consisted notably of statistically significant increases in absolute and relative liver weights and in relative (but not absolute) kidney weights of males and females at 1500/2100/3000 and 5000/7000/10 000 ppm.

Table 7. Organ weights in the 90-day oral toxicity study in rats

	0 ppm		**500/700/1 000 ppm**		**1 500/2 100/3 000 ppm**		**5 000/7 000/10 000 ppm**	
	Males	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
Terminal body weight (g)	479	263	475	256	445	249	337*	224*
Absolute liver weight (g)	12.18	6.46	13.63	7.06	15.68*	8.17*	19.75*	11.30*
Relative liver weight[a]	254	246	287	276*	352*	329*	581*	504*
Absolute kidney weight (g)	3.20	1.90	3.39	1.87	3.51	1.97	3.10	1.81
Relative kidney weight[a]	67.0	72.1	71.4	73.1	79.0*	79.2*	91.9*	80.8*

ppm: parts per million; *: $P < 0.05$

[a] g × 10 000/body weight.

Source: O'Hara & DiDonato (1984)

At macroscopic examination, prominent or accentuated lobular architecture of the liver was seen at 1500/2100/3000 ppm in males and at 5000/7000/10 000 ppm in both sexes, correlating with clinical chemistry or histopathological changes. Darkened livers were seen at 1500/2100/3000 ppm in males and at 5000/7000/10 000 and 15 000/21 000/30 000 ppm in both sexes. Swollen livers were seen only at 5000/7000/10 000 ppm in both sexes. Enlarged livers were seen in males at 1500/2100/3000 and 5000/7000/10 000 ppm. Darkened kidneys were seen in males at 5000/7000/10 000 ppm and in both sexes at 15 000/21 000/30 000 ppm. Histopathological changes are summarized in Table 8. Centrilobular or panlobular hepatocellular hypertrophy, including minimal to mild single-cell necrosis of hepatocytes, was seen in both sexes at 1500/2100/3000 ppm and above. Increased Kupffer cell pigmentation was seen at 5000/7000/10 000 ppm. Zones of swollen, vacuolated hepatocytes were seen in males at 5000/7000/10 000 ppm. In the kidneys, a minimal to mild pigmentation of the convoluted tubular epithelium was seen in males at 1500/2100/3000 and above. In the adrenals, there was increased vacuolation of the adrenal cortex in males at 1500/2100/3000 ppm and above. In the thyroid, an increased number of small follicles was seen in males at 1500/2100/3000 and above. An increase in the severity of pigmentation (haemosiderosis) in the red pulp of the spleen was observed in both sexes at 1500/2100/3000 ppm and above, and an increased incidence of chronic alveolitis was seen in males at 5000/7000/10 000 ppm. No treatment-related changes were seen in the 500/700/1000 ppm dose group.

The NOAEL for 90-day toxicity in rats was 500/700/1000 ppm (equal to 51.5 mg/kg bw per day), based on increased liver and kidney weights, hepatocellular hypertrophy, single-cell necrosis in the liver and pigmentation in tubular epithelium in the kidneys at 1500/2100/3000 ppm (equal to 158 mg/kg bw per day) (O'Hara & DiDonato, 1984).

Table 8. Summary of histopathological changes in the 90-day oral toxicity study in rats

	Incidence of histopathological changes									
	0 ppm		150/210/300 ppm		500/700/1 000 ppm		1 500/2 100/3 000 ppm		5 000/7 000/10 000 ppm	
	M	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
No. of mice examined	*10*	*10*	*10*	*0*	*10*	*10*	*10*	*10*	*10*	*10*
Liver										
Centrilobular or panlobular hepatocellular hypertrophy	0	0	0	NE	0	0	10	7	10	10
Zones of swollen, vacuolated hepatocytes	0	0	0	NE	0	0	0	0	9	0
Coagulative necrosis	1	0	0	NE	0	0	0	0	2	0
Necrosis, hepatocellular, single cell	0	0	0	NE	0	0	1	3	1	1
Pigmented Kupffer cells	0	0	0	NE	0	0	0	0	1	4
Adrenal										
Vacuolation in the zona fasciculata cells	2	0	NE	NE	3	0	5	2	5	3
Kidney										
Pigmentation in tubular epithelium	0	0	0	NE	0	NE	7	0	8	0
Thyroid										
Increase in small follicles	1	1	NE	NE	2	0	3	2	4	1
Spleen										
Pigmentation in the red pulp	1	0	NE	NE	NE	NE	9	10	10	10

F: females; M: males; NE: not examined at this dose; ppm: parts per million

Source: O'Hara & DiDonato (1984)

Dogs

A dose-finding study was conducted in dogs. Myclobutanil (TD 83-087; lot no. LSPL 83-0017E; purity 84.5%) was administered in the diet to Beagle dogs (two of each sex per group) for 4 weeks at a dietary concentration of 0, 50, 250 or 1000 ppm (equal to 0, 2.0, 10.5 and 45.3 mg/kg bw per day for males and 0, 2.2, 10.6 and 39.3 mg/kg bw per day for females, respectively) and for 2 weeks at 4000 ppm (equal to 45.0–47.0 mg/kg bw per day for males and females, respectively). Acetone (100 mL) was used to facilitate premixing of appropriate amounts of myclobutanil into the diet. During the second week of treatment, granular beef bouillon was added to the 4000 ppm diets in an attempt to increase palatability. No organ weights were assessed, and histopathology was not conducted. Physical examinations included evaluation of external structure, behaviour, posture, gait, mucous membranes, body temperature, muscle tone and the presence or absence of reflexes. The thorax was examined by stethoscope for irregularities of respiration or heart rate.

There were no treatment-related effects on mortality, clinical signs, heart rate or body temperature. During both weeks of exposure to 4000 ppm, a 20% decrease in body weight was observed in both sexes. A slight decrease in body weight gain was observed in females at 1000 ppm during the first week of treatment, but their weights were comparable to those of controls during the remaining 3 weeks of treatment. Feed consumption was reduced in both sexes at 4000 ppm during the 2 weeks of treatment, by less than 4% of control values during week 2. The addition of beef bouillon to the diet did not increase feed consumption at 4000 ppm. The transitory reductions of feed consumption and corresponding decreases in body weight gains at the beginning of the treatment were considered to be due to low palatability of the test material, rather than a general toxic effect. Feed consumption for females at 1000 ppm was slightly decreased during the first week of treatment (28% less consumption than during the week prior to treatment), but was comparable to control values for the remainder of the study.

There were no treatment-related effects on haematology or clinical chemistry parameters monitored at 2 or 4 weeks. Glucose was decreased at 4000 ppm in both sexes at 12 days, but this was considered to be a reflection of the dogs' decreased feed consumption. No treatment-related gross lesions were observed at necropsy (Goldman & Emmons, 1986).

A 3-month dietary toxicity study was conducted in dogs. This study was not conducted in accordance with test guidelines, but, with the exception of urine analysis, almost all examinations required by test guidelines were conducted. Myclobutanil (lot no. LSPL0016/E; TD 83-076; purity 81.1%) was administered in the diet to five groups of 5-month-old Beagle dogs (four of each sex per group) for 3 months at a dietary concentration of 0, 10, 200, 800 or 1600 ppm (equal to 0, 0.34, 7.26, 29.1 and 56.8 mg/kg bw per day for males and 0, 0.42, 7.88, 32.4 and 58.0 mg/kg bw per day for females, respectively).

There were no deaths or signs of treatment-related toxicity. There were no treatment-related effects on heart rate or body temperature or any other unusual signs in the treated dogs when compared with controls. Myclobutanil at 1600 ppm caused a statistically significant decrease in body weight for males during the first 2 weeks and for females during the first 3 weeks. Both sexes showed an approximately 300 g reduction in body weight at day 7. After these decreases, the weight gains were comparable to those of controls. The reduced body weights at the beginning of the study in both sexes were reflective of low palatability at 1600 ppm. Feed consumption was decreased for males during the first 7 weeks (approximately 85% of controls) and for females during the entire exposure period (13–25% of controls); however, the decreases were not accompanied by body weight changes in both sexes. There were no observable ocular abnormalities attributed to treatment with myclobutanil.

In haematology, increased platelets in females at 1600 ppm (16% increases above control values at 3 months) were considered to be treatment related, as similar increases were observed in the 1-year oral toxicity study at the same dose level (see below). In blood chemistry, serum ALP in females at 1600 ppm was at least twice as high as control values (246% and 346% compared with control values at 1 and 3 months, respectively). Slight (< 50% compared with control values) and/or transitory increases for males at 1600 ppm and for males and females at 800 ppm were not considered adverse.

Absolute and relative liver weights at 800 ppm (24% increase in relative weight in males only) and 1600 ppm (42% and 31% increases in relative weight in males and females, respectively) were statistically significantly increased at 3 months. No treatment-related lesions were evident at necropsy. Microscopically, centrilobular or midzonal hepatocellular hypertrophy occurred with a dose-related severity (minimal to mild) at 200 ppm (males only, 3/4), 800 ppm (8/8) and 1600 ppm (8/8). Periportal hepatocytes were minimally enlarged in severely hypertrophied livers. Liver hypertrophy, including increased weights and/or centrilobular/midzonal hepatocellular hypertrophy, without co-expressed hepatotoxicity in males at 200 and 800 ppm was considered to be adaptive. There is a possibility that the liver hypertrophy with clearly increased ALP in females at 1600 ppm was adverse.

The NOAEL for 90-day oral toxicity in dogs was 800 ppm (equal to 29.1 mg/kg bw per day in males), based on liver hypertrophy, increased alkaline phosphatase and increased platelets at 1600 ppm (equal to 56.8 mg/kg bw per day in males) (McLaughlin & DiDonato, 1984).

A 1-year oral toxicity study was conducted in dogs. Although this study was GLP compliant, it was not conducted in accordance with test guidelines. However, it is considered that the data were adequate for the toxicological evaluation. In this study, myclobutanil (TD 84-063; lot no. 83159-7; purity 91.4%) was administered in the diet to five groups of Beagle dogs (six of each sex per group) for 1 year at a dietary concentration of 0, 10, 100, 400 or 1600 ppm (equal to 0, 0.34, 3.09, 14.3 and 54.2 mg/kg bw per day for males and 0, 0.40, 3.83, 15.7 and 58.2 mg/kg bw per day for females, respectively). Haematological and clinical biochemical examinations were conducted at predosing weeks −2 and −1 and dosing weeks 13, 25, 39 and 53.

There were no deaths or clinical signs of treatment-related toxicity. Body weight gain of male dogs at 1600 ppm was statistically significantly decreased after 1 week of treatment (80% of controls), but was comparable to that of controls throughout the remainder of the study. Group mean body weights for females at 1600 ppm were significantly below control values during the first 5 weeks of the study, with a loss in actual weight (109 g) during the first week and a reduced body weight gain during the second week (8 g compared with a gain of 194 g in controls). In males, feed consumption was reduced during the first week (approximately 200 g lower than controls), but otherwise was comparable with that of controls during the study. Feed consumption in females at 1600 ppm was consistently lower than control values throughout the study. The decreased feed consumption throughout the study in females at 1600 ppm was not accompanied by body weight changes. There were no ocular abnormalities attributed to the treatment.

In male dogs at 1600 ppm, a slight but statistically significant decrease in red blood cells (9.3–10.4%) and a constant increase in platelets were observed throughout the study. These changes were not detected in females at the same dose. In males at 400 ppm, slight but statistically significant increases in platelet counts were found at a few time points, but the individual values were consistent during the experimental period, including pretest in males, at this dose; the control values were incidentally low. No other changes were seen at 400 ppm. Therefore, the increased platelets in males at 400 ppm were not considered to be treatment related. Although statistically significant increases in mean corpuscular haemoglobin (MCH) and MCV during weeks 13–53 were seen primarily in males, the increases were very slight (within 4–6% and 2–3% of control values for MCH and MCV, respectively). The absence of any progression in these parameters with increased treatment period indicated that they were not toxicologically significant.

The activity of serum ALP in females at 1600 ppm was consistently increased to 2-fold above the control group and pretreatment values. About half the number of males in this group showed slight but consistent increases in ALP compared with pretest values, and most dogs did not indicate an age-matched decrease at 1600 ppm. Although an age-matched decrease in ALP was not observed in females at 400 ppm, the pretest values were high in two dogs in this group. ALT activity was also slightly increased in males in weeks 25 and 53 at 1600 ppm. Although the consistent increases in ALP in both sexes at 1600 ppm might indicate adverse effects on the liver, there was no progression in ALP values. The increase in ALP in females at 400 ppm was not considered adverse because there were no other parameters indicating hepatotoxicity at this dose level. Serum albumin levels were decreased in both sexes at 1600 ppm. Slight but statistically significant changes in GGT at week 53 or non-dose-dependent changes in inorganic phosphorus concentrations in serum were not considered toxicologically significant because they were slight or transitory. No treatment-related changes were observed in urine analysis.

Increased absolute and relative liver weights were observed in both sexes at 1600 ppm and in females at 100 and 400 ppm. Macroscopically, enlarged livers and/or accentuated lobular architecture were observed in one male and three females at 1600 ppm. Treatment-related histopathological changes were observed in the livers in both sexes at 400 ppm and above. Minimal to mild

hepatocellular hypertrophy was noted in males at 1600 ppm. Mild to moderate hepatocellular hypertrophy was noted in females at 400 ppm and above. No treatment-related changes were observed at 100 ppm. The distribution of the hepatocellular hypertrophy was predominantly centrilobular. At 1600 ppm, the more severely affected females showed ballooned hepatocytes in the centrilobular area of the liver. This change might indicate a degenerative process of hepatocytes resulting from severe hepatocellular hypertrophy. Hepatocellular hypertrophy and/or increase in liver weights not accompanied by indicators of hepatotoxicity in both sexes at 400 ppm were considered adaptive. Other hepatic changes and changes in other tissues were considered to be incidental and unrelated to treatment. Treatment-related liver effects are summarized in Table 9.

The NOAEL for 1-year oral toxicity in dogs was 400 ppm (equal to 14.3 mg/kg bw per day), based on increased ALP, hepatocellular hypertrophy, ballooned hepatocytes and increased platelets in males at 1600 ppm (equal to 54.2 mg/kg bw per day) (Goldman & Harris, 1986a).

(b) Dermal application

A 4-week dermal toxicity study of two formulations of myclobutanil, namely myclobutanil 2EC (TD no. 85-109; lot no. EG-0807-1) and myclobutanil 40WP (TD no. 85-110; lot no. EG-0809-1), was conducted in rats. This study complied with GLP. Three aqueous dilutions of myclobutanil 2EC at 0.07%, 0.67% and 6.67% w/v, corresponding to doses of 1, 10 and 100 mg/kg bw per day, and one aqueous dilution of myclobutanil 40WP at 6.67% w/v, corresponding to 100 mg/kg bw per day, were applied to the intact skin of six Sprague-Dawley rats of each sex per group. The doses were applied (1.5 mL/kg bw), non-occluded, once daily for 6 hours, for a total of 19–20 exposures over a 4-week period. Two additional groups of six rats of each sex were similarly treated with either distilled water or a myclobutanil 2EC formulation blank.

There were no deaths or signs of general toxicity during this study. Treatment-related effects were limited to the site of application. Skin irritation, scored as slight (days 2–25 in males and days 2–29 in females) to moderate (days 26–29), was observed in the 6.67% myclobutanil 2EC group. Females in the 2EC vehicle control group showed slight irritation on day 2 and days 20–29, and males in the 2EC vehicle control group showed slight irritation only on day 2. There were no treatment-related effects on body weight, feed consumption, haematological parameters, blood chemistry parameters or organ weights, except liver. Slight but statistically significant increases (13%) in relative liver weights were seen, but there was no dose–response relationship. Macroscopically, skin treated with both formulations of 6.67% myclobutanil and the 2EC vehicle control group showed eschar (sloughing of dead skin) and desiccation, one male in the control group had pinpoint red discolorations and another had thickening of the skin. Microscopic examination of the treated skin of the 6.67% myclobutanil 2EC group showed significant dermal irritation. Similar, but less severe, findings were seen in the 2EC vehicle control group. The skin of rats in the myclobutanil 40WP group exhibited a minimal degree of epidermal necrosis, epidermal thickening and/or subacute/chronic inflammation of the dermis. There were no treatment-related changes in any other organ. Skin irritation and/or minimal gross and microscopic changes in the treated skin were observed at the treated site with both formulations.

The NOAEL for systemic toxicity of both formulations via dermal exposure was 100 mg/kg bw per day, the highest dose tested (Bonin & Hazelton, 1986).

(c) Exposure by inhalation

Inhalation studies were not performed.

2.3 Long-term studies of toxicity and carcinogenicity

Mice

To assess chronic toxicity and carcinogenicity, a 2-year dietary study in mice was conducted. This study complied with GLP and was conducted according to test guidelines authorized by the United States Environmental Protection Agency and the Organisation for Economic Co-operation and Development. Myclobutanil (TD no. 83-260; lot no. LAP 0298; purity 90.4%) was administered to

Table 9. Summary of blood biochemistry and liver changes in 1-year oral toxicity study in dogs

	Males					Females				
	0 ppm	**10 ppm**	**100 ppm**	**400 ppm**	**1 600 ppm**	**0 ppm**	**10 ppm**	**100 ppm**	**400 ppm**	**1 600 ppm**
No. of dogs	*6*	*6*	*6*	*6*	*6*	*6*	*6*	*6*	*6*	*6*
Platelets (10^3/mm^3)										
Week –2	292.3	298.0	296.2	313.2	320.2	285.7	321.7	361.8	386.7	316.7
Week –1	290.2	286.3	289.5	316.5	318.2	281.2	329.0	372.0	310.2	310.2
Week 13	245.7	284.5	266.5	334.3*	396.3*	304.8	309.5	369.0	368.7	368.7
Week 25	272.5	309.8	259.2	330.2	374.2*	322.3	341.2	389.8	362.0	362.0
Week 39	326.5	347.7	301.0	366.7	409.2*	371.7	390.0	385.7	425.0	418.3
Week 53	286.8	371.3*	291.8	345.7*	402.3*	337.0	367.7	363.5	397.2	384.5
ALP (U/L)										
Week –2	90.5	111.6	95.5	103.7	83.2	92.7	106.6	148.5	109.3	107.8
Week –1	88.2	107.8	91.7	98.7	81.3	85.8	103.5	139.5	106.3	103.0
Week 13	68.5	73.2	59.3	78.2	109.3*	72.2	89.0	102.2	102.2	213.7*
Week 25	55.5	60.5	41.8	66.0	104.5*	68.5	73.2	81.2	101.3	211.0*
Week 39	43.5	49.8	29.0	53.7	90.8*	57.2	57.5	58.9	90.5	218.2*
Week 53	40.0	53.2	27.7	53.2	97.3*	57.5	60.7	59.0	91.7*	187.0*
ALT (U/L)										
Week –2	22.0	19.2	19.3	19.0	19.5	22.0	15.0	20.5	23.0	19.8
Week –1	22.5	20.2	18.8	18.2	18.5	21.6	17.2	20.2	21.5	22.3
Week 13	19.8	22.7	20.2	21.5	22.0	23.2	18.3	22.8	17.7	21.8
Week 25	22.3	23.2	23.2	18.2	30.5* (36)[a]	19.8	16.3	19.8	18.2	21.0
Week 39	21.0	21.0	20.2	16.7	25.7	19.7	19.8	20.6	18.0	23.8
Week 53	23.7	23.2	23.2	20.5	29.3* (27)[a]	22.7	18.0	20.8	19.7	25.3

	Males					Females				
	0 ppm	**10 ppm**	**100 ppm**	**400 ppm**	**1 600 ppm**	**0 ppm**	**10 ppm**	**100 ppm**	**400 ppm**	**1 600 ppm**
Liver weight										
Absolute[b]	299	265	291	291	389*	226	260	281*	295*	349*
Relative[c]	295	300	294	337	424*	290	346*	330	370*	441*
Histopathology										
Hepatocellular hypertrophy, centrilobular	0	0	0	1	5	0	0	0	2	6
Ballooned hepatocyte, centrilobular	0	0	0	0	0	0	0	0	0	4

ALP: alkaline phosphatase; ALT: alanine aminotransferase; ppm: parts per million; U: units; *: $P < 0.05$

[a] Percentage of the control value.
[b] Absolute weight (g).
[c] Relative weight (absolute weight (g) / body weight (kg) × 100.

Source: Goldman & Harris (1986a)

CD-1 mice (110 of each sex per group) in the diet at a dose of 0, 20, 100 or 500 ppm (equal to 0, 2.7, 13.7 and 70.2 mg/kg bw per day for males and 0, 3.2, 16.5 and 85.2 mg/kg bw per day for females, respectively) for up to 2 years. At 3, 6 and 12 months, 10 mice of each sex per group were examined.

There were no treatment-related effects on survival or clinical signs in the treated groups. Significant decreases in body weight were seen almost throughout the study in females and after week 11 in males at 500 ppm. No treatment-related changes in feed consumption or ocular changes were seen in either sex in any dose group.

Blood samples were taken at 3, 6, 12 and 24 months for haematology and blood chemistry examinations. At all time points tested, no haematological parameters were affected in either sex of any treated group. In blood chemistry at 3 months of treatment, ALT was increased in females at 500 ppm (60% increase compared with controls). In males, total protein values were decreased at 100 ppm and above, and total bilirubin was increased at 500 ppm. However, these changes were not seen at later sampling times, suggesting that the toxicological significance of these findings is questionable. Other differences seen in blood chemistry parameters were not treatment related after 3 months. No treatment-related changes in blood chemistry parameters were seen at 6, 12 or 24 months in either sex. No treatment-related effects were found in urine analysis at any time point.

Hepatic MFO activity was significantly increased in females at 100 ppm and in both sexes at 500 ppm at 3 months. This increase was approximately 2-fold in males at 500 ppm and 1.3- and 1.5-fold in females at 100 and 500 ppm, respectively. At 6 months, hepatic MFO activity was increased 1.3- and 2.8-fold in males and 1.4- and 2.9-fold in females at 100 and 500 ppm, respectively. At 12 months, hepatic MFO activity was increased 1.5- and 3.7-fold in females at 100 and 500 ppm, respectively. MFO activity of male mice at 500 ppm was increased 1.6-fold. The increases in males at 500 ppm and in females at 100 ppm were not statistically significant.

Myclobutanil had no effect on hepatic microsomal protein content at concentrations up to and including 500 ppm at 3 months. At 6 months, hepatic microsomal protein content was increased by 28% and 21% in males and females, respectively. Hepatic microsomal protein content was not affected in males or females at 12 months.

Hepatic palmitoyl coenzyme A oxidase activity was not affected by the 12-month treatment.

Liver weight (absolute and relative) was significantly increased in both sexes at 500 ppm at 3 months. No treatment-related changes were seen in any of the organ weights after 6, 12 and 24 months of treatment.

In histopathology for non-neoplastic changes, some treatment-related changes were observed in the liver of males at 500 ppm. Hepatocellular hypertrophy was the main change seen from 3 to 12 months, but it was no longer evident at 24 months of treatment. After 6 months, pigmentation of Kupffer cells, single-cell necrosis of hepatocytes or vacuolated hepatocytes were observed in males at 500 ppm, but no progression was found in these lesions with increasing age. The total incidence of focus of hepatocellular alteration was slightly increased in males at 500 ppm at 24 months, but there was no clear difference in the type of focus among the groups. The incidence and severity of these changes were similar to those seen in control females. The treatment-related changes in the liver are summarized in Table 10. Numerous microscopic findings were encountered in mice of all groups, including the controls, but these changes were of types that are common incidental findings in laboratory mice of this age.

In histopathology for neoplastic changes, various tumours occurred at similar incidences and onset times among the control and treated groups in both sexes or at single or very low incidence. None of them was considered to be treatment related.

The NOAEL for 2-year oral toxicity in mice was 100 ppm (equal to 13.7 mg/kg bw per day), based on the histopathology related to hepatotoxicity at 500 ppm (equal to 70.2 mg/kg bw per day). The NOAEL for carcinogenicity was 500 ppm (equal to 70.2 mg/kg bw per day), the highest dose tested (Goldman & Harris, 1986b).

Table 10. Summary of histopathology in the liver in a 2-year oral study in mice

	Incidence of finding							
	Males				Females			
Time point / findings	0 ppm	20 ppm	100 ppm	500 ppm	0 ppm	20 ppm	100 ppm	500 ppm
3 months / No. of mice examined	10	10	10	10	10	10	10	10
Hypertrophy, hepatocellular, centrilobular	1	1	1	9	0	0	0	0
6 months / No. of mice examined	10	10	10	10	10	10	10	10
Hypertrophy, hepatocellular, centrilobular	2	2	1	9	0	0	0	0
Pigmentation, Kupffer cells	0	0	0	5	0	0	0	0
12 months / No. of mice examined	20	20	20	20	20	20	20	20
Hypertrophy, hepatocellular, centrilobular	5	6	5	16	1	0	1	2
Pigmentation, Kupffer cells	4	1	4	12	4	2	1	4
Individual hepatocellular necrosis, multifocal	2	1	1	6	0	0	1	2
Vacuolation, punctate, multifocal	0	0	0	4	0	1	1	3
12–24 months / No. of mice examined	66	63	65	62	64	66	66	67
Hypertrophy, hepatocellular, centrilobular[a]	8	6	5	11	1	3	2	1
Hypertrophy, hepatocytes, diffuse	5	5	1	3	2	1	1	0
Hypertrophy, hepatocytes, midzonal	0	0	0	0	0	1	0	0
Periportal punctate vacuolation	1	0	2	7	3	2	0	7
Total incidence of cellular alterations[b]	4	4	6	11	2	2	2	6

ppm: parts per million

[a] Combined minimal, slight and moderate due to no enhancement with increasing dose.

[b] Combined basophilic, clear cell, eosinophilic and vacuolated cell types.

Source: Goldman & Harris (1986b)

To confirm the carcinogenicity of myclobutanil at higher doses, a second dietary carcinogenicity study was conducted, but using only female mice. This study complied with GLP and was conducted according to test guidelines, with the deviation that only one dose level was tested. Myclobutanil (TD no. 90-016; lot no. 2-2943; purity 92.9%) was administered to 60 CD-1 female mice per group in the diet at a concentration of 0 or 2000 ppm (equal to 394 mg/kg bw per day) for up to 18 months to determine the carcinogenic potential at the MTD. Physical examinations including palpation were performed weekly.

Survival in the treated group was similar to that of controls. Numerous clinical signs were noted throughout the study, but the type and incidence were similar in control and treated mice. Treatment-related, statistically significant effects on body weight were seen in treated mice, with decreases in body weight gain (12–26%) throughout the study. Overall, the body weight gain for treated mice was 20% less than that of controls. Feed consumption was generally lower in treated mice than in controls throughout the study. Statistically significant decreases in feed consumption were seen during the first 2 weeks of the study.

A statistically significant increase (36%) in white blood cell count at 2000 ppm at 18 months was considered incidental because of a lack of increase in related lesions, including inflammation. No treatment-related changes were seen in blood chemistry. Urine analysis was not conducted. Statistically significant increases in absolute (30% and 23%, respectively) and relative (32% and 33%, respectively) liver weights were noted at 12 and 18 months. There was no notable difference in effect between the two time points. Changes observed in brain and kidney weights were considered to be a reflection of decreased terminal body weight.

No treatment-related macroscopic findings were observed at 12 or 18 months. Treatment-related microscopic changes were observed in the liver and adrenal glands at 2000 ppm at 12 and 18 months, with incidence increasing with time. Liver changes were primarily slight to moderate hepatocellular hypertrophy in most of the treated mice, minimal to slight single-cell necrosis of hypertrophied hepatocytes in several mice and slight to moderate vacuolation (fatty change) of hepatocytes. Changes in the adrenal glands included hypertrophy of the zona fasciculata of the cortex. There was no effect on the incidence or type of neoplastic changes.

Myclobutanil demonstrated no carcinogenicity up to the MTD in mice. The major target of myclobutanil in mice was the liver. Effects at 2000 ppm were qualitatively similar to those observed at lower doses in the earlier study of Goldman & Harris (1986b) described above (Anderson, O'Hara & Brown, 1993).

Rats

A 2-year oral toxicity study was conducted to investigate the chronic toxicity and carcinogenicity of myclobutanil in rats. Although this study complied with GLP, it was not conducted according to test guidelines. However, it is considered that the data were adequate for toxicological evaluation. Myclobutanil (weeks 1–15: TD no. 83-260; lot no. LAP 0298; purity 90.4%; week 16 to termination: TD no. 84-038; lot no. 83159-7; purity 91.4%) was administered to male and female Sprague-Dawley rats in the diet for up to 2 years. Hepatic MFO activity was determined in six rats of each sex per group at 3, 6 and 12 months, by in vitro enzyme assay of demethylation of aminopyrine. Hepatic palmitoyl coenzyme A activity was determined in six rats of each sex per group at 12 months. Organ weights, except liver weights, were measured at necropsy at 12, 17 and 24 months.

The experimental design is summarized in Table 11.

Table 11. Experimental design of 2-year oral toxicity study in rats[a]

Concentration of myclobutanil in diet (ppm)			Chemical intake (mg/kg bw per day)[b]		No. of rats killed at month:				
Weeks 1 & 2	**Weeks 3 & 4**	**Week 5 – termination**	**Males**	**Females**	**3**	**6**	**12**	**17**	**24**
0	0	0	0	0	10	10	20	18/10[c]	All surviving for postmortem
25	35	50	2.5	3.2	10	10	20	18/10	
100	140	200	9.8	12.8	10	10	20	18/10	
400	560	800	39.2	52.3	10	10	20	18/10	

bw: body weight; ppm: parts per million

[a] Total number of males: 114; total no. of females: 106.
[b] Mean compound intake over 24 months.
[c] Males/females.

Source: Shellenberger (1986)

Myclobutanil did not affect the survival of males or females. Although various clinical signs were found throughout the study in both sexes, all clinical signs were considered to be unrelated to the treatment. Myclobutanil treatment caused approximately 30–40 g lower body weights in males at 800 ppm between 6 and 18 months, compared with controls, but had no significant effect on body weights at 50 and 200 ppm. In females, body weights during the first and second weeks of treatment were decreased at 200 ppm and above, an effect that may be related to the small decreases (6–8 g per rat per week) in feed consumed, rather than an acutely toxic effect of exposure. Body weights in all treated groups were comparable with those of the control group throughout the first 52 weeks. During the second year, body weights in females at 800 ppm were generally lower than the control values. Feed consumption of males at 800 ppm was decreased to week 78 as a result of treatment. Feed consumption of females at 800 ppm was significantly decreased compared with that of controls at weeks 1, 14, 48 and 66. In ophthalmological examination, no treatment-related changes were observed in either sex in any treated group.

At 12 months, red blood cell counts, haemoglobin and haematocrit were slightly lower (approximately 10% in each) in males at 800 ppm than in the controls, but the differences were not statistically significant. There were no apparent differences between control and treated males at 17 months and prior to termination of the study. Haematological parameters determined in control and treated female rats were similar at all time points. The marginal decreases in haematological parameters at 12 months were considered transitory and not toxicologically significant. The blood chemistry revealed no consistent differences between control and compound-treated males or females. In urine analysis, there were no consistent differences in parameters attributed to treatment at 3, 5, 11 and 17 months and prior to termination.

A slight increase in MFO activity (34–47%) was seen in males at 800 ppm during 3–12 months. In female rats, MFO activity was statistically significantly increased by 61% and 78% at 200 and 800 ppm, respectively, at 3 months. At 6 and 12 months, slight increases (not statistically significant) were seen in females at 800 ppm at 6 and 12 months (35% and 40%). Myclobutanil had no effects on hepatic peroxisomal [^{14}C]palmitoyl coenzyme A oxidase activity in any treated group after 12 months.

There were no statistically significant differences in absolute or relative liver weights in males at any time point. In females, relative weights were slightly (approximately 10%), but statistically significantly, increased at 3 months. At other time points, statistically significant changes were not seen in relative liver weights in females. After 12 months, the absolute and relative testicular weights at 800 ppm were lower than the control values. The decreases were enhanced with increased age. At termination, absolute testes weights were statistically significantly reduced at 200 ppm and higher. The reduced testes weights at 800 ppm were correlated with testicular size reduction or soft testes (24 months only) at necropsy at 12, 17 and 24 months.

At 12 months and termination, absolute and relative organ weights for brain, heart, adrenals, kidneys and spleen were comparable with those of controls. Increases in absolute and relative ovarian weights were seen at 12 months, but were not statistically significant. There was no effect at termination. Other gross lesions were considered to be incidental findings in males and females.

In histopathology for non-neoplastic findings, a treatment-related finding was observed in the testes (Table 12). The incidence of testicular atrophy was increased at 200 ppm and above at termination after 12 months of treatment. Incidences of the atrophy in the control and 50 ppm groups were similar, but the incidence was slightly increased at 200 ppm and above, with dose dependency. Testicular atrophy was characterized as the frequent lack of spermatid formation and germ epithelium in the seminiferous tubules. In severe cases, only Sertoli cells remained in the tubules. No treatment-related effects were observed in the livers of rats at any of the time points examined. A slight increase in hepatocellular fatty change in the centrilobular area in males at 800 ppm at 17 months only was not considered to be treatment related because of its transitory nature. Various non-neoplastic and neoplastic changes observed throughout the study in either sex were considered to be incidental and not treatment related.

Table 12. Summary of testicular changes in a 2-year oral toxicity study in rats

	0 ppm	50 ppm	200 ppm	800 ppm
Testicular weight				
12 months / No. of rats examined	20	19	20	20
Absolute (g)	3.751	3.661	3.524	3.300*
Relative (testis to body weight ratio, g/100 g)	0.556	0.512	0.516	0.507
17 months / No. of rats examined	18	18	18	18
Absolute	3.341	3.393	3.655	3.017
Relative (testis to body weight ratio, g/100 g)	0.4334	0.449	0.470	0.389
24 months / No. of rats examined	17	19	20	22
Absolute	3.223	3.006	2.491*	2.430*
Relative (testis to body weight ratio, g/100 g)	0.492	0.488	0.444	0.399
Histopathology				
12 months / No. of rats examined	20	19	20	20
Atrophy – unilateral	0	1	0	0
Atrophy – bilateral	0	0	1	3
17 months / No. of rats examined	18	18	18	18
Atrophy – unilateral	2	2	2	1
Atrophy – bilateral	2	2	0	4
24 months / No. of rats examined	17	19	20	22
Atrophy – unilateral	2	3	6	2
Atrophy – bilateral	2	1	5	13

ppm: parts per million; *: $P < 0.05$

Source: Shellenberger (1986)

The NOAEL for 2-year oral toxicity in rats was 50 ppm in males (equal to 2.5 mg/kg bw per day), based on the testicular toxicity found after 12 months of treatment at 200 ppm (equal to 9.8 mg/kg bw per day). The NOAEL for carcinogenicity was 800 ppm (equal to 39.2 mg/kg bw per day), the highest dose tested (Shellenberger, 1986).

To confirm the apparent lack of carcinogenicity of myclobutanil, a second dietary carcinogenicity study was conducted using male and female rats in which myclobutanil was administered at a single dose higher than the top dose used in the earlier study. This study complied with GLP and was conducted according to test guidelines, with a deviation in that a single dose level was tested. Myclobutanil (lot no. 2-2943; purity 92.9%) was administered to 60 Sprague-Dawley rats of each sex per group at a dietary concentration of 0 or 2500 ppm (equal to 0 and 106 or 136 mg/kg bw per day for males and females, respectively) for 104 weeks. These conditions represented an anticipated MTD based on a previous 90-day toxicity study in rats. An interim kill was conducted at 52 weeks using 10 rats of each sex per group. Ophthalmological and blood chemistry examinations were not determined.

There was no apparent compound-related effect on survival throughout the study. No treatment-related clinical signs were observed in either sex throughout the study. Slight but statistically significant decreases in body weight were noted in males at weeks 26 and 52 and in

females at week 52 at 2500 ppm. No statistically significant differences in body weight were noted at week 104. There were no treatment-related changes seen in feed consumption. In haematology, statistically significant decreases in nucleated red blood cells were observed in males at 2500 ppm at week 53.

Treatment-related findings were limited to the liver and testes. A significant increase was noted in absolute and relative liver weights in males and relative liver weight in females at 2500 ppm at 12 months. At 24 months, no changes were noted in either sex. Absolute and relative weights of left testis and bilateral testes were significantly lower in males at 2500 ppm at 12 months. At 24 months, absolute weights of left testis and bilateral testes were also increased, but their relative (to body weight) weights were not different from those in control rats. Macroscopically, enlarged liver, thickened lobes in the liver and small and soft testes were increased in rats at 2500 ppm at the 12- and 24-month kills and in unscheduled deaths. The histopathological changes in the liver consisted of statistically significant increases in centrilobular to midzonal hepatocellular enlargement (equal to hypertrophy) and vacuolation (equal to fatty change) at 2500 ppm of both sexes. The changes were present at the 12-month interim kill and were not appreciably different at study termination. The liver changes were consistent with increased absolute and relative liver weights in treated rats. The incidence of liver neoplasia or foci of cellular alteration was not different in control and treated groups. Compound-related testicular changes occurred as bilateral aspermatogenesis in 22/60 treated rats, compared with 2/60 control rats; the increase was statistically significant. Normal bilateral spermatogenic activity was observed in 49/60 control and 24/60 treated rats. Decreased spermatogenic activity was associated with a significantly increased incidence of hypospermia and cellular debris in the epididymides of treated rats. Other treatment-related effects on incidences of non-neoplastic and neoplastic lesions were not seen in either sex. Liver and testicular changes are summarized in Table 13.

Myclobutanil was not carcinogenic in rats at dietary concentrations up to 2500 ppm (equal to 106 mg/kg bw per day). The primary toxicity targets of myclobutanil were liver and testis in rats, and the testicular toxicity at 2500 ppm was qualitatively in line with that detected at lower doses in the previous study (Wolfe, 1993).

2.4 Genotoxicity

The genotoxicity of myclobutanil was investigated in a comprehensive array of studies. Five in vitro and two in vivo genotoxicity tests were conducted, including a bacterial assay for reverse mutations, an in vivo chromosomal aberration assay in rat bone marrow cells, an in vitro assay for unscheduled DNA synthesis in primary cultures of rat hepatocytes, an in vitro forward mutation assay in Chinese hamster ovary (CHO) cells and in vivo mouse micronucleus tests in bone marrow (Table 14). These studies complied with GLP. Although most of studies were not conducted according to current test guidelines, they were considered to be adequate for the risk assessment. The second micronucleus test was conducted according to test guidelines.

Myclobutanil did not demonstrate any mutagenic properties in the TA98, TA100, TA1535 or TA1537 strains of *Salmonella typhimurium* tested in this study. Myclobutanil does not induce chromosomal aberrations in CHO cells in the presence or absence of metabolic activation. Treatment of male rat primary hepatocytes with myclobutanil did not result in the induction of unscheduled DNA synthesis. Myclobutanil did not induce mutations at the *Hgprt* locus in CHO cells in culture when tested in either the presence or absence of metabolic activation. In in vivo genotoxicity testing, under the conditions of this study, myclobutanil did not induce chromosomal aberrations in mouse bone marrow cells.

In conclusion, myclobutanil was negative in all genotoxicity studies in vitro and in vivo.

Table 13. Summary of organ weights and histopathological changes in the liver and testes in the second 2-year oral toxicity study in rats

	Males				Females			
	12 months		All animals		12 months		All animals	
	0 ppm	2 500 ppm	0 ppm	2 500 ppm	0 ppm	2 500 ppm	0 ppm	2 500 ppm
No. of rats examined	*10*	*10*	*60*	*60*	*10*	*10*	*60*	*60*
Liver weights								
Absolute (g)	19.57	23.48*	19.53	20.41	11.26	11.27	12.86	13.94
Relative (weight (g)/bw (g))	2.457	3.136*	2.733	2.963	2.478	3.023*	3.065	3.195
Testes weights								
Absolute (g)	3.73	2.25*	3.45	2.66*	–	–	–	–
Relative (weight (g)/bw (g))	0.470	0.308*	0.476	0.379	–	–	–	–
Histopathology, liver								
Centrilobular to midzonal hepatocellular enlargement	0	10	0	52	0	10	0	45
Centrilobular to midzonal hepatocellular vacuolation	0	8	0	32	0	2	0	13
Histopathology, testes								
Hypospermia, unilateral	0	0	4	8	–	–	–	–
Hypospermia, bilateral	0	2	5	3	–	–	–	–
Aspermatogenesis, unilateral	0	0	1	8	–	–	–	–
Aspermatogenesis, bilateral	0	6	2	22	–	–	–	–
Histopathology, left epididymis								
Immature abnormal sperm forms	0	1	12	9	–	–	–	–
Hypospermia	0	7	8	31	–	–	–	–
Lumen, debris, cellular	0	6	2	25	–	–	–	–
Histopathology, right epididymis								
Immature abnormal sperm forms	0	0	10	10	–	–	–	–
Hypospermia	0	6	6	27	–	–	–	–
Lumen, debris, cellular	0	6	2	22	–	–	–	–

ppm: parts per million; *: $P < 0.05$

Source: Wolfe (1993)

Table 14. Summary of genotoxicity studies with myclobutanil

Test	Test object	Concentration	Lot no. / purity	Result	Reference
In vitro					
Bacterial reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537	75, 250, 750, 2 500 and 7 500 µg/plate	LSPL 83/0017E / 84.5%	Negative ±S9	Byers & Chism (1983)
Bacterial reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537	75, 250, 750, 2 500 and 7 500 µg/plate	LAP-0298 / 90.4%	Negative ±S9	Byers & Chism (1984)
Mammalian cytogenetics	CHO cells	0.034–1 020 µg/mL	83159 / 91.9%	Negative ±S9	Ivett (1985)
Mammalian forward mutation	UDS: Primary rat hepatocytes	0.1–1 000 µg/mL	83159-5 / 91.9%	Negative	Muller (1986)
Mammalian forward mutation	CHO cells (CHO/*Hgprt*)	25–1 000 µg/mL	LSPL-0016/E / 81.1%	Negative ±S9	O'Neill, Foxall & Byers (1984)
In vivo					
Micronucleus	Mouse bone marrow polychromatic erythrocytes	0, 65, 260 and 650 mg a.i./kg bw	LSPL-0016/E / 81.1%	Negative	McLeod & McCarthy (1984)
Micronucleus	Mouse bone marrow polychromatic erythrocytes	0, 117, 585 and 1 170 mg a.i./kg bw	83159-7 / 91.4%	Negative	Sames & Frank (1987)

a.i.: active ingredient; bw: body weight; CHO: Chinese hamster ovary; Hgprt; hypoxanthine–guanine phosphoribosyltransferase; S9: 9000 × *g* supernatant fraction from rat liver homogenate; UDS: unscheduled deoxyribonucleic acid synthesis

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

A two-generation (two litters per generation) reproduction study was conducted in rats. This study complied with GLP, although it was not conducted according to test guidelines. However, this study generally met the requirements of current guidelines. Myclobunatil (TD no. 83-155; lot no. LSPL 83/0017E; purity 84.5%) was administered in the diet to three groups of 25 Crl:CD(SD)BR rats of each sex in the first (P_1) and second (P_2) parental generations at 0, 50, 200 or 1000 ppm. The chemical intake averaged for each generation and each group is shown in Table 15. Necropsies were performed on all P_1 and P_2 rats after pups from their second matings (F_{1b} and F_{2b} pups) were weaned. All organs, tissues and body cavities were examined, and any gross abnormalities were noted. The livers were weighed. Histopathological evaluation of the liver and reproductive organs was performed on all treated and control P_1 and P_2 rats. Necropsies were performed on all F_1 and F_2 pups that were found dead after 14 days of age. Gross observations were recorded, but no tissues were preserved.

Table 15. Average chemical intakes in two-generation reproductive toxicity study in rats

	Chemical intake (mg/kg bw per day)		
Generation	**50 ppm**	**200 ppm**	**1 000 ppm**
P_1 males	3.67	14.3	70.7
P_1 females	4.42	17.2	85.9
P_2 males	3.64	15.1	76.4
P_2 females	4.17	17.5	88.0

bw: body weight; P_1: first parental generation; P_2: second parental generation; ppm: parts per million

Source: Costlow & Harris (1985)

There were no treatment-related changes in mortality or clinical signs in either generation. Body weights in males and females at all treated doses were comparable with those of the controls in both generations. At 1000 ppm, feed consumption in males for weeks 1–4 and in females for weeks 1–2 of both generations was slightly, but not significantly, lower than in controls. The slight decreases in feed consumption by both sexes at 1000 ppm during the first few weeks were judged to be due to lower palatability at this dose, because of the lack of the same change when administration was by gavage and the lack of detected effects on the gastrointestinal tract in the present study. Feed consumption in the other treated groups was comparable with control values.

The litter data for the P_1 generation are summarized in Table 16. In reproductive performance of P_1/F_{1a}, the number of mated females that delivered was slightly, but statistical significantly, lower than in controls at 1000 ppm, whereas the number of P_1 females expressing positive signs of mating (sperm in vaginal smear), the mean number of days needed for mating to occur and the mean length of gestation were comparable with control values. This dose induced testicular toxicity; therefore, the lower number of females that delivered was possibly treatment related. Myclobutanil at 50 and 200 ppm did not affect the number of P_1 females that had positive signs of mating (sperm in vaginal smear) or the number of mated females that delivered.

In the second mating of the P_1 animals, there was no suggestion of an adverse effect on fertility in male or female rats.

In the P_1 males and females, dose-dependent increases in liver weight were observed in males (14%) and females (9%) at 1000 ppm. At 200 ppm, relative liver weights (7%) were increased in males only. In the P_1 rats, a treatment-related microscopic change was observed in the liver, consisting of centrilobular hypertrophy of hepatocytes in males (10/25) and females (8/25) and centrilobular vacuolation in males (8/25) at 1000 ppm. Increased liver weights at 200 ppm were considered to be adaptive, as there was no indication of hepatotoxicity at this dose.

In litter data for P_1/F_{1a} animals, the number of dead pups (12) was significantly higher than in controls (3) at 1000 ppm, and survival to day 4 was reduced as a consequence. Neither mean pup weight at birth (males, females or combined sexes) nor sex ratio was significantly different from control values at this dose, but the increase in the number of dead pups was judged to be treatment related. Litter data at 200 ppm and lower were comparable with those of the control group. At 1000 ppm, female pup weight was significantly less than that of controls on day 4 of lactation. By day 7 of lactation, pups of both sexes at 1000 ppm showed lower body weights. The reduced weight gain was persistent through day 21 of lactation, and the difference in weight between controls and pups at 1000 ppm was increased.

In P_1/F_{1b} animals, the number of pups born dead at 200 ppm and above was statistically significantly elevated over controls. The increase at 1000 ppm, similar to the data for the P_1/F_{1a} and P_2 animals, was judged to be treatment related. However, the number at 200 ppm was the same as for the P_1/F_{1a} animals, which was not statistically significant. The control value for F_{1b} animals was 0,

Table 16. Summary of litter data for P_1 animals in a reproductive toxicity study in rats

	F_{1a}				F_{1b}			
Litter data	**0 ppm**	**50 ppm**	**200 ppm**	**1 000 ppm**	**0 ppm**	**50 ppm**	**200 ppm**	**1 000 ppm**
No. of pups/litter at birth	13.7	12.8	13.7	12.3	13.0	13.3	13.7	14.2
Sex ratio (M/(M + F)) at birth	0.44	0.47	0.50	0.53*	0.45	0.46	0.45	0.52
No. of pups born dead	3	4	9	12*	0	6	9*	16*
Viability index[a]	98.4	97.1	96.4	92.7*	89.9	85.3	77.1	86.2
Litter size – live pups								
Birth	313	302	293	233	287	292	315	327
Day 4 pre-cull	311	297	291	227	258	249	243	282
Day 4 post-cull	226	224	209	177	208	200	202	211
Day 7	226	224	209	177	206	198	200	211
Day 14	225	224	209	177	205	198	199	210
Day 21 (weaning)	225	224	209	177	204	198	196	210
Body weight (g)/pup								
Day 0	6.0	6.1	6.2	6.3	5.9	6.0	6.1	5.9
Day 4 pre-cull	9.6	9.9	9.6	9.4	9.5	9.0	9.4	8.5
Day 7	15.3	15.1	15.0	14.3**	15.2	14.3	14.9	13.1**
Day 14	29.6	29.6	29.2	26.7**	30.4	29.1	29.8	26.9**
Day 21 (weaning)	45.7	45.9	44.4	41.9**	46.6	45.6	46.2	42.2**

F: females; F_{1a}: first mating of P_1 parents; F_{1b}: second mating of P_1 parents; M: males; ppm: parts per million; *: $P < 0.05$ for combined sex; **: $P < 0.05$ for each sex

[a] No. of pups alive at day 4/total no. born.

Source: Costlow & Harris (1985)

whereas the values for P_1/F_{1a}, P_2/F_{2a} and P_2/F_{2b} animals were 3, 6 and 5, respectively. The increase at 200 ppm was not considered to be treatment related, but was influenced by the low control value.

The second mating of the P_1 dams produced a growth response that was nearly identical to that in the first mating, but the effect was more pronounced. Pups at 200 ppm and lower were comparable with controls throughout the lactation period.

In the second generation, no treatment-related changes in mortality or clinical signs were detected in P_2 males and females. Body weights of male P_2 rats at 1000 ppm were significantly decreased throughout the 8 weeks of dosing prior to mating. Although the body weight at this dose was lower (16 g lower than in controls) prior to the treatment, the lower body weights during the first 3 weeks of postnatal growth were ascribed to treatment. At 1000 ppm, slight but consistent and statistically significant differences noted at 1000 ppm may reflect a modest degree of poor palatability of the diet of both the P_1 and P_2 animals.

A summary of reproductive performance is shown in Table 17. Although there were no statistically significant differences, the numbers of females giving birth and females weaning litters were slightly lower than control values in both the P_2/F_{2a} and F_{2b} animals. The period to mating (in days) took longer than the control value for F_{2b} animals at 1000 ppm. Gestation period was not prolonged by the treatment.

Table 17. Summary of reproductive indices for P_2 animals in a reproductive toxicity study in rats

	F_{2a}				F_{2b}			
Indices	**0 ppm**	**50 ppm**	**200 ppm**	**1 000 ppm**	**0 ppm**	**50 ppm**	**200 ppm**	**1 000 ppm**
No. of males	25	25	25	25	25	25	25	25
No. of females	25	24	25	25	25	24	25	25
No. of females mating	25	23	23	22	24	21	25	21
No. of females giving birth	23	23	24	20	23	22	25	17
No. of females weaning litters	22	23	23	18	22	21	24	15
Period to mating (days)	2.2	3.1	2.8	3.0	3.0	3.4	2.9	4.4
Gestation period (days)	21.7	21.7	21.9	22.2	21.7	22.0	21.8	21.7

F_{2a}: first mating of P_2 parents; F_{2b}: second mating of P_2 parents; ppm: parts per million

Source: Costlow & Harris (1985)

Relative liver weights were statistically significantly increased in males at 200 ppm (4% increase) and 1000 ppm (13% increase) and in females at 1000 ppm (8% increase) in P_2 rats. In the P_2 rats, centrilobular hepatocellular hypertrophy occurred in males at 200 ppm (2/25) and 1000 ppm (18/25) and in females at 1000 ppm (4/25). Centrilobular hepatocytic vacuolation was found in males at 200 ppm and above without dose dependency (3/25 and 1/25 at 200 and 1000 ppm, respectively), indicating that this change was not treatment related. Centrilobular hypertrophy associated with increased liver weights at 200 ppm was considered to be treatment related but adaptive, owing to a lack of indication of hepatotoxicity at this dose.

An increased incidence of rats with multifocal or diffuse testicular atrophy was observed in P_2 males at 1000 ppm. Associated changes, such as decreased amounts of spermatozoa, the presence of necrotic spermatocytes and spermatids in the epididymal tubules and prostatic atrophy, were noted at this dose. Testicular effects are summarized in Table 18.

In litter data for P_2/F_{2a} rats, litter size at 1000 ppm for this mating was lower than that in controls, and the number of pups born dead was higher than in controls. This response and the increase in dead pups were considered to be treatment related. Body weights for F_{2a} pups were not significantly less than those of controls at birth, but reduced body weight gain was readily apparent at day 7 and persisted through day 21 of lactation at 1000 ppm. In litter data for P_2/F_{2b} rats, litter size at 1000 ppm was less than that of controls, and the number of dead pups was higher than in controls. The increase in dead pups was not statistically significant, but was considered to be treatment related. Survival to day 4 was significantly reduced at 1000 ppm only because of the pups born dead, but there was no increased mortality between days 0 and 4 among pups born alive. Body weights for F_{2b} pups were not significantly different from those of controls at birth, but the reduced body weight gain of pups was clearly present by day 7 and persisted through day 21 of lactation. Summarized litter data are shown in Table 19.

In this two-generation reproductive toxicity study in rats, the NOAEL for parental toxicity was 200 ppm (equal to 15.1 mg/kg bw per day), based on lower body weights, histopathological changes in vacuolation and hypertrophy of hepatocytes in the liver and testicular atrophy in P_2 males at 1000 ppm (equal to 76.4 mg/kg bw per day). The NOAEL for reproductive toxicity was 200 ppm (equal to 17.5 mg/kg bw per day), based on reduced reproductive ability, including number of females mating, number of females giving birth, number of females weaning litters and prolongation of the time to mating in P_2 females at 1000 ppm (equal to 88.0 mg/kg bw per day). The NOAEL for offspring toxicity was 200 ppm (equal to 17.2 mg/kg bw per day), based on increased number of pups

born dead for both generations at 1000 ppm (equal to 85.9 mg/kg bw per day) (Costlow & Harris, 1985).

Table 18. Summary of histopathological changes in testes of the P_2 males in a reproductive toxicity study in rats

	Incidence of finding			
Findings	**0 ppm**	**50 ppm**	**200 ppm**	**1 000 ppm**
Testes (number of tissues examined)	25	25	25	25
Multifocal atrophy – unilateral	0	2	1	2
Multifocal atrophy – bilateral	3	2	3	3
Diffuse atrophy – unilateral	0	0	1	4
Diffuse atrophy – bilateral	0	1	0	4
Diffuse necrosis – unilateral	0	1	0	0
Number of rats with testicular lesions	3	5	5	11
Epididymides (number of tissues examined)	25	25	25	25
Necrotic spermatocytes/spermatids – unilateral	0	0	0	5
Necrotic spermatocytes/spermatids – bilateral	2	3	2	8
Decreased spermatozoa – unilateral	0	0	1	1
Decreased spermatozoa – bilateral	1	2	0	8
Number of rats with epididymal lesions	2	3	3	13
Prostate (number of tissues examined)	25	25	25	25
Atrophy	2	1	0	11

ppm: parts per million

Source: Costlow & Harris (1985)

Table 19. Summary of litter data for P_2 animals in a reproductive toxicity study in rats

	F_{2a}				F_{2b}			
Litter data	**0 ppm**	**50 ppm**	**200 ppm**	**1 000 ppm**	**0 ppm**	**50 ppm**	**200 ppm**	**1 000 ppm**
No. of pups/litter at birth	13.8	13.8	13.1	11.4*	15.4	14.8	13.8	13.4*
Sex ratio (M/(M + F)) at birth	0.53	0.51	0.45	0.46	0.49	0.51	0.51	0.46
No. of pups born dead	6	3	1	13*	5	6	3	12
Viability index[a]	86.8	84.9	86.7	84.6	96.9	94.2	98.5	90.8
Litter size – live pups								
Birth	314	314	314	216	349	319	341	218
Day 4 pre-cull	276	269	273	193	343	306	339	207*
Day 4 post-cull	209	215	216	169	230	207	240	155
Day 7	209	213	216	169	230	202	240	154
Day 14	208	213	216	169	230	202	240	154

Litter data	F_{2a}				F_{2b}			
	0 ppm	**50 ppm**	**200 ppm**	**1 000 ppm**	**0 ppm**	**50 ppm**	**200 ppm**	**1 000 ppm**
Day 21 (weaning)	208	213	216	169	220	202	239	144
Body weight (g)								
Day 0	5.8	6.1	6.2	6.2	6.0	5.9	6.1	5.8
Day 4 pre-cull	9.2	9.6	9.9	9.2	9.1	9.5	9.1	8.7
Day 7	14.9	15.1	15.2	13.4	14.5	15.0	14.7	13.3**
Day 14	29.1	29.2	29.2	25.3**	29.4	30.2	28.7	26.2**
Day 21 (weaning)	45.3	45.5	44.6	40.2**	46.5	48.1	46.0	41.8**

F: females; M: males; ppm: parts per million; *: $P < 0.05$ for combined sex; **: $P < 0.05$ for each sex

[a] The number of pups alive at day 4/total number born.

Source: Costlow & Harris (1985)

(b) Developmental toxicity

Rats

A developmental toxicity study was conducted with rats. This study complied with GLP, although it was not conducted according to test guidelines. However, most of the parameters tested in this study generally meet the requirements of current guidelines. Myclobutanil (TD no. 83-087; lot no. LSPL 0017/E; purity 84.5%) was administered orally in corn oil to 25 presumed-pregnant Sprague-Dawley rats from day 6 through day 15 of gestation at doses of 0, 31.3, 93.8, 313 and 469 mg/kg bw per day. Reanalysis for skeletal variations observed was conducted in compliance with GLP.

There were no deaths in this study. Treatment-related clinical signs or reactions to treatment were observed at 313 mg/kg bw per day and above, mainly at 469 mg/kg bw per day: rough hair coat, desquamation and salivation at 313 mg/kg bw per day and above and red exudate from the mouth and scant or soft faeces at 469 mg/kg bw per day. These clinical signs were found during days 8–13, mainly days 8–10. The distribution of their occurrences indicated a low possibility that they were induced by single-dose exposure. At 469 mg/kg bw per day, a statistically significantly lower maternal body weight was observed on day 10 of gestation, but not thereafter.

The litter data are summarized in Table 20. Fetal weights were not affected by the treatment in both sexes. The fertility index (the number of pregnant rats/the number of presumed-pregnant rats) was not affected by the treatment. The number of fetuses per litter was slightly, but statistically significantly, decreased at 93.8 mg/kg bw per day and above, but the litter sizes were within the range of historical control data (average, 13.46; range, 12.10–14.6). The litter size in the control group (15.3) was slightly above the historical control range. In addition, the numbers of corpora lutea and implantations in the control group were slightly above historical control values. Therefore, the decrease in litter size was not considered to be treatment related, but was influenced by the control value.

The number of resorptions (early plus late) per litter and the number of early resorptions per litter were slightly increased at 93.8 mg/kg bw per day and above. Although the decrease in the number of early resorptions per litter was very slight and showed no dose–response relationship between 93.8 and 313 mg/kg bw per day, these values were above the historical control range (mean, 5.6; range, 0.32–0.82). The numbers of late resorptions per litter and litters with late resorptions were increased at 313 and 469 mg/kg bw per day. In addition, increased resorptions were observed at 68 mg/kg bw per day and above in Sprague-Dawley rats in the dose-finding developmental toxicity study previously reviewed by JMPR in 1992, indicating the similar effect induced by myclobutanil at

similar doses in the same strain of rat. Therefore, the slight increase in early resorptions at 93.8 mg/kg bw per day and above was considered to be treatment related.

Table 20. Summary of litter data (means) in a developmental toxicity study in rats

Parameter	0 mg/kg bw per day	31.3 mg/kg bw per day	93.8 mg/kg bw per day	313 mg/kg bw per day	469 mg/kg bw per day
Number bred	25	25	25	25	25
Number non-pregnant	3	1	4	2	2
Number dead/killed moribund	0	0	0	0	0
Number of total resorptions	0	0	0	0	1
Number of viable litters	22	24	21	23	22
Number of corpora lutea/dam[a]	17.9[b]	15.2	16.6	16.4	16.8
Number of implantations/dam	16.1	14.3	15.2	15.0	15.7
Preimplantation loss (%)	10	6	8	8	6
Number of fetuses/litter	15.3[c]	13.5	13.3*	13.2*	13.1*
Number of resorptions/litter[a]	0.82	0.79	1.86	1.78	2.57
Early[a]	0.82[d]	0.71	1.76	1.35	2.04
Late[a]	0[e]	0.08	0.10	0.43	0.52
Number of litters with resorptions[a]	12	17	16[f]	18	19[g]
Early[a]	12	16	16	15	17
Late[a]	0	2	1	8	9
Fetal weights (g)	3.23	3.30	3.25	3.39	3.26
Fetal sex ratio M:F	0.87	0.98	0.92	0.99	0.88

bw: body weight; F: female; M: male; *: $P < 0.05$

[a] Not statistically analysed.
[b] Historical control data; average, 15.45; range, 14.82–17.4.
[c] Historical control data; average, 13.82; range, 12.91–14.92.
[d] Historical control data; average, 0.56; range, 0.32–0.82.
[e] Historical control data; average, 0.02; range, 0.00–0.05.
[f] One dams showed 10 early and two late resorptions.
[g] One dam showed all implantations resorbed (14 early resorptions).

Source: Costlow & Kane (1984a)

In fetal examinations, the numbers of 7th cervical ribs and 14th rudimentary ribs per litter were statistically significantly increased at 313 mg/kg bw per day and above in a dose-dependent manner. These increases in variation frequencies were considered to be treatment related. When all malformations were considered together, a marginally significant dose-related trend was noted. The control incidence was zero and required the use of Fisher's exact test for analysis of the data. Although the analysis indicated a significant increase in malformations (fetuses only) at the 469 mg/kg bw per day dose, it was not considered to be toxicologically significant. A summary of variations and total number of all malformations is shown in Table 21.

Table 21. Summary of variations and total number of all malformations in a developmental toxicity study in rats

	0 mg/kg bw per day		31.3 mg/kg bw per day		93.8 mg/kg bw per day		313 mg/kg bw per day		469 mg/kg bw per day	
Observations	**Fetus**	**Litter**	**Fetus**	**Litter**	**Fetus**	**Litter**	**Fetus**	**Litter**	**Fetus**	**Litter**
Number of fetuses (number of litters) examined										
External	337 (22)		324 (24)		280 (21)		303 (23)		301 (22)	
Visceral	114		111		95		103		100	
Skeletal	223		213		185		200		201	
Skeletal variations										
7th cervical ribs	3	2	0	0	3	3	17	10*	45	14*
14th rudimentary rib(s)	1	1	4	3	1	1	17	8*	72	18*
14th full rib(s)	0	0	0	0	0	0	0	0	1	1
Any rib variation[a]	8	5	7	6	11	7	34	16*	72	20*
Any reduced ossification[b]	150	22	103	24	93	18	123	18	125	22
Total malformed	0	0	2	2	3	2	0	0	4*	4

bw: body weight; *: $P < 0.05$

[a] Includes 7th cervical, 14th rudimentary, 14th full or 13th rudimentary rib.

[b] Includes skull bones, hyoid, vertebrae, sternebrae.

Source: Costlow & Kane (1984a)

A reanalysis of data from Costlow & Kane (1984a), focusing on the evaluation and interpretation of the aforementioned skeletal alterations, 7th cervical rib and 14th rudimentary rib, both of which are common in rats, was conducted by Carney, Tornesi & Passage (2005). The reason for the reanalysis was that the interpretation of supernumerary ribs has been controversial for many years (Kimmel & Wilson, 1973). Many investigators discount such effects based on their known association with maternal toxicity/maternal stress (Khera, 1981; Kavlock, Chernoff & Rogers, 1985; Beyer & Chernoff, 1986) and their reversibility through the process of skeletal remodelling during postnatal development (Wickramaratne, 1988; Marr et al., 1992; Foulon et al., 2000). In contrast, certain developmental toxicants (e.g. salicylate, acetozolamide, bromoxynil, actinomycin D, dinoseb) induce supernumerary ribs that persist into adulthood (reviewed in Chernoff & Rogers, 2004), which has led some to assign greater significance to such effects. Interestingly, the incidence of cervical ribs in live human fetuses (19–39%) is much higher than in the adult human (0.04–1.2%), indicating that cervical ribs are usually transient structures in humans. Whereas extra ribs in the lumbar region are relatively common in the rat, they exhibit a low incidence (0.04–2%) in both the human fetus and adult (reviewed in Chernoff & Rogers, 2004). Using a more recent criterion, supernumerary ribs were classified as such by Carney, Tornesi & Passage (2005) when their length was more than twice their width. The reanalysis on this basis confirmed that the incidences of both skeletal alterations were just slightly above expected control incidences based on published data using similar rib length criteria and therefore were considered to be a treatment-related effect (Carney, Tornesi & Passage, 2005).

The NOAEL for maternal toxicity was 93.8 mg/kg bw per day, based on clinical signs indicating ill-health or reaction to treatment at 313 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 31.3 mg/kg bw per day, based on an increased number of early resorptions per litter at 93.8 mg/kg bw per day. Myclobutanil was not teratogenic in rats (Costlow & Kane, 1984a; Carney, Tornesi & Passage, 2005).

Rabbits

A developmental toxicity study of myclobutanil in rabbits was conducted in compliance with GLP, although it was not conducted according to test guidelines. However, examinations used in this study generally meet the current guidelines. Myclobutanil (TD no. 83-260; lot no. LAP-0298; purity 90.4%) was administered orally (adsorbed onto a silica carrier [Hi-Sil 233] and suspended in 1% aqueous [w/v] methyl cellulose) to 18 artificially inseminated New Zealand White rabbits from day 7 through day 19 of gestation at a dose of 0, 20, 60 or 200 mg/kg bw per day. There were two control groups, one with distilled water and one with the methyl cellulose vehicle. Feed consumption was not assessed.

Two rabbits at 200 mg/kg bw per day died due to intubation errors. No deaths occurred in the other groups. The does of the 200 mg/kg bw per day group showed signs indicative of a toxic response, such as increased incidences of irregular-shaped faeces, bloody urine, bloody urogenital or anal area and blood and/or aborted material in the drop pan. The signs did not appear at the beginning of the treatment, and their incidences showed peaks in the middle of the treatment period. All other clinical observations occurred sporadically and were not related to treatment with myclobutanil. At 200 mg/kg bw per day, three does aborted and two does died (one pregnant). Decreased body weight gain persisted during the treatment period at 200 mg/kg bw per day. Whereas body weight changes in the other treatment groups, including the vehicle control, were slightly decreased or constant during the period, mean maternal body weight at 60 mg/kg bw per day was slightly lower than in the vehicle control and 20 mg/kg bw per day groups. The body weight on day 11 in the 60 mg/kg bw per day group was significantly lower than that in the control group. Total body weight gains during the treatment period were 0.03, −0.02, 0.04, −0.06 and −0.28 kg in the control, vehicle control, 20, 60 and 200 mg/kg bw per day groups, respectively. The decreases in maternal body weight at 60 mg/kg bw per day and above were considered treatment related.

The viability index (number of fetuses/number of implantations) summarized an increase in the number of resorptions per litter, the number of litters with more than two resorptions and the number of litters totally resorbed at 200 mg/kg bw per day. Early but not late resorptions were increased at this dose. These were treatment-related adverse effects. In fetal data, litter size (viable fetuses per litter – combined sexes) was decreased at 200 mg/kg bw per day. This resulted in a significantly lower viability index. Pairwise comparisons of the 20 and 60 mg/kg bw per day groups with combined controls revealed no significant differences in viability index or litter size. In spite of the lack of statistically significant differences, fetal weights at 200 mg/kg bw per day were below historical control values, and this depression was considered to be related to the treatment. There were no external variations in any group. The treatment with myclobutanil did not affect fetal or soft tissue variations.

The NOAEL for maternal toxicity was 20 mg/kg bw per day, based on decreased body weight gain at 60 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 60 mg/kg bw per day, based on an increased number of resorptions per litter, an increased number of litters totally resorbed and lower fetal weights at 200 mg/kg bw per day. Myclobutanil was not teratogenic in rabbits (Costlow & Kane, 1984b).

2.6 Special studies

(a) Supplementary studies for reproductive toxicity

Myclobutanil has been shown to exert some reproductive toxicity, causing testicular atrophy and also an increased number of stillborn pups. A supplementary study was conducted to determine whether or not the early embryo loss may be induced by chromosomal abnormalities of the

spermatozoa in the myclobutanil-treated rats, leading to the death of conceptuses. This study complied with GLP. Myclobutanil (TD no. 86-77; lot no. 83 159-7; purity 91.4%) was administered to groups of 25 male Crl:COBS CD(SD)BR rats at a dose of 0, 10, 100 or 735 mg/kg bw once via gavage in Kodak stripped corn oil. After the single-dose treatment, each male rat was assigned to 8 consecutive weeks of mating with intact females. On day 14 of presumed gestation, each female was caesarean-sectioned and examined for corpora lutea, implantations, resorptions and live (beating embryonic heart) or dead conceptuses. Males were examined for gross lesions and effects on the testes and epididymides.

One male was found dead at 735 mg/kg bw with severe body weight loss 2 days after the single dose of myclobutanil. Clinical signs attributed to the treatment were evident in males during week 1 post-dosing at 735 mg/kg bw. These clinical signs included chromodacryorrhoea, chromorhinorrhoea, salivation, red oral exudate or dried red perioral material and/or urine-stained abdominal fur. In other males during weeks 2 through 8 post-dosing, no treatment-related clinical signs were observed. In females, no clinical signs were detected. Body weight gains at 735 mg/kg bw showed a consistent decrease but were not remarkable at the study termination. Female body weights and body weight gains during gestation were comparable among all sets of females mated by males in all groups. At termination, no treatment-related change was observed in males or females. No differences were seen in testes weights in any group. The percentage of pregnant female rats from the first mating with males treated at 735 mg/kg bw was slightly low compared with that which occurred during any other mating of the same male rats, and the decrease was considered to be the result of decreased mating performance of these rats prior to recovery from systemic toxic effects of the test substance. There were no other differences in pregnancy incidences or in the analysis of male fertility among all groups. The caesarean-sectioning data for each week of mating did not demonstrate any dose-dependent or significant differences. Averages for corpora lutea, implantations, litter sizes (live and dead embryos), resorptions and percentage of dead conceptuses per litter were similar for females among all groups.

Whereas a single oral gavage dose of 735 mg/kg bw produced systemic toxicity in male rats, including death in one male, there was no indication of a dose-dependent effect on the incidence of embryo death, even at a dose lethal to adults. No treatment-related changes were observed at 100 mg/kg bw (Dearlove, Hoberman & Christian, 1986).

(*b*) *Toxicity studies on metabolites and impurities*

Only very limited studies on metabolites were submitted.

Acute toxicity

An oral acute toxicity study of two main metabolites of myclobutanil in plants (RH-9090 and RH-9089) and two impurities in the myclobutanil preparation (RH-8812 and RH-8813) was carried out in mice. RH-9090 and RH-9089 were also detected in rat, hen and cow. These studies complied with GLP. Male and female CD-1 mice (five of each sex per group) were given a single oral gavage dose of RH-9090 (TD 87-089; lot no. LTN 2074; purity 98%) in polyethylene glycol 400, RH-9089 (TD 87-090; lot no. LN 2616; purity 99.4%), RH-8812 (TD 87-088; lot no. WJZ 2121; purity 99.6%) or RH-8813 (TD 87-091; lot no. WJZ 2122; purity 99.8%) at 0, 300, 1000 or 3000 mg/kg bw in corn oil, following a 4-hour fast. Two vehicle control groups (polyethylene glycol 400 and corn oil) were maintained. Animals were observed for 14 days post-dosing. Body weights were measured prior to dosing, on days 1, 2, 6, 9, 12 and 14, and just prior to termination. All premature decedents were necropsied as soon as practical. All surviving animals were necropsied after the final observation period.

Their mortalities and LD_{50}s are shown in Table 22. The LD_{50} value of the parent compound, myclobutanil, was 1910 mg/kg bw in male mice and 1360 mg/kg bw in female mice (see Table 4). The LD_{50}s for RH-9090 were equal to or lower than the parent value, and those for RH-9089 showed lower values than the parent. There were no remarkable differences in mortalities between the impurities and the parent (Shimizu, Tokiwa & Nakayoshi, 1987).

Table 22. Results of acute toxicity studies of myclobutanil metabolites and impurities in mice

Test material	Lot no. / purity	Dose (mg/kg bw)	Mortality[a]		LD_{50}
			Males	Females	
RH-9090	LTN 2074 / 98%	300	0/5	0/5	300 < LD_{50} < 1 000 mg/kg bw for males, 1 000 < LD_{50} < 3 000 mg/kg bw for females
		1 000	4/5 – 2 on day 0 and 2 on day 1	3/5 – Day 0	
		3 000	5/5 – Day 0	5/5 – 4 on day 0 and 1 on day 1	
RH-9089	LN 2616 / 99.4%	300	0/5	0/5	300 < LD_{50} < 1 000 mg/kg bw for both sexes
		1 000	3/5 – Day 0	2/5 – 1 on day 0 and 1 on day 1	
		3 000	5/5 – Day 0	4/5 – Day 0	
RH-8812	WJZ 2121 / 99.6%	300	0/5	0/5	1 000 < LD_{50} < 3 000 mg/kg bw for both sexes
		1 000	1/5 – Day 2	2/5 – Day 2	
		3 000	5/5 – 3 on day 1, 1 on day 2 and 1 on day 3	5/5 - 1 on day 0 and 4 on day 1	
RH-8813	WJZ 2122 / 99.8%	300	0/5	0/5	LD_{50} > 3 000 mg/kg bw for both sexes
		1 000	0/5	0/5	
		3 000	1/5 – Day 1	1/5 – Day 1	

[a] The number of mice dead/the number of mice examined.

Source: Shimizu, Tokiwa & Nakayoshi (1987)

Treatment-related clinical signs were as follows:

- *RH-9090*: The 300 mg/kg bw males displayed piloerection of the tail and soft faeces after 3 hours. All survivors returned to normal from 5 hours post-dosing. Females showed no signs at this dose. The males given 1000 mg/kg bw displayed erecting tail after 30 minutes, which was followed by sedation, depression of spontaneous movement, prone position and piloerection. One survivor returned to normal from day 2. The females given 1000 mg/kg bw displayed depression of spontaneous movement and erecting tail just after administration, which was followed by sedation. Two survivors returned to normal from day 1. The males and females given 3000 mg/kg bw displayed depression of spontaneous movement and sedation just after administration, which were followed by salivation, convulsion, erecting tail and prone position.
- *RH-9089*: The males given 300 mg/kg bw displayed piloerection of the tail after 30 minutes. All survivors returned to normal from 4 hours. The males given 1000 mg/kg bw displayed convulsion, piloerection of the tail and prone position after 30 minutes, which were followed by sedation. The females given 1000 mg/kg bw displayed sedation after 30 minutes, which was followed by convulsion, piloerection of the tail, depression of spontaneous movement and prone position. Two male and three female survivors returned to normal from day 1. The males given 3000 mg/kg bw displayed piloerection of the tail just after administration, which was followed by depression of spontaneous movement, convulsion and prone position. The females given 3000 mg/kg bw displayed salivation and piloerection of the tail just after administration, which were followed by sedation, convulsion and depression of spontaneous movement. One female survivor returned to normal from day 1.

- *RH-8812*: The males given 1000 mg/kg bw displayed sedation after 1 hour, which was followed by depression of spontaneous movement and prone position. Females displayed depression of spontaneous movement after 30 minutes, which was followed by sedation, prone position and convulsion. Four male and three female survivors returned to normal from day 2. The males given 3000 mg/kg bw displayed depression of spontaneous movement, sedation and prone position after 1 hour. Females displayed depression of spontaneous movement after 30 minutes, which was followed by convulsion, prone position and sedation.
- *RH-8813*: The females given 300 mg/kg bw displayed piloerection of the tail after 30 minutes. All animals returned to normal from 2 hours post-dosing. The males given 1000 mg/kg bw displayed sedation after 2 hours. All animals returned to normal from day 2. The females given 1000 mg/kg bw displayed erecting tail after 30 minutes, which was followed by depression of spontaneous movement and sedation. All females returned to normal from 4 hours post-dosing. The males given 3000 mg/kg bw displayed piloerection of the tail and prone position after 2 hours, which were followed by sedation and convulsion. Females given 3000 mg/kg bw displayed piloerection of the tail after 30 minutes, which was followed by depression of spontaneous movement. Male and female survivors returned to normal from day 1.

Treatment-related body weight changes were also detected. For RH-9090 and RH-9089, body weights were decreased in both sexes given 1000 mg/kg bw and females given 3000 mg/kg bw on day 1, but increased normally thereafter. For RH-8812, body weights were decreased in males given 1000 and 3000 mg/kg bw on days 1 and 2 and in females given 1000 mg/kg bw on day 1, but increased normally thereafter. For RH-8813, body weights were decreased in both sexes given 1000 and 3000 mg/kg bw on day 1, but increased normally thereafter. No macroscopic change was observed in either sex of any group treated with each test article at necropsy at termination (Shimizu, Tokiwa & Nakayoshi, 1987).

Short-term studies of toxicity

To determine the repeated oral dose toxicity potential of myclobutanil butyric acid, a metabolite in soil, female Crl:CD(SD) rats were administered myclobutanil butyric acid by gavage once daily for 14 days at a dose of 0, 50, 150, 450, 750 or 1000 mg/kg bw per day. Clinical observations, body weight and feed consumption measurements, and urinary metabolite identification (1000 mg/kg bw per day only) were performed, followed by gross necropsy on test day 15. In addition, a separate group of four female Crl:CD(SD) rats was administered 100 mg/kg bw per day of myclobutanil via gavage for 4 days to determine if myclobutanil is metabolized to myclobutanil butyric acid in rats. Urine from all four rats, as well as two female control rats, was collected during the last 24 hours of exposure and analysed for myclobutanil butyric acid.

All animals survived. There were no treatment-related effects on clinical observations, body weights, feed consumption or gross necropsy observations at any dose level tested. Urinary metabolite identification indicated that myclobutanil butyric acid was mainly excreted unchanged, although 3–4 minor metabolites were found. In rats given myclobutanil, levels of myclobutanil butyric acid were less than 1% of the administered dose of myclobutanil, compared with the results from the myclobutanil butyric acid group.

In conclusion, myclobutanil butyric acid has no toxicity in a 2-week oral toxicity study in rats. Myclobutanil butyric acid was not a metabolite in rats (Rasoulpour & Zablotny, 2009).

Genotoxicity

The genotoxicity studies on RH-9090, RH-9089 and myclobutanil butyric acid are summarized in Table 23.

Table 23. Summary of genotoxicity studies on RH-9090, RH-9089 and myclobutanil butyric acid

Test	Test object	Concentration	Lot no. / purity	Result	Reference
In vitro					
RH-9089					
Bacterial reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537 *Escherichia coli* WP2 uvrA	156–5 000 µg/plate	Not described	Negative ±S9	Food Safety Commission (2011)
DNA repair test	*Bacillus subtilis* H17, M45	200–10 000 µg/mL	Not described	Negative ±S9	Food Safety Commission (2011)
RH-9090					
Bacterial reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537 *E. coli* WP2 uvrA	313–5 000 µg/plate	Not described	Negative ±S9	Food Safety Commission (2011)
DNA repair test	*B. subtilis* H17, M45	100–5 000 µg/mL	Not described	Negative ±S9	Food Safety Commission (2011)
Myclobutanil butyric acid					
Bacterial reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537 *E. coli* WP2 uvrA	100–5 000 µg/plate	V43-037424-86 / 98%	Negative ±S9	Michael & Mecchi (2009)
Mammalian forward mutation	CHO cells (CHO/*Hgprt*)	181.7–2 907 µg/mL	V43-037424-86 / 98%	Negative ±S9	Schisler & Geter (2009)
In vivo					
Myclobutanil butyric acid					
Micronucleus	Mouse peripheral blood	500, 1 000 and 2 000 (limit dose) mg/kg bw	V43-037424-86 / 98%	Negative	Schisler & LeBaron (2009)

bw: body weight; CHO: Chinese hamster ovary; DNA: deoxyribonucleic acid; Hgprt: hypoxanthine–guanine phosphoribosyltransferase; S9: 9000 × *g* supernatant fraction from rat liver homogenate

In conclusion, RH-9090 and RH-9089, which are the major metabolites of myclobutanil in rats, plant, hen and cow, were not genotoxic in vitro, and myclobutanil butyric acid, a degradate in soil, was not genotoxic in vitro or in vivo (Schisler & LeBaron, 2009; Michael & Mecchi, 2009; Schisler & Geter, 2009; Food Safety Commission, 2011).

3. Observations in humans

Myclobutanil is a triazole fungicide that was manufactured previously by contract manufacturer Rhodia Chirex in the United Kingdom and, since late 2002, by contract manufacturer Kemira Fine Chemicals in Finland. Myclobutanil is repackaged in Barranquilla, Colombia. Medical surveillance data on eight employees have not shown any abnormalities to suggest adverse health

effects; there have been no incidents or allegations of adverse effects in this operation. Myclobutanil was bottled briefly in San Lorenzo, Argentina, in 2002. No medical surveillance has been conducted on the seven workers involved. No medical surveillance data on manufacturing personnel are available.

Medical surveillance data are available from the manufacturing/formulation of myclobutanil at Mozzanica, Italy, over the time span 2000–2005 and cover 25 workers. For all 25 workers, there were no health effects related to working with myclobutanil.

In conclusion, in reports on manufacturing plant personnel, no adverse health effects were noted.

Comments

Biochemical aspects

Myclobutanil was rapidly and extensively absorbed in rats (> 89%). Peak plasma and tissue concentrations of radiolabelled myclobutanil were achieved within 1 hour after oral administration. Plasma elimination was biphasic; the half-lives were 5 hours for the rapid phase and 26 hours for the slow phase in rats exposed to a single dose. Myclobutanil was widely distributed, and no significant tissue accumulation was observed 96 hours post-dosing.

Metabolism was extensive and appeared to occur mainly through a variety of oxidation reactions of the butyl group. The major unconjugated phenethyl triazole–containing metabolites were RH-9090 ((2*RS*,5*RS*)-2-(4-chlorophenyl)-5-hydroxy-2-(1*H*-1,2,4-triazol-1-ylmethyl)hexanenitrile) and RH-9089 ((2*RS*)-2-(4-chlorophenyl)-5-oxo-2-(1*H*-1,2,4-triazol-1-ylmethyl)hexanenitrile). Myclobutanil was rapidly and mostly excreted in urine and faeces within 48 hours in rats.

Toxicological data

The oral LD_{50} for myclobutanil was greater than or equal to 1600 mg/kg bw in rats. The dermal LD_{50} was greater than 5000 mg/kg bw in rats and rabbits. The inhalation LC_{50} was greater than 5.1 mg/L in rats. Myclobutanil was not irritating to the skin but was moderately irritating to the eye of rabbits. Myclobutanil was not sensitizing in the guinea-pig maximization test or the mouse local lymph node assay and was mildly sensitizing using the Buehler method.

The liver was the interspecies target of myclobutanil in short- and long-term toxicity studies. The testis was also a target of myclobutanil in long-term toxicity studies in rats. Reductions of feed consumption and corresponding decreases in body weight gains at the beginning of treatment in short-term dietary studies in mice, rats and dogs and a reproductive toxicity study in rats are considered to be due to low palatability, rather than an adverse effect, as no similar change in feed consumption was observed in gavage studies and no effects on the gastrointestinal tract were observed.

In a 90-day toxicity study in mice administered myclobutanil in the diet at a concentration of 0, 3, 10, 30, 100, 300, 1000, 3000 or 10 000 ppm (equal to 0, 0.40, 1.54, 4.79, 14.1, 42.7, 132, 542 and 2035 mg/kg bw per day for males and 0, 0.62, 2.11, 6.94, 22.9, 65.5, 232, 710 and 2027 mg/kg bw per day for females, respectively), the NOAEL was 300 ppm (equal to 42.7 mg/kg bw per day), based on fatty changes and necrosis of hepatocytes at 1000 ppm (equal to 132 mg/kg bw per day).

In a 90-day oral toxicity study in rats, myclobutanil was administered in the diet at a concentration of 0, 5, 15, 50, 150, 500, 1500, 5000 or 15 000 ppm for weeks 1 and 2; at 0, 7, 21, 70, 210, 700, 2100, 7000 or 21 000 ppm for weeks 3 and 4; and at 0, 10, 30, 100, 300, 1000, 3000, 10 000 or 30 000 ppm for the remainder of the study. These dietary concentrations were equal to doses of 0, 0.52, 1.60, 5.22, 15.3, 51.5, 158, 585 and 1730 mg/kg bw per day for males and 0, 0.67, 2.03, 6.85, 19.7, 65.8, 195.2, 665 and 1811 mg/kg bw per day for females, respectively. The NOAEL was 500/700/1000 ppm (equal to 51.5 mg/kg bw per day), based on increased liver and kidney weights, hepatocellular hypertrophy, single-cell necrosis in the liver and pigmentation in tubular epithelium in the kidneys at 1500/2100/3000 ppm (equal to 158 mg/kg bw per day).

In a 90-day oral toxicity study in dogs administered myclobutanil in the diet at 0, 10, 200, 800 or 1600 ppm (equal to 0, 0.34, 7.26, 29.1 and 56.8 mg/kg bw per day for males and 0, 0.42, 7.88, 32.4 and 58.0 mg/kg bw per day for females, respectively), the NOAEL was 800 ppm (equal to 29.1 mg/kg bw per day), based on liver hypertrophy, increased ALP and increased platelets at 1600 ppm (equal to 56.8 mg/kg bw per day).

In a 1-year oral toxicity study in dogs administered myclobutanil in the diet at a concentration of 0, 10, 100, 400 or 1600 ppm (equal to 0, 0.34, 3.09, 14.3 and 54.2 mg/kg bw per day for males and 0, 0.40, 3.83, 15.7 and 58.2 mg/kg bw per day for females, respectively), the NOAEL was 400 ppm (equal to 14.3 mg/kg bw per day), based on hepatocellular hypertrophy, ballooned hepatocytes, increased ALP and increased platelets in males at 1600 ppm (equal to 54.2 mg/kg bw per day).

The Meeting concluded that the overall NOAEL for oral toxicity in dogs was 800 ppm (equal to 29.1 mg/kg bw per day), and the overall LOAEL was 1600 ppm (equal to 54.2 mg/kg bw per day).

In a 2-year toxicity and carcinogenicity study in mice administered myclobutanil in the diet at a concentration of 0, 20, 100 or 500 ppm (equal to 0, 2.7, 13.7 and 70.2 mg/kg bw per day for males and 0, 3.2, 16.5 and 85.2 mg/kg bw per day for females, respectively), the NOAEL for toxicity was 100 ppm (equal to 13.7 mg/kg bw per day), based on histopathological signs of hepatotoxicity at 500 ppm (equal to 70.2 mg/kg bw per day). Myclobutanil was not carcinogenic in this study.

In a second 2-year carcinogenicity study conducted to confirm the absence of carcinogenicity at high doses, female mice were administered myclobutanil in the diet at a concentration of 2000 ppm (equal to 394 mg/kg bw per day), the MTD. No carcinogenicity was observed at this dose.

In a 2-year carcinogenicity study in rats administered myclobutanil in the diet at a concentration of 0, 50, 200 or 800 ppm (equal to 0, 2.5, 9.8 and 39.2 mg/kg bw per day for males and 0, 3.2, 12.8 and 52.3 mg/kg bw per day for females, respectively), the NOAEL for non-neoplastic effects was 50 ppm (equal to 2.5 mg/kg bw per day), based on testicular toxicity found after 12 months of treatment at 200 ppm (equal to 9.8 mg/kg bw per day). Myclobutanil was not carcinogenic in this study.

A second 2-year carcinogenicity study in rats confirmed the absence of carcinogenicity of myclobutanil at a higher dietary concentration, 2500 ppm (equal to 106 mg/kg bw per day for males and 136 mg/kg bw per day for females).

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

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

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

In a two-generation reproductive toxicity study in rats administered myclobutanil in the diet at a concentration of 0, 50, 200 or 1000 ppm (equal to 0, 3.67, 14.3 and 70.7 mg/kg bw per day for P_1 males, 0, 4.42, 17.2 and 85.9 mg/kg bw per day for P_1 females, 0, 3.64, 15.1 and 76.4 mg/kg bw per day for P_2 males and 0, 4.17, 17.5 and 88.0 mg/kg bw per day for P_2 females, respectively), the NOAEL for parental toxicity was 200 ppm (equal to 15.1 mg/kg bw per day), based on lower body weights, histopathological changes of vacuolation and hypertrophy of hepatocytes and testicular atrophy in P_2 males at 1000 ppm (equal to 76.4 mg/kg bw per day). The NOAEL for reproductive toxicity was 200 ppm (equal to 17.5 mg/kg bw per day), based on reduced reproductive ability, including number of females mating, number of females giving birth, number of females weaning litters or prolonged time to mating, in P_2 females at 1000 ppm (equal to 88.0 mg/kg bw per day). The NOAEL for offspring toxicity was 200 ppm (equal to 17.2 mg/kg bw per day), based on an increased number of pups born dead for both generations at 1000 ppm (equal to 85.9 mg/kg bw per day).

In a developmental toxicity study in rats administered myclobutanil by gavage at 0, 31.3, 93.8, 313 or 469 mg/kg bw per day, the NOAEL for maternal toxicity was 93.8 mg/kg bw per day,

based on clinical signs of rough hair coat, desquamation and salivation at 313 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 31.3 mg/kg bw per day, based on an increased number of early resorptions per litter at 93.8 mg/kg bw per day.

To determine whether treatment-related early resorptions in rats were caused by chromosomal abnormalities of the spermatozoa, leading to death of conceptuses, male rats were administered a single dose of myclobutanil at 0, 10, 100 or 735 mg/kg bw and mated with untreated females. There was no evidence of treatment-related embryo death, even at a dose lethal to adults.

In a developmental toxicity study in rabbits administered myclobutanil by gavage at 0, 20, 60 or 200 mg/kg bw per day, the NOAEL for maternal toxicity was 20 mg/kg bw per day, based on decreased body weight gain at 60 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 60 mg/kg bw per day, based on an increased number of resorptions per litter, an increased number of litters totally resorbed and lower fetal weights at 200 mg/kg bw per day.

The Meeting concluded that myclobutanil is not teratogenic.

There were no studies submitted that specifically investigated neurotoxicity or immunotoxicity.

Toxicological data on metabolites and/or degradates

The oral LD_{50} ranges for RH-9090 and RH-9089, major metabolites in plants, rats, hens and cows, were between 300 and 1000 mg/kg bw in mice.

In a 2-week oral toxicity study on myclobutanil butyric acid ((3*RS*)-3-(4-chlorophenyl)-3-cyano-4-(1*H*-1,2,4-triazol-1-yl)butanoic acid), a degradate in soil, no toxicity was observed at doses up to 1000 mg/kg bw administered by gavage to rats.

Tests of the in vitro genotoxicity of RH-9089, RH-9090 and myclobutanil butyric acid and an in vivo genotoxicity assay on myclobutanil butyric acid showed no evidence of genotoxicity.

The Meeting concluded that RH-9090 and RH-9089, which are major metabolites in rats, are covered by the reference doses for myclobutanil. Myclobutanil butyric acid is of no toxicological concern, as it is of lower toxicity than the parent.

Human data

In reports on manufacturing plant personnel, no adverse health effects were noted.

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

Toxicological evaluation

The Meeting reaffirmed the ADI of 0–0.03 mg/kg bw on the basis of the NOAEL of 2.5 mg/kg bw per day in a 2-year study in rats, based on testicular atrophy at 9.8 mg/kg bw per day. A safety factor of 100 was applied. This ADI is based on the same end-point as in 1992.

The Meeting established an ARfD of 0.3 mg/kg bw for women of childbearing age only, on the basis of the NOAEL of 31.3 mg/kg bw per day in a developmental toxicity study in rats, based on an increased number of early resorptions at 93.8 mg/kg bw per day. A safety factor of 100 was applied. The Meeting concluded that it is not necessary to establish an ARfD for the remainder of the population in view of the low acute oral toxicity of myclobutanil and the absence of any other toxicological effects that would be likely to be elicited by a single dose.

Levels relevant to risk assessment of myclobutanil

Species	Study	Effect	NOAEL	LOAEL
Mouse	Two-year studies of toxicity and carcinogenicity[a,b]	Toxicity	100 ppm, equal to 13.7 mg/kg bw per day	500 ppm, equal to 70.2 mg/kg bw per day
		Carcinogenicity	2 000 ppm, equal to 394 mg/kg bw per day[c]	–
Rat	Two-year studies of toxicity and carcinogenicity[a,b]	Toxicity	50 ppm, equal to 2.5 mg/kg bw per day	200 ppm, equal to 9.8 mg/kg bw per day
		Carcinogenicity	2 500 ppm, equal to 106 mg/kg bw per day[c]	–
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	200 ppm, equal to 17.5 mg/kg bw per day	1 000 ppm, equal to 88.0 mg/kg bw per day
		Parental toxicity	200 ppm, equal to 15.1 mg/kg bw per day	1 000 ppm, equal to 76.4 mg/kg bw per day
		Offspring toxicity	200 ppm, equal to 17.2 mg/kg bw per day	1 000 ppm, equal to 85.9 mg/kg bw per day
	Developmental toxicity study[d]	Maternal toxicity	93.8 mg/kg bw per day	313 mg/kg bw per day
		Embryo and fetal toxicity	31.3 mg/kg bw per day	93.8 mg/kg bw per day
Rabbit	Developmental toxicity study[d]	Maternal toxicity	20 mg/kg bw per day	60 mg/kg bw per day
		Embryo and fetal toxicity	60 mg/kg bw per day	200 mg/kg bw per day
Dog	Thirteen-week and 1-year studies of toxicity[a,b]	Toxicity	800 ppm, equal to 29.1 mg/kg bw per day	1 600 ppm, equal to 54.2 mg/kg bw per day

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

Estimate of acceptable daily intake (ADI)

0–0.03 mg/kg bw

Estimate of acute reference dose (ARfD)

0.3 mg/kg bw (applies to women of childbearing age only)

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 myclobutanil

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapidly absorbed (> 89%)
Dermal absorption	No data
Distribution	Extensive
Potential for accumulation	No significant tissue accumulation
Rate and extent of excretion	Rapidly excreted
Metabolism in animals	Extensively metabolized, mainly through a variety of oxidation reactions
Toxicologically significant compounds in animals and plants	Myclobutanil, unconjugated phenethyl triazole–containing metabolites (RH-9089, RH-9090) (rat, hen, cow, plants)
Acute toxicity	
Rat, LD_{50}, oral	≥ 1 600 mg/kg bw
Rat, LD_{50}, dermal	> 5 000 mg/kg bw
Rat, LC_{50}, inhalation	> 5.1 mg/L
Rabbit, dermal irritation	Not irritating to skin
Rabbit, ocular irritation	Moderately irritating to eye
Guinea-pig, dermal sensitization	Not sensitizing (maximization test and local lymph node assay); mildly sensitizing (Buehler method)
Short-term studies of toxicity	
Target/critical effect	Liver / increases in ALP and platelets (dog)
Lowest relevant oral NOAEL	29.1 mg/kg bw per day (dog)
Lowest relevant dermal NOAEL	100 mg/kg bw per day, highest dose tested (rat)
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Testes/atrophy (rat)
Lowest relevant NOAEL	2.5 mg/kg bw per day (rat)
Carcinogenicity	Not carcinogenic (rat and mouse); unlikely to pose a carcinogenic risk to humans
Genotoxicity	
	Unlikely to be genotoxic

Reproductive toxicity	
Target/critical effect	Testicular atrophy, increased number of pups born dead, reduced reproductive ability
Lowest relevant parental NOAEL	15.1 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	17.2 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	17.5 mg/kg bw per day (rat)
Developmental toxicity	
Target/critical effect	Fetal toxicity / increased number of early resorptions and lower fetal weights
Lowest relevant maternal NOAEL	20 mg/kg bw per day (rabbit)
Lowest relevant embryo/fetal NOAEL	31.3 mg/kg bw per day (rat)
Neurotoxicity	
Acute neurotoxicity NOAEL	No data
Subchronic neurotoxicity NOAEL	No data
Developmental neurotoxicity NOAEL	No data
Other toxicological studies	
Immunotoxicity	No data
Studies on toxicologically relevant metabolites	*RH-9089 and RH-9090*: Oral LD_{50}: 300–1 000 mg/kg bw (mice) Unlikely to be genotoxic *Myclobutanil butyric acid*: NOAEL: 1 000 mg/kg bw, highest dose tested (2-week study in rats) Unlikely to be genotoxic
Studies on impurities	Studies on RH-8812 and RH-8813 not relevant for dietary risk assessment
Medical data	
	No adverse effects noted in medical surveillance reports on manufacturing plant personnel

Summary

	Value	Study	Safety factor
ADI	0–0.03 mg/kg bw	Two-year study of toxicity and carcinogenicity (rat)	100
ARfD	0.3 mg/kg bw[a]	Developmental toxicity study (rat)	100

[a] Applies to women of childbearing age only.

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PYMETROZINE

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Explanation

Pymetrozine is the International Organization for Standardization (ISO)–approved common name for (*E*)-4,5-dihydro-6-methyl-4-(3-pyridylmethyleneamino)-1,2,4-triazin-3(2*H*)-one (International Union of Pure and Applied Chemistry), with Chemical Abstracts Service number 123312-89-0. Pymetrozine, which was developed under the code CGA 215944, is an insecticide that acts by inhibiting feeding, but the precise molecular targets are uncertain.

Pymetrozine 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

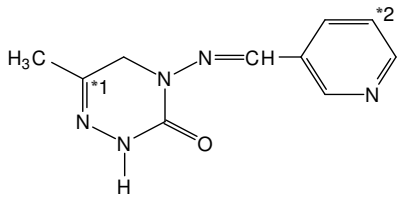
1. Biochemical aspects

1.1 Absorption, distribution and excretion

(a) Oral route

Two toxicokinetic studies were performed. In the first study, single oral doses of [6-^{14}C]triazine-labelled and [5-^{14}C]pyridine-labelled test substance (Fig. 1) were administered to groups of male and female Tif:RAIf rats. Urine, faeces, expired air and blood were collected at various time points. Tissues were collected either 7 days after administration of the radiolabelled dose or at various time points. Distribution of dose groups, administration route and sample collection are shown in Table 1. In the second study, the fate of pymetrozine was investigated in male and female Sprague-Dawley rats using [6-^{14}C]triazine-labelled and [5-^{14}C]pyridine-labelled material. The main objectives of this study were to extend the blood and tissue depletion kinetics determined only for males in the previous study to female rats and to assess the role of biliary elimination. Single oral doses of the labelled test substance were administered at dose levels of 0.5 mg/kg body weight (bw) (low dose) and 100 mg/kg bw (high dose) to several groups of male and female rats, including bile duct–cannulated animals, as outlined in Table 2.

Fig. 1. Structure of pymetrozine with position of radiolabels



*1: [6-^{14}C]triazine label; *2: [5-^{14}C]pyridine label

Following oral administration in the first study, radioactivity was rapidly and almost completely absorbed from the gastrointestinal tract into the general circulation. Independent of the label, maximum concentrations in the blood were reached 15 minutes and 4 hours after administration at the low and high dose levels, respectively (Bissig, 1993).

In the second study, absorption was rapid in female rats at both dose levels. At the low dose, mean maximum blood concentrations were observed at approximately 1 hour (T_{max}) post-dosing for both labels. At the high dose, after a rapid increase, the high blood concentrations were maintained until approximately 8 hours post-dosing. Areas under the blood concentration–time curve (AUCs) were proportionately greater at the high dose level, indicating a saturation of clearance. Half-lives in blood were longer with the [5-^{14}C]pyridine label than with the [6-^{14}C]triazine label (Table 3).

The calculated half-lives ($t_{½}$) for the depuration of the residual activity from the tissues were in the range of 1–2 hours at the low dose for both labels and up to 11 hours at the high dose level. Seven days after a single oral administration of [6-^{14}C]triazine-labelled pymetrozine at the low dose, tissue residues were low. The mean concentrations were below 0.025 parts per million (ppm) pymetrozine equivalents for all tissues except for the heart, with 0.038 ppm.

The principal route of excretion was urine, with a total of 56–80% of the dose. Excretion was rapid. Within 24 hours, 52–74%, 10–37% and 0.2–1.4% of the administered dose were detected in urine, faeces and expired air, respectively. The excretion pattern was essentially independent of sex, route of administration and pretreatment. Both sexes eliminated significantly more via kidneys at the high dose level than at the low dose level (Table 4).

Table 1. General outline of the first toxicokinetic study on pymetrozine, in Tif:RAIf rats

Group	Animals	Dose, label	Sample collection
Group A1	5 males 5 females	Low dose, intravenous [6-^{14}C]triazine	Collection of urine, faeces and expired air at different time points and of various tissues after 7 days
Group B1	5 males 5 females	Low dose, oral [6-^{14}C]triazine	Collection of urine, faeces and expired air at different time points and of various tissues after 7 days
Group C1	5 males 5 females	Low dose, oral [6-^{14}C]triazine pretreated[a]	Collection of urine and faeces at different time points and of various tissues 7 days after the radiolabelled dose
Group D1	5 males 5 females	High dose, oral [6-^{14}C]triazine	Collection of urine, faeces and expired air at different time points and of various tissues after 7 days
Group D2	5 males 5 females	High dose, oral [5-^{14}C]pyridine	Collection of urine, faeces and expired air at different time points and of various tissues after 7 days
Group E1	4 males	Low dose, oral [6-^{14}C]triazine	Collection of blood at different time points
Group E2	4 males	High dose, oral [6-^{14}C]triazine	Collection of blood at different time points
Group E3	4 males	Low dose, oral [5-^{14}C]pyridine	Collection of blood at different time points
Group E4	4 males	High dose, oral [5-^{14}C]pyridine	Collection of blood at different time points
Group F1	12 males	Low dose, oral [6-^{14}C]triazine	Collection of various tissues at different time points
Group F2	12 males	High dose, oral [6-^{14}C]triazine	Collection of various tissues at different time points
Group F3	12 males	Low dose, oral [5-^{14}C]pyridine	Collection of various tissues at different time points
Group F4	12 males	High dose, oral [5-^{14}C]pyridine	Collection of various tissues at different time points

[a] Single oral dose of [6-^{14}C]triazine-labelled pymetrozine preceded by 14 daily low doses of non-radiolabelled pymetrozine.

Source: Bissig (1993)

Biliary excretion contributed 12–30% to the clearance from the general circulation (Table 5). Clearance of total radioactivity from whole blood at the low dose was rapid for [6-^{14}C]triazine-labelled pymetrozine, with total elimination within about 24 hours. For [5-^{14}C]pyridine-labelled pymetrozine, elimination slowed down following an initial rapid phase, with radioactivity persisting in whole blood up to 168 hours post-dosing. A similar biphasic pattern of elimination was observed at the high dose for both labels, with pyridine-related residues being significantly higher. The sustained high blood concentration and the dose-disproportional increase in the AUC at the high dose are assumed to be due to saturation of the distribution and/or elimination processes (Jack & Dunsire, 1995).

At T_{max}, $T_{max}/2$, $T_{max}/4$ and $T_{max}/8$ at both dose levels and for both sites of labelling, the highest residues of radioactivity were observed in the liver and kidney. This is consistent with the fact that urine and bile are the main routes of excretion. Elimination of the radioactivity from the tissues was rapid in the early stages post-dosing, followed by slower and apparently more variable elimination in the later stages. At both dose levels, pyridine-related residues were more persistent (Jack & Dunsire, 1995).

Table 2. General outline of the second toxicokinetic study on pymetrozine, in Sprague-Dawley rats

Group	Animals	Dose, label	Sample collection
Group E5	3 females	Low dose, oral [6-^{14}C]triazine	Collection of blood at different time points
Group E6	3 females	High dose, oral [6-^{14}C]triazine	Collection of blood at different time points
Group E7	3 females	Low dose, oral [5-^{14}C]pyridine	Collection of blood at different time points
Group E8	3 females	High dose, oral [5-^{14}C]pyridine	Collection of blood at different time points
Group F5	12 females	Low dose, oral [6-^{14}C]triazine	Collection of various tissues at different time points
Group F6	12 females	High dose, oral [6-^{14}C]triazine	Collection of various tissues at different time points
Group F7	12 females	Low dose, oral [5-^{14}C]pyridine	Collection of various tissues at different time points
Group F8	12 females	High dose, oral [5-^{14}C]pyridine	Collection of various tissues at different time points
Group G1	4 males[a]	Low dose, oral [6-^{14}C]triazine	Collection of urine, bile and faeces at different time points and gastrointestinal tract and carcass after 2 days
Group G2	4 males[a]	High dose, oral [6-^{14}C]triazine	Collection of urine, bile and faeces at different time points and gastrointestinal tract and carcass after 2 days
Group G3	4 males[a]	Low dose, oral [5-^{14}C]pyridine	Collection of urine, bile and faeces at different time points and gastrointestinal tract and carcass after 2 days
Group G4	4 males[a]	High dose, oral [5-^{14}C]pyridine	Collection of urine, bile and faeces at different time points and gastrointestinal tract and carcass after 2 days

[a] Bile duct cannulated.

Source: Jack & Dunsire (1995)

Table 3. Selected toxicokinetic parameters in female Sprague-Dawley rats exposed to radiolabelled pymetrozine

	[6-^{14}C]Triazine		**[5-^{14}C]Pyridine**	
	E5	**E6**	**E7**	**E8**
	0.5 mg/kg bw	**100 mg/kg bw**	**0.5 mg/kg bw**	**100 mg/kg bw**
AUC (ppm·h)	0.47 ± 0.05	534 ± 37	2.8 ± 0.2	995 ± 112
$t_{½}$	3.7 ± 0.2 (4–12 h)	3.0 ± 0.1 (8–28 h) 80 ± 11 (72–168 h)	6.7 ± 0.2 (4–12) 147 ± 38 (24–168 h)	4.3 ± 0.1 (8–24 h) 155.7 ± 21.1 (24–168 h)
Radioactivity in whole blood after 1 h (µg equiv/mL)	0.115 ± 0.015	37.626 ± 3.843	0.104 ± 0.014	26.783 ± 5.168

AUC: area under the blood concentration–time curve; bw: body weight; equiv: equivalent; ppm: parts per million; $t_{½}$: half-life

Source: Jack & Dunsire (1995)

Table 4. Excretion pattern in Tif rats exposed to radiolabelled pymetrozine via oral and intravenous administration

	Excretion (% of dose)									
	[6-^{14}C]Triazine								**[5-^{14}C]Pyridine**	
	Intravenous		**Oral**						**Oral**	
	A1		**B1**		**C1**		**D1**		**D2**	
	M	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
	0.5 mg/kg bw	**0.5 mg/kg bw**	**0.5 mg/kg bw**	**0.5 mg/kg bw**	**0.5 mg/kg bw**	**0.5 mg/kg bw**	**100 mg/kg bw**	**100 mg/kg bw**	**100 mg/kg bw**	**100 mg/kg bw**
Urine										
0–24 h	63.6	68.3	52.0	58.4	55.4	63.0	69.6	73.5	70.2	73.5
24–48 h	0.9	0.9	1.8	1.8	0.9	0.8	2.0	3.9	3.0	3.1
48–168 h	1.1	0.7	2.5	1.9	0.5	0.4	0.9	1.0	3.7	3.7
Subtotal	65.6	69.9	56.3	62.1	56.8	64.2	72.5	78.3	76.9	80.3
Faeces										
0–24 h	24.9	20.3	26.6	23.0	36.6	27.1	20.8	11.6	15.6	10.2
24–48 h	1.1	1.2	2.7	3.7	1.8	2.7	3.9	5.0	2.8	4.3
48–168 h	0.5	1.1	1.1	1.1	0.5	0.6	0.7	1.4	1.0	1.0
Subtotal	26.5	22.6	30.3	27.8	38.9	30.3	25.4	18.0	19.5	15.4
Expired air	0.3	0.6	0.2	0.4	0.2	0.5	1.2	1.4	0.4	0.6
Cage wash	0.2	0.7	0.5	0.4	0.3	0.4	0.3	0.4	0.3	0.3
Total excretion	92.7	93.6	87.3	90.7	96.1	95.4	99.4	98.1	97.0	96.6

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

Source: Bissig (1993)

(b) Dermal route

Dermal absorption is estimated to be 1% (USEPA, 2000).

1.2 Biotransformation

Samples of urine and faeces from group D rats from the kinetic study of Bissig (1993) were analysed by high-performance liquid chromatography (HPLC), nuclear magnetic resonance and mass spectrometry. HPLC analysis revealed complex urinary and faecal metabolite patterns. The chromatograms were qualitatively similar for both sexes, both dose levels, both routes of administration and the pretreated animals. The most unpolar urinary fraction, accounting for 1–2% of the low dose and 15–18% of the high dose, co-chromatographed with unchanged pymetrozine. In the faeces, 1% of the administered dose was characterized as unchanged pymetrozine (Bissig, 1993; Schulze-Aurich, 1994).

The main metabolic reactions were identified as 1) oxidation reactions (approximately 19%) at the methyl substituent, leading, via the alcohol, to the corresponding carboxylic acid (Metabolite 3U, Metabolite 5U); 2) oxidation reactions (approximately 7% of the dose) at the triazine-methylene group, leading to the corresponding alcohol (Metabolite 2U); and 3) cleavage reactions between the triazine and the pyridine ring systems (estimated to represent approximately 20% of the dose). The initial cleavage products were further metabolized.

Table 5. Excretion pattern in male Sprague-Dawley rats exposed to pymetrozine via oral administration

	Excretion (% of dose)			
	[6-^{14}C]Triazine		[5-^{14}C]Pyridine	
	G1	G2[a]	G3	G4
	0.5 mg/kg bw	100 mg/kg bw	0.5 mg/kg bw	100 mg/kg bw
Bile				
0–8 h	27.42	6.17	22.52	3.15
8 –24 h	2.94	11.27	2.06	7.87
24–48 h	0.07	0.29	0.37	0.92
Subtotal	30.43	17.73	24.94	11.94
Urine				
0–24 h	58.19	61.09	50.30	53.29
24–48 h	1.23	2.45	1.49	5.72
Subtotal	59.41	63.54	51.79	59.00
Faeces				
0–24 h	9.70	8.69	6.43	3.74
24–48 h	1.47	2.56	0.61	2.05
Subtotal	11.17	11.25	7.04	5.79
Cage wash	1.99	2.51	1.43	3.95
Total excretion	90.49	81.94	86.24	81.96

bw: body weight

[a] Mean data from three animals only.

Source: Jack & Dunsire (1995)

A subsequent (non-GLP) review of these data by Thanei (1996) reported that because of inaccurate characterization of the metabolites in the earlier studies, the metabolite profile had been modified. The main changes were that levels of CGA 215525 were increased from < 1% in the original assessment to a reported maximum of 16.7%, and levels of CGA 294849 were increased from < 1% to a reported maximum of 9%. However, there was great variation between the values in the studies, and it is considered that the quantitative aspects of the metabolism of pymetrozine have a high degree of associated uncertainty.

In a study to investigate the comparative metabolism of pymetrozine, groups of female Tif RAIf rats (n = 3) and Tif:MAGf mice (n = 8) received diets containing non-labelled pymetrozine for 14 days followed by a single radiolabelled dose. The dietary levels were 10, 100, 500, 2000 and 5000 ppm in mice and 20, 100, 300, 1000 and 3000 ppm in rats. The radiolabelled gavage dose (12 mg/kg bw in mice and 7 mg/kg bw in rats), given in 0.5% carboxymethyl cellulose/Tween 80, consisted of a mixture of [5-^{14}C]pyridine-labelled and [6-^{14}C]triazine-labelled pymetrozine. The excreta were collected over 96 hours and analysed by HPLC and radio–thin-layer chromatography. The results showed that the pattern of excretion and metabolism was similar in rats and mice and was not influenced significantly by pretreatment with pymetrozine over a dose range covering 2 orders of magnitude (Table 6) (Muller, 1995; this study was not cited in Thanei, 1996).

A proposed metabolic pathway is presented in Fig. 2.

Table 6. Metabolite profile from rats and mice receiving pymetrozine in the diet

	Mean % of administered dose											
	Mouse						Rat					
Assignment of metabolite codes[a]	**10 ppm**	**100 ppm**	**500 ppm**	**2 000 ppm**	**5 000 ppm**	**Mean ± SD (ppm)**	**20 ppm**	**100 ppm**	**300 ppm**	**1 000 ppm**	**3 000 ppm**	**Mean ± SD (ppm)**
Pymetrozine (CGA 215944)	6.3	8.2	10.5	8.9	9.5	8.7 ± 0.9	16.1	13.8	11.7	10.9	6.8	11.9 ± 2.6
Metabolite 2U	4.6	5.5	5.1	4.5	6.8	5.3 ± 0.9	10.4	10.6	9.7	13.8	14.6	11.8 ± 2.1
Metabolite 3U	21.5	21.1	21.6	21.3	17.5	20.6 ± 1.7	23.1	24.9	21.1	24.9	20.3	22.9 ± 2.1
Metabolite 5U	11.5	9.4	6.8	6.8	6.6	8.2 ± 1.2	7.7	7.8	8.6	8.4	9.9	8.5 ± 0.8
Sum (containing both ring systems)	43.9	44.2	44.0	41.5	40.4	42.8 ± 1.6	57.3	57.1	51.1	58.0	51.6	55.0 ± 3.1
Metabolite 4U	3.4	2.8	3.2	2.1	2.6	2.8 ± 0.4	1.1	2.0	1.2	2.1	1.9	1.7 ± 0.4
Metabolite 6U	0.2	0.2	0.3	0.3	0.3	0.2 ± < 0.1	0.4	0.4	0.5	0.5	0.7	0.5 ± 0.1
CGA 180777	0.9	0.8	0.8	0.7	0.8	0.8 ± < 0.1	0.6	0.6	0.8	0.7	0.7	0.7 ± < 0.1
Sum (pyridine-derived metabolites)	4.5	3.8	4.2	3.1	3.7	3.8 ± 0.4	2.1	3.0	2.5	3.2	3.3	2.8 ± 0.3
CGA 215525	0.4	0.3	0.5	0.4	0.5	0.4 ± < 0.1	0.4	0.4	0.5	0.4	0.6	0.4 ± < 0.1
CGA 294849	0.1	0.1	0.1	0.1	0.1	0.1 ± < 0.1	0.1	0.1	0.1	0.1	0.2	0.1 ± < 0.1
CGA 249257	0.3	0.2	0.3	0.3	0.3	0.3 ± < 0.1	0.1	0.1	0.1	<0.1	0.1	0.1 ± < 0.1
CGA 259168	0.2	0.2	0.2	0.2	0.2	0.2 ± < 0.1	0.7	0.7	0.9	0.8	1.2	0.9 ± 0.2
GS 23199	0.3	0.3	0.4	0.3	0.4	0.3 ± < 0.1	0.4	0.4	0.5	0.4	0.7	0.5 ± 0.1
Sum (triazine-derived metabolites)	1.2	1.0	1.4	1.3	1.5	1.3 ± 0.2	1.7	1.5	2.1	1.7	2.6	1.9 ± 0.4
Unknown	34.9	35.9	32.1	32.4	32.4	33.5 ± 1.6	25.0	26.8	29.2	26.3	29.5	27.4 ± 1.4
Total	84.5	84.9	81.7	78.2	78.0	81.5 ± 2.9	86.1	88.5	84.9	89.2	87.0	87.1 ± 1.7

ppm: parts per million; SD: standard deviation

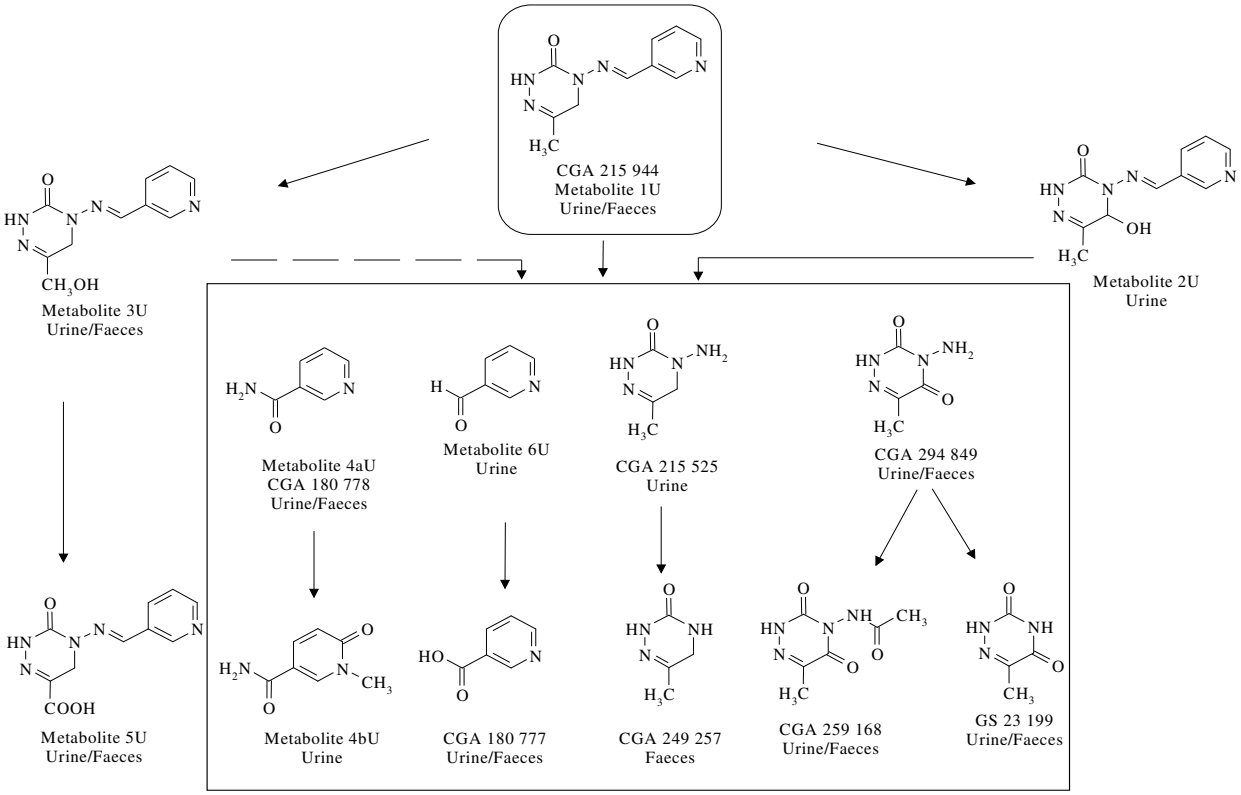
[a] See Fig. 2 for metabolite structures.

Source: Muller (1995)

1.3 Effects on enzymes and other biochemical parameters

Studies on the induction of xenobiotic metabolizing enzymes are presented in section 2.6(b) as part of the considerations of potential mechanisms for tumour formation.

Fig. 2. Proposed metabolic pathways of pymetrozine in rats and mice



2. Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

Pymetrozine was of low acute toxicity by the oral, dermal and inhalation routes (Table 7).

Table 7. Summary of acute toxicity studies with pymetrozine

Species	Strain	Sex	Route	Purity (%)	LD_{50}/LC_{50}	Reference
Rat	HSD: Sprague-Dawley	M & F	Oral gavage	98	5 820 mg/kg bw	Glaza (1991d)
Rat	HSD: Sprague-Dawley	M & F	Dermal	98	> 2 000 mg/kg bw	Glaza (1991a)
Rat	Tif:RAIf	M & F	Inhalation (aerosol)	98	> 1.8 mg/L[a] (MMAD < 3 µm)	Hartmann (1991)

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

[a] Highest achievable concentration.

(b) Dermal irritation

Pymetrozine (batch no. P102002; purity 98%) produced no erythema or oedema of the skin when tested in six New Zealand White rabbits (Glaza, 1991b).

(c) Ocular irritation

Pymetrozine (batch no. P102002; purity 98%) produced transient iridial reaction (scores 0–1; resolved at 24 hours) and conjunctival redness and chemosis (scores 1–2; resolved at 96 hours) when tested in six New Zealand White rabbits (Glaza, 1991c).

(d) Dermal sensitization

Pymetrozine (batch no. P301005; purity 99.3%) produced a reaction in 1/10 males and 0/10 females when tested in a Magnusson and Kligman maximization test in guinea-pigs (Winkler, 1997).

2.2 Short-term studies of toxicity

(a) Oral administration

Mice

Groups of 10 male and 10 female Tif:MAGf (SPF) mice were given diets containing pymetrozine (batch no. P.102002; purity 98.0%) at 0, 1000, 3000 or 7000 ppm for 3 months. The approximate mean daily intakes were calculated to be 0, 143, 429 and 1000 mg/kg bw per day for males and 0, 252, 589 and 1240 mg/kg bw per day for females, respectively.

There were no compound-related clinical signs and no mortality. Overall body weight gains in males were markedly lower (41%) than those of the controls at 7000 ppm, and there was a tendency for decreased mean body weight gain in males at 3000 ppm from week 6 onwards. Body weights of females were unaffected by treatment. Feed consumption was reduced by 32% at week 1 and continued to be slightly reduced (up to 18%) at 7000 ppm. In males, feed intake was increased in groups treated with 1000 and 3000 ppm, indicating that the body weight deficits are not entirely due to reduced feed intake.

Males at 7000 ppm had slightly lower white blood cell counts. Absolute and relative liver weights were dose-dependently increased in all groups. Relative spleen weights in both sexes and relative kidney weights in males were elevated at 3000 and 7000 ppm (Table 8).

Table 8. Organ weights in the 90-day study in mice receiving pymetrozine in the diet

	Males				Females			
Parameter	**0 ppm**	**1 000 ppm**	**3 000 ppm**	**7 000 ppm**	**0ppm**	**1 000 ppm**	**3 000 ppm**	**7 000 ppm**
Liver, absolute (g)	2.251	2.530*	2.700*	2.854**	1.814	2.021	2.395**	2.681**
Liver, relative[a]	49.23	54.68**	62.21**	77.20**	57.24	65.15	74.79**	86.59**
Spleen, relative[a]	1.878	2.109	2.346*	2.892**	4.317	4.583	5.071*	5.027*
Kidneys, relative[a]	13.10	13.51	14.39*	15.39**	14.69	15.55	15.03	13.60

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$

[a] Relative to body weight (mg/g).

Source: Gerspach (1992a)

Histopathological changes were observed in all groups. They consisted of hypertrophy of centrilobular hepatocytes, increased incidences of focal single-cell necrosis and lymphocytic

infiltration in the liver and increased severity of extramedullary haematopoiesis in the spleen (Table 9).

Table 9. Histopathological findings in the 90-day study in mice receiving pymetrozine in the diet

	Incidence of finding (n = 10)			
Finding	**0 ppm**	**1 000 ppm**	**3 000 ppm**	**7 000 ppm**
Males				
Liver: Hepatocyte hypertrophy	0	3	7*	10*
Liver: Hepatic parenchyma single-cell necrosis	1	3	3	6*
Liver: Lymphocytic infiltration	0	2	5*	10*
Spleen: Extramedullary haematopoiesis, moderate	1	2	3	4
Spleen: Extramedullary haematopoiesis, marked	0	0	0	0
Females				
Liver: Hepatocyte hypertrophy	0	2	5	10*
Liver: Hepatic parenchyma single-cell necrosis	3	7*	8*	9*
Liver: Lymphocytic infiltration	0	3	8*	10*
Spleen: Extramedullary haematopoiesis, moderate	3	3	2	1
Spleen: Extramedullary haematopoiesis, marked	6	7	8	9

ppm: parts per million

*: $P < 0.05$

Source: Gerspach (1992a)

No no-observed-adverse-effect level (NOAEL) can be determined for this study, based on increased hepatocellular necrosis at 1000 ppm (equal to 252 mg/kg bw per day), the lowest dose tested, in females (Gerspach, 1992a).

Rats

Groups of 10 male and 10 female Tif:RAIf (SPF) rats were administered pymetrozine (batch no. 102002; purity 98%) at 0, 10, 100 or 600 mg/kg bw per day for 28 days via gavage in distilled water containing 0.5% carboxymethyl cellulose and 0.1% Tween 80.

Transient reddening of the ears was noted in seven males and five females at 600 mg/kg bw per day. No compound-related mortality was reported. Body weight gains were lower than in the controls at 600 mg/kg bw per day, linked to reduced feed consumption. Water consumption was increased in females at 600 mg/kg bw per day, and a similar trend was also noted in males. Ophthalmoscopy revealed no changes. Haematological and blood chemistry changes were seen in the high-dose groups of both sexes: a mild anaemia with a tendency to macrocytosis (increased mean corpuscular volume values) and hyperchromasia (increased mean corpuscular haemoglobin values), associated with higher reticulocyte counts, and slightly increased white blood cell counts. There were increases in plasma bilirubin, albumin and cholesterol levels and a decrease in plasma chloride level in both sexes.

Relative liver weights were increased at 100 mg/kg bw per day (by 10–12%) and 600 mg/kg bw per day (by 40–72%). Increased kidney weight to body weight ratios (12–23%), increased spleen weights (22–42%) and decreased thymus weights (30–43%) were recorded for animals of both sexes at 600 mg/kg bw per day. Mottled livers were seen in males of the 600 mg/kg bw per day group. Histopathological changes consisted of hypertrophy of centrilobular hepatocytes at 100 and 600 mg/kg bw per day, atrophy of the thymus in males at 100 mg/kg bw per day and in both sexes at

600 mg/kg bw per day, and hyperplasia of the splenic white pulp in both sexes at 100 and 600 mg/kg bw per day. Spermatogenesis and the number of spermatozoa in the epididymis were reduced at 600 mg/kg bw per day.

The NOAEL was 10 mg/kg bw per day, based on thymic atrophy and hyperplasia of the splenic white pulp at 100 mg/kg bw per day (Fankhauser, 1992).

Groups of five male and five female Tif:RAIf (SPF) rats were given diets containing pymetrozine (batch no. HK-6989; purity 100%) at 0, 100, 500, 2000 or 10 000 ppm (equal to 0, 10, 55, 203 and 691 mg/kg bw per day for males and 0, 10, 55, 212 and 699 mg/kg bw per day for females, respectively) for 28 days.

Under the conditions of the study, no effects were observed at dietary dose levels of 100 or 500 ppm. At a dose level of 2000 ppm and above, slight changes in body weight (9%), feed intake and urine volume were observed, but these are not considered adverse. At 10 000 ppm, transient clinical signs (reddening of ears, piloerection) were detected. Additionally, several clinical chemistry, haematology and urine analysis parameters were changed, indicating anaemia and hepatotoxicity. Histopathological findings included hepatocyte necrosis and centrilobular hypertrophy, splenic congestion, fatty changes in adrenal cortex and thymic atrophy. Spermatogenesis in the testis as well as the spermatozoa in the epididymis were reduced.

The NOAEL was 2000 ppm (equal to 203 mg/kg bw per day), based on a range of findings at 10 000 ppm (equal to 691 mg/kg bw per day) (Gerspach, 1991).

Groups of 10 male and 10 female Tif:RAIf (SPF) rats were given diets containing pymetrozine (batch no. P.102002; purity 98.0%) at 0, 50, 500 or 5000 ppm for 3 months. Mean daily intakes were 0, 3.4, 33 and 360 mg/kg bw per day for males and 0, 3.6, 34 and 370 mg/kg bw per day for females, respectively. An additional 10 animals of each sex of the control and high-dose groups were kept for a 4-week follow-up recovery period.

There were no clinical signs or mortalities during the study. There were no adverse effects in the 50 or 500 ppm dose groups.

At 5000 ppm, body weights were markedly lower than those of the controls during the course of the study (by 10% in week 1, by 26% at week 13), with a slight recovery when dosing ceased. Feed consumption was reduced during treatment (17–23% below control), but was similar to control values during the recovery period. Water consumption was also reduced (18–24% below control) during the treatment period. A reversible increase in white blood cell counts was noted. There were increases in plasma bilirubin and cholesterol levels and higher activities of alkaline phosphatase in both sexes. Lower plasma glucose and potassium concentrations and a slight increase of the albumin to globulin ratio were observed in males. Reversibility was seen for most effects on clinical chemistry parameters (Table 10).

Bilirubin in the urine of females and a slight decrease in urine volume in males were noted. Relative liver weights were increased at the end of treatment (40–42% above control values) and still slightly increased at the end of the recovery period (9–12%). Thymus weights were decreased (19–42%) and relative spleen weights increased (26–40%) in both sexes, with partial recovery in females, but no recovery in males. At the end of the treatment period, histopathological changes consisted of hypertrophy of centrilobular hepatocytes in males (8/10), slight focal calcification of the kidneys in males (3/10) and atrophy of the thymus in animals of both sexes (7/10 males and 5/10 females). After the recovery period, thymic atrophy was still present in 4/10 males.

The NOAEL was 500 ppm (equal to 33 mg/kg bw per day), on the basis of thymic atrophy, hepatocellular hypertrophy and renal calcification at 5000 ppm (equal to 360 mg/kg bw per day) (Gerspach, 1992b).

Table 10. Clinical chemistry parameters in rats receiving pymetrozine for 90 days

	Males				Females			
	0 ppm	**50 ppm**	**500 ppm**	**5 000 ppm**	**0 ppm**	**50 ppm**	**500 ppm**	**5 000 ppm**
Bilirubin (µmol/L)	2.493	2.776	2.599	3.682*	2.764	2.532	2.567	4.448*
Recovery[a]	8.502	–	–	7.459	2.865	–	–	2.466
Albumin/globulin ratio (L)	1.123	1.127	1.131	1.163*	1.236	1.245	1.262	1.334
Recovery	1.129	–	–	1.150	1.239	–	–	1.232
Cholesterol (fmol)	1.832	1.632	1.904	2.685*	2.108	2.120	2.223	2.612*
Recovery	1.862	–	–	1.864	2.193	–	–	2.159
Glucose (mmol/L)	8.365	8.533	8.099	7.053*	7.625	7.076	7.798	7.554
Recovery	8.502	–	–	7.459	7.206	–	–	6.465
Sodium (mmol/L)	144.5	143.1*	144.1	144.1	142.3	141.8	142.0	141.4*
Recovery	144.0	–	–	143.8	142.3	–	–	141.5
Potassium (mmol/L)	3.715	3.677	3.606	3.293*	3.350	3.190	3.328	3.381
Recovery	3.484	–	–	3.319	3.070	–	–	3.092
Alkaline phosphatase (U/L)	100.4	105.9	108.8	140.5*	58.77	59.49	55.87	89.82*
Recovery	92.38	–	–	107.2	55.80	–	–	64.24

ppm: parts per million; U: units; *: $P < 0.05$

[a] Recovery: all results refer to week 18.

Source: Gerspach (1992b)

Dogs

Groups of two male and two female Beagle dogs were given diets containing pymetrozine (batch no. KGL 4193/6; purity 98%) at 0, 100, 500 or 2500 ppm (equal to 0, 3.2, 15 and 55 mg/kg bw per day for males and 0, 2.8, 16 and 50 mg/kg bw per day for females, respectively) for 28 days.

No effects were observed at 100 ppm. At a dose level of 500 ppm and above, thymus weight was decreased by 20% in females. At 2500 ppm, thymus atrophy, body weight loss and low feed intake were observed. The body weight effects were not evident in the first week of dosing. Alkaline phosphatase activity and white blood cell counts were increased.

The NOAEL was 100 ppm (equal to 2.8 mg/kg bw per day), based on a decrease in thymus weights in females at 500 ppm (equal to 16 mg/kg bw per day) (Altmann, 1991).

Groups of four male and four female Beagle dogs were given diets containing pymetrozine (batch no. P.102002; purity 98.0%) at 0, 100, 500 or 2500 ppm for 3 months. The mean daily intakes were 0, 3.1, 14 and 53 mg/kg bw per day for males and 0, 3.2, 15 and 60 mg/kg bw per day for females, respectively.

One high-dose female dog was terminated in extremis in week 5. Clinical signs were seen sporadically in the high-dose group. Body weights of animals in the high-dose group were lower than those of controls, with high interindividual variation ranging from severe loss to normal development. Mean feed consumption was reduced for the high-dose males during the whole treatment period and for females from week 2 onwards (decreased by 4–59%). Ophthalmoscopic examination did not

reveal any reaction to treatment. Red blood cell parameters (erythrocyte count, haemoglobin, haematocrit) were decreased in males and females of the high-dose group. A severe anaemia associated with macrocytosis, hyperchromasia of red blood cells and increased reticulocyte counts was noted in two high-dose females, which also had markedly increased levels of plasma bilirubin. Plasma protein levels were elevated from week 8 in females at 500 and 2500 ppm owing to an increase in the globulin fraction.

Bilirubinuria, which correlated with the increased plasma bilirubin levels, was observed in the dogs of the high-dose group. Liver weights were increased at 500 and 2500 ppm, and spleen weights were elevated at 2500 ppm (Table 11). Reduced weights of testes were noted in males and of heart, thymus and thyroid in both sexes of the high-dose group (Table 11).

Table 11. Mean organ weights in the 90-day study of pymetrozine in dogs

	Organ weight (g)							
	Males				**Females**			
Parameter	**0 ppm**	**100 ppm**	**500 ppm**	**2 500 ppm**	**0 ppm**	**100 ppm**	**500 ppm**	**2 500 ppm**
Body weight (kg)	*12.9*	*12.1*	*12.4*	*9.8**	*11.5*	*11.7*	*11.8*	*8.0**
Heart	113.8	105.4	111.5	82.39*	100.4	98.66	96.67	75.98*
Liver absolute	341.0	348.4	398.2*	386.2	330.8	378.6	391.5*	369.5
Liver relative (g/100 g bw)	28.53	30.26	33.90	40.01*	29.45	33.59	34.50	50.87*
Thymus	11.26	10.11	8.58	5.28	11.16	12.94	10.73	3.21*
Spleen	30.38	25.18	30.36	30.86	27.70	26.90	31.39	113.9
Thyroid	1.202	0.910*	1.016	0.758*	0.979	1.096	1.154	0.700*
Testes	20.83	18.43	20.28	14.28*	–	–	–	–

ppm: parts per million; *: $P < 0.05$

Source: Altmann (1992)

At necropsy, the body was reported as having either yellowish or whitish coloration in two females, and the liver was mottled in some dogs of the high-dose group.

Histopathological findings were wide ranging at 2500 ppm and included the spleen, thymus, testes and liver at 500 ppm:

- hepatocellular necrosis, fibrosis and/or inflammatory changes of the liver and proliferation of intrahepatic bile ducts at 500 and 2500 ppm. Extramedullary haematopoiesis, haemosiderosis and cholestasis were observed among high-dose animals;
- inflammatory and/or degenerative changes in the skeletal muscle (myopathy), gastrointestinal wall (inflammation, oedema), thyroid, salivary gland and prostate (lymphohistiocytic infiltration) at 500 and 2500 ppm. Additionally, at the high dose, similar changes occurred in the myocardium (inflammation), gallbladder of females (oedema), parathyroid gland of males (lymphohistiocytic infiltration) and autonomic ganglions in females (perivascular cell infiltration);
- dilatation of prostatic tubuli (reported as cystic dilatation of glandular tissue) and minimal tubular atrophy of the testis or reduced spermatogenesis at 500 and 2500 ppm;
- uterine atrophy at 2500 ppm;

- atrophy of the thymic cortex at 500 and 2500 ppm, atrophic lymphatic follicles and an increased occurrence of phagocytic cells in the mesenteric lymph nodes at 2500 ppm;
- hypocellularity of the bone marrow in one high-dose male and hypercellularity of the bone marrow in females of the mid- and high-dose groups;
- splenic haemosiderosis in males and extramedullary haematopoiesis in females at 500 and 2500 ppm.

The NOAEL was 100 ppm (equal to 3.1 mg/kg bw per day), based on thymic atrophy, testicular tubular atrophy, reduced spermatogenesis, hepatocellular necrosis and inflammatory changes in several organs at 500 ppm (equal to 14 mg/kg bw per day) (Altmann, 1992).

Groups of Beagle dogs (four animals of each sex) received pymetrozine (batch no. P.102002; purity 98.0%) at a dietary level of 0, 20, 200 or 1000 ppm for 1 year. Additionally, two male and two female dogs in each of the control and high-dose groups were treated identically and maintained for a treatment-free recovery period of 4 weeks. The average compound intakes were 0, 0.57, 5.3 and 28 mg/kg bw per day for males and 0, 0.57, 5.0 and 27 mg/kg bw per day for females, respectively.

Vomiting was reported in three top-dose males; five top-dose females were reported to show apathy. One male of the 1000 ppm group was found dead in week 33, which was attributed to acute bronchopneumonia.

Decreases in feed consumption and body weight were noted at 1000 ppm, with reversibility during the recovery period. No ophthalmological changes were observed. Changes in electrocardiography were slight and considered to be not compound related. After 26 weeks of treatment, one female had a severe anaemia with macrocytosis and hypochromasia of red blood cells and bilirubinaemia; results were normal at 52 weeks. Red blood cell parameters were also slightly decreased in males at 200 and 1000 ppm (Table 12).

Table 12. Haematology and clinical chemistry values in a 1-year study of pymetrozine in dogs

	0 ppm	20 ppm	200 ppm	1 000 ppm
Males				
Red blood cells ($\times 10^{12}$/L), week 52	6.978	6.985	6.293	6.511
Haemoglobin (mmol/L), week 52	9.967	10.04	8.875*	8.950*
Haematocrit (L), week 52	0.486	0.500	0.442	0.446*
Females				
Cholesterol (mmol/L)				
Pretest	3.550	3.505	3.353	3.788
Week 13	3.262	3.250	3.605	3.975
Week 26	3.442	3.183	3.680	4.213
Week 52	3.353	3.298	4.248*	4.611*
Recovery	3.285	–	–	4.025
Phospholipids (mmol/L)				
Pretest	3.648	3.598	3.520	3.805
13	3.552	3.518	3.883	4.105
26	3.662	3.543	4.140	4.158
52	3.665	3.753	4.390	4.598*
Recovery	3.570	–	–	3.980

ppm: parts per million; *: $P < 0.05$

Source: Altmann (1994)

Higher plasma cholesterol and phospholipid levels were recorded in females at 1000 ppm and also at the end of treatment in the 200 ppm females. In the high-dose females, these minor changes persisted up to the end of the recovery period (Table 12).

Liver weights were increased at 200 and 1000 ppm in both sexes. In females, liver weight was still increased after the recovery period (Table 13). A reduction of the testes weight was evident at 20 ppm and was statistically significant at 200 and 1000 ppm (Table 13).

Table 13. Absolute organ weights in a 1-year study of pymetrozine in dogs

	Absolute organ weight (g)			
Parameter	**0 ppm**	**20 ppm**	**200 ppm**	**1 000 ppm**
Males				
Body weight (kg)	*13.58*	*12.85*	*12.78*	*12.76**
Liver	384	396	428	407
Testis	22.08	19.81	18.00*	18.40*
Females				
Body weight (kg)	*12.18*	*13.45*	*14.25**	*12.88*
Liver	390	404	463*	461

ppm: parts per million; *: $P < 0.05$

Source: Altmann (1994)

Histopathological findings were limited to animals receiving 1000 ppm. They consisted of myopathy in two male dogs, increased inflammatory cell infiltration in the liver in the males, associated with a focal fibrosis in one case, and increased splenic or hepatic haemosiderosis in some animals. The inflammatory cell infiltration of the liver or increased haemosiderosis and myopathy were still present at the end of the recovery period. Bone marrow investigation revealed no treatment-related findings.

In males at 1000 ppm, testicular lesions were reported (unilateral tubular atrophy in one male; bilateral occurrence of spermatic giant cells in the testicular spermatogenic epithelium as well as atrophy of prostatic glandular tissue of another).

The NOAEL was 20 ppm (equal to 0.57 mg/kg bw per day), based on changes in clinical chemistry parameters in females as well as reduced haemoglobin and significant reductions in testis weight in males at 200 ppm (equal to 5.0 mg/kg bw per day) (Altmann, 1994).

(b) Dermal application

Rats

Groups of five male and five female Tif:RAIf (SPF) rats were administered pymetrozine (batch no. P.102002; purity 98.0%) at a dose level of 0, 10, 100 or 1000 mg/kg bw per day to the shaved skin under occlusive dressing for 6 hours/day, 5 days/week, for 4 weeks. There was no evidence of systemic or local toxicity.

The NOAEL was 1000 mg/kg bw per day, the highest dose tested (Hagemann, 1993).

(c) Exposure by inhalation

No data were submitted.

2.3 Long-term studies of toxicity and carcinogenicity

Mice

Groups of mice (Tif:MAGf (SPF)), 60 of each sex per dose level, received pymetrozine (batch no. P.102002; purity 98%) at a dietary level of 0, 10, 100, 2000 or 5000 ppm for 78 weeks. The average compound intakes were 0, 1.2, 12, 254 and 678 mg/kg bw per day for males and 0, 1.2, 11, 243 and 673 mg/kg bw per day for females, respectively. Fifty animals of each sex per group were utilized for evaluation of carcinogenic potential, and 10 animals of each sex per group were used for evaluation of haematological parameters (week 53 and week 78).

There were no effects on mortality, appearance or behaviour. Survival was greater than 70% in all groups.

Lower mean body weights were recorded for males and females at 5000 ppm (Table 14). Relative liver, kidney, adrenal and spleen weights were altered in males at 2000 and 5000 ppm; liver, spleen and kidney weights were changed in top-dose females (Table 14).

Table 14. Body weights and organ to body weight ratios in mice receiving pymetrozine for 78 weeks

	0 ppm	10 ppm	100 ppm	2 000 ppm	5 000 ppm
Mean body weight (g)					
Males					
Week –1	27.54	27.29	27.45	27.40	27.57
Week 51	52.92	51.14	52.90	52.38	47.39*
Week 75	52.96	50.46	52.70	50.99	47.07*
Females					
Week –1	23.62	23.78	23.41	23.51	23.72
Week 51	49.74	51.21	51.48	49.19	40.60*
Week 75	53.36	52.05	51.20	48.66	40.40*
Relative organ weight (mg/g bw)					
Males					
Liver	56.25	55.73	55.34	76.51*	100.1*
Kidney	16.94	17.81	17.30	16.08	16.13
Spleen	2.656	3.105	2.885	3.336	3.455*
Adrenals	0.119	0.140	0.132	0.152*	0.161*
Females					
Liver	55.72	51.67	52.59	61.45*	85.35*
Kidney	11.00	11.22	11.17	11.29	13.09*
Spleen	6.365	5.442	6.941	5.999	5.614
Adrenals	0.351	0.354	0.363	0.374	0.379

bw: body weight; ppm: parts per million; *: $P < 0.05$

Source: Gerpach (1995b)

Necropsy revealed a higher incidence of liver findings in both sexes at 2000 and 5000 ppm. Splenic enlargement was noted in males of the 2000 and 5000 ppm groups. Histopathological examination revealed a significantly increased incidence and severity of liver cell hypertrophy in

males and females at 2000 and 5000 ppm, associated with a significantly increased incidence of benign and malignant liver tumours in both sexes at 5000 ppm and of malignant tumours also in males of the 2000 ppm group (Table 15). There were higher incidences of chronic inflammation of the glandular stomach and hyperplasia of the gastric mucosa in males at 5000 ppm, haemosiderosis of the spleen in males at 5000 ppm and females at 2000 and 5000 ppm, extramedullary haematopoiesis in the spleen of males and females in the 2000 and 5000 ppm groups, and hypercellularity of the bone marrow in males at 2000 and 5000 ppm and females at 5000 ppm (Table 15).

Table 15. Selected incidences of histopathological lesions in mice receiving pymetrozine for 78 weeks

	Incidence of lesion									
	Males					**Females**				
	0 ppm	**10 ppm**	**100 ppm**	**2 000 ppm**	**5 000 ppm**	**0 ppm**	**10 ppm**	**100 ppm**	**2 000 ppm**	**5 000 ppm**
Liver / Total examined	50	50	50	49	50	49	50	50	50	50
Hypertrophy	29	30	28	49**	50**	12	11	12	47**	46**
Necrosis	3	3	2	1	3	2	1	0	3	5
Stomach / Total examined	50	49	49	50	50	49	50	50	50	50
Chronic inflammation	13	14	8	12	20	13	10	12	10	11
Mucosal hyperplasia	10	8	9	11	19	6	9	8	7	6
Spleen / Total examined	50	50	50	50	50	49	50	50	50	50
Extramedullary haematopoiesis	30	28	29	38**	44**	37	37	35	43	46**
Haemosiderosis	24	21	17	18	35**	30	29	31	41**	43**
Bone marrow / Total examined	50	50	50	50	50	49	50	50	50	50
Hypercellularity	29	28	27	40**	42**	22	21	20	29	33*
Liver / Total examined	50	50	50	49	50	49	50	50	50	50
Benign hepatoma	10	3	12	9	11	4	5	4	1	14*
Carcinoma	5	5	5	9**	23**	0	0	0	0	4**
Hepatoma + carcinoma	15	8	17	18	34**	4	5	4	1	18**
Lung / Total examined	50	49	49	50	50	49	50	50	50	50
Adenoma[a]	14	8	11	14	13	6	3	3	9	8
Carcinoma[b]	1	1	3	1	0	1	1	5	7	2
Adenoma + carcinoma[c]	15	9	14	15	13	7	4	8	16*	10*

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$

[a] Historical control incidence in females: 3–13%; mean 7% (in five studies, 1988–1991).
[b] Historical control incidence in females: 2–7%; mean 4% (in five studies, 1988–1991).
[c] Historical control incidence in females: 5–18%; mean 5% (in five studies, 1988–1991).

Source: Gerpach (1995b)

There were no differences in the incidences of benign and/or malignant tumours in the lung of male mice. In females at 2000 and 5000 ppm, the incidence of combined benign and malignant pulmonary tumours was statistically significant increased, and both the lung adenomas and the

combined pulmonary tumour incidences were above the historical control range. Although there is no clear dose–response relationship, there is only a 2.5-fold dose spacing gap, and a treatment-related effect cannot be discounted. An increase in lung carcinomas in females at 100 ppm is not considered to be an adverse effect of treatment when considered along with the incidence of lung adenoma at this dose level and the absence of an increase in carcinomas at 5000 ppm (Table 15).

The NOAEL for non-neoplastic toxicity was 100 ppm (equal to 11 mg/kg bw per day), based on liver hypertrophy, splenic haematopoiesis and haemosiderosis and alterations in kidney, liver and spleen weights at 2000 ppm (equal to 243 mg/kg bw per day). The NOAEL for carcinogenicity was 100 ppm (equal to 11 mg/kg bw per day), based on the increased incidence of malignant hepatocellular carcinomas in males at 2000 ppm (equal to 254 mg/kg bw per day) and benign and malignant lung tumours in females at 2000 ppm (equal to 243 mg/kg bw per day) (Gerspach, 1995b).

Rats

Groups of Tif:RAIf (SPF) rats (80 of each sex per dose) received pymetrozine (batch no. P.102002; purity 98%) at a dietary level of 0, 10, 100, 1000 or 3000 ppm. The mean compound intakes were 0, 0.4, 3.7, 39 and 128 mg/kg bw per day for males and 0, 0.4, 4.5, 47 and 154 mg/kg bw per day for females, respectively. Fifty rats of each sex per group were used for evaluation of carcinogenic potential, and 10 animals of each sex per group were used for the analysis of haematological, biochemical and urine parameters. Additionally, 10 animals of each sex per group were utilized for the analysis of haematological parameters, and another 10 animals of each sex per group were used for interim termination at 12 months.

Survival in treated animals, particularly males, was better than that of controls. Treatment had no adverse effect on mortality, appearance or behaviour.

Lower mean body weights by the end of the study were reported for males (15%) and females (24%) at 3000 ppm. Slightly lower mean body weights by the end of the study were also recorded at 1000 ppm (by 4% in males and 7% in females). These changes were paralleled by decreases in feed consumption in females at 1000 ppm and in both sexes at 3000 ppm. No ophthalmological changes were observed. A range of clinical chemistry changes was noted at 3000 ppm, but there was no consistency across the sexes. Sodium levels were reduced at all dose levels in both sexes, but without a clear dose–response relationship. Sodium levels are normally very well regulated, but, in the absence of a dose–response relationship or any related findings, this is not considered adverse (Table 16). In addition, compared with values for sodium in the 28- and 90-day rat studies, the control values appear to be unusually high. There were no treatment-related effects on any urine parameters.

Table 16. Haematological and clinical chemistry findings in the 24-month study of pymetrozine in rats

Parameter	0 ppm	10 ppm	100 ppm	1 000 ppm	3 000 ppm
Males					
Red blood cells ($\times 10^{12}$/L)					
Week 13	8.790	8.549	8.597	8.572	8.399*
Glucose (mmol/L)					
Week 13	7.669	8.143	7.793	7.627	6.369
Week 53	9.568	9.406	9.295	9.231	7.828*
Chloride (mmol/L)					
Week 13	98.57	98.85	98.49	97.66	96.42
Week 53	102.8	102.9	101.5	101.8	99.83*
Week 105	104.9	105.1	104.1	103.5	102.9

Parameter	0 ppm	10 ppm	100 ppm	1 000 ppm	3 000 ppm
Sodium (mmol/L)					
Week 13	145.5	144.8	144.1*	143.9*	144.0*
Week 27	144.1	143.6	142.4	142.4*	142.6
Week 53	145.8	144.2	143.4*	143.0*	143.9
Week 78	144.7	144.8	144.0	143.9	143.8
Week 104	143.1	143.3	142.5	141.4	142.2
Albumin (g/L)					
Week 13	37.93	37.89	38.38	38.64	39.80
Week 53	36.05	36.71	36.78	36.75	38.09
Week 105	33.76	35.30	35.91	35.50	37.75
Bilirubin (µmol/L)					
Week 13	2.643	2.719	2.709	2.798	3.493*
Week 53	2.284	2.938	2.633	2.435	3.731*
Week 105	3.358	3.034	8.630	3.149	4.610*
ALT (U/L)					
Week 13	30.88	30.56	31.92	27.36	22.44
Week 105	48.86	39.27	44.60	41.29	36.86
Females					
Cholesterol (mmol/L)					
Week 13	1.854	2.040	2.029	2.324	2.583
Week 105	3.314	3.219	2.689	3.404	4.722
Sodium (mmol/L)					
Week 13	143.3	143.0	142.5	142.5	142.6
Week 27	142.6	144.1	141.6	141.2*	141.0*
Week 53	144.0	143.5	143.7	142.5	142.1
Week 78	145.1	143.7	144.1	143.5	141.8*
Week 104	141.2	138.7	139.9	138.6	139.3
Phosphorus (mmol/L)					
Week 13	1.355	1.423	1.312	1.445	1.681
Week 53	0.900	1.072	0.918	1.191*	1.240*
Week 105	1.405	1.235	1.355	1.308	1.597
ALT (U/L)					
Week 13	25.12	29.36	27.67	23.68	21.12
Week 53	60.22	54.89	92.51	39.66	27.27*
Week 105	42.47	44.01	34.44	34.04	28.14

ALT: alanine aminotransferase; ppm: parts per million; U: units; *: $P < 0.05$

Source: Gerspach (1995a)

The weights of liver, kidneys and spleen were elevated in both sexes at 3000 ppm and in males at 1000 ppm after 1 year of treatment. The relative testes weights were significantly increased after 53 and 104 weeks at 3000 ppm. At termination, significantly higher organ to body weight ratios were obtained for liver, kidney and spleen of males and females and for ovaries at 3000 ppm (Table 17).

Table 17. Relative organ weights in a 24-month study of pymetrozine in rats

	Relative organ weight (g/kg bw)				
Parameter	**0 ppm**	**10 ppm**	**100 ppm**	**1 000 ppm**	**3 000 ppm**
Males					
Liver					
Interim	29.76	32.59	32.63	37.55*	42.68*
Terminal	34.85	32.83	32.62	34.96	39.53*
Kidney					
Interim	5.109	5.199	5.399	5.508	6.253*
Terminal	7.345	7.065	7.588	7.434	7.362*
Testes					
Interim	6.535	6.004	6.360	6.402	7.568^{+}
Terminal	8.330	6.621	7.376	7.748	9.133^{+}
Spleen					
Interim	1.240	1.395	1.263	1.539*	2.198*
Terminal	2.160	1.645	1.892	2.166	2.303^{+}
Females					
Liver					
Interim	33.51	31.83	31.19	34.52	43.06*
Terminal	37.80	35.40	36.38	38.99	46.92*
Kidney					
Interim	6.549	6.311	6.393	6.843	7.865
Terminal	7.410	6.757	6.952	6.990	8.076*
Spleen					
Interim	1.614	1.529	1.522	1.760	2.423*
Terminal	1.679	1.656	1.713	1.861	2.238*
Ovaries					
Interim	0.458	0.478	0.423	0.452	0.562
Terminal	0.501	0.484	0.538	0.896	0.626*

bw: body weight; ppm: parts per million; *: $P < 0.01$ (Lepage pairwise comparison with controls); $^{+}$: $P < 0.01$ (Jonckheere trend test)

Source: Gerspach (1995a)

Necropsy examination revealed a higher incidence of female rats with liver cysts and a slight increase in the number of females with liver masses and of males with mottled livers at 3000 ppm. Histopathological examination revealed the liver to be the primary target organ, as indicated by a significantly increased incidence of hepatocellular hypertrophy in both sexes at 1000 and 3000 ppm, an increased incidence of foci of cellular change in both sexes at 3000 ppm and in females at 1000

ppm (Table 18) and increased incidences of hepatocellular adenoma and biliary cysts in females at 3000 ppm (Table 18). The increase in foci of cellular change at 100 ppm in females was well within the reported historical control range, and a subsequent evaluation of the density and area of the foci (Kobel, 2009) indicated that the finding at 100 ppm was consistent with the pattern of spontaneous findings; therefore, it is not considered to be a treatment-related adverse effect. Hyperplasia of thyroid follicular epithelium was observed in a higher number of males at 1000 and 3000 ppm and females at 3000 ppm; there were no increases in thyroid gland tumours in either sex. The statistically significant increase in dilatation of the uterus in females at 3000 ppm is reported to be within the historical control range for this strain of rats used in this laboratory. The incidence of malignant adrenal medullary tumours was increased in males at 3000 ppm but did not achieve statistical significance; on pooling both the benign and malignant medullary tumours, the combined tumour incidence is within the historical control range for this strain of rats used in this laboratory and overall is considered an equivocal finding. The incidence of benign granular cell tumours seen in the cerebral meninges of males at 3000 ppm is within the historical control range for this strain of rats used in this laboratory.

Table 18. Incidences of microscopic lesions in a 24-month study of pymetrozine in rats

	Incidence of lesions									
	Males					**Females**				
	0 ppm	**10 ppm**	**100 ppm**	**1 000 ppm**	**3 000 ppm**	**0 ppm**	**10 ppm**	**100 ppm**	**1 000 ppm**	**3 000 ppm**
Non-neoplastic										
Liver / Total examined	60	60	60	60	60	60	60	60	59	60
Biliary cyst	1	2	0	0	4	2	2	0	3	13**
Hypertrophy	0	1	5	22**	37**	2	1	0	12*	40**
Focus of cellular change[a]	10	15	16	12	30**	9	8	14**	19**	35**
Thyroid / Total examined	60	59	57	59	60	59	60	60	59	60
Follicular epithelium hyperplasia	2	3	1	9**	10**	1	1	4	3	9**
Uterus / Total examined	–	–	–	–	–	60	60	60	60	60
Dilatation	–	–	–	–	–	1	3	3	4	7*
Neoplastic										
Liver / Total examined	50	50	50	50	50	50	50	50	50	50
Hepatocellular adenoma[b]	2	0	2	0	2	0	0	0	2	7**
Adrenal medulla / Total examined	50	50	50	50	50	50	50	50	50	50
Benign medullary tumour	2	0	3	2	1	0	1	0	0	1
Malignant medullary tumour[c]	0	0	1	0	3	0	0	0	0	0
Cerebral meninges / Total examined	50	50	50	50	50	50	50	50	50	50
Benign granular cell tumour[d]	0	0	0	1	2*	1	0	1	0	1

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Peto-Test)

[a] Historical control incidence range in females: 2.5–46% (mean 14%) (10 studies, 1989 and 1993).
[b] Historical control incidence range: 0–3% (mean 0.7%) (10 studies, 1989 and 1993).
[c] Historical control incidence range in males: 0–3% (mean 1%) for malignant medullary tumour and 4–12% (mean 6%) for benign and malignant medullary tumours (10 studies, 1989 and 1993).
[d] Historical control incidence range in males: 0–5% (mean 2.4%) (10 studies, 1989 and 1993).

Source: Gerspach (1995a)

The NOAEL for non-neoplastic findings was 100 ppm (equal to 3.7 mg/kg bw per day), based on altered organ weights, foci of cellular change in the liver and increased thyroid hyperplasia in males at 1000 ppm (equal to 39 mg/kg bw per day). The NOAEL for tumours was 1000 ppm (equal to 47 mg/kg bw per day), based on a statistically significant increase in benign hepatocellular tumours in females at 3000 ppm (equal to 154 mg/kg bw per day) (Gerspach, 1995a).

2.4 Genotoxicity

(a) In vitro studies

Pymetrozine was not genotoxic in an Ames test or in assays for gene mutations in mammalian cells, chromosome aberrations and unscheduled deoxyribonucleic acid (DNA) synthesis (Table 19).

(b) In vivo studies

Pymetrozine was not genotoxic in assays for micronucleus induction or unscheduled DNA synthesis (Table 19).

Table 19. Genotoxicity studies with pymetrozine

Test	Target	Concentration or dose tested	Purity (%)	Results	Reference
In vitro					
Gene mutations in bacteria	*Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 *Escherichia coli* WP2uvrA	312.5–5 000 µg/plate (±S9)	98	Negative	Hertner (1991c)
Gene mutations in mammalian cells	Chinese hamster cells V79	5.21, 20.83, 83.33 and 333.33 µg/mL (±S9)	98	Negative	Geleick (1991)
Unscheduled DNA synthesis	Rat hepatocytes	2.78, 8.33, 25, 75, 150 and 300 µg/mL	98	Negative	Hertner (1991a)
Chromosome aberrations	Chinese hamster ovary cells CCL 61	82.5, 165 and 330 µg/mL (±S9)	98	Negative	Hertner (1991b)
In vivo					
Micronucleus test	Male and female TifIbm:MAG mice	200, 600 and 2 000 mg/kg bw	98.8	Negative	Ogorek (1998)
Unscheduled DNA synthesis	Male B6C3F1 mice	625, 1 250 and 2 000 mg/kg bw	99.3	Negative	Clay (2006)

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

In an adequate range of tests, there were no indications that pymetrozine was genotoxic in vitro or in vivo.

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

Groups of Tif:RAIf (SPF) rats (30 animals of each sex per group) received diets containing pymetrozine (batch no. P.102002; purity 98%) at 0, 20, 200 or 2000 ppm. Achieved intakes were reported to be 0, 1.4, 14 and 127 mg/kg bw per day for males and 0, 1.6, 16 and 152 mg/kg bw per day for females, respectively. Parents were mated 1:1. After weaning and a premating period of 10 weeks, F_1 animals were mated to produce the F_2 generation. Litters were culled to four male and four

female pups, where possible, on day 4 postpartum. Clinical signs, body weights, feed consumption, mating parameters, gestation and delivery parameters, pup survival, and physical and behavioural development (surface righting and eye opening) were recorded. No evaluation of date of sexual maturation was included in the protocol. A gross necropsy examination was performed on all pups not selected for mating. All parental animals were necropsied after weaning of their offspring and subjected to pathological examination. Histopathology was performed on the sexual organs and the apparent target organs, liver, spleen and pituitary.

In the first mating, there were no treatment-related mortalities or clinical signs in parent animals. Body weights were about 10% lower than control values at 2000 ppm in both sexes from the second week of treatment onwards. Feed consumption was reduced at 2000 ppm in both sexes except in females during the lactation period. Parental males had increased incidences of liver hypertrophy at 200 and 2000 ppm; hyperplasia of the splenic lymphatic follicles was increased in top-dose females. There were no effects on mating, reproductive outcome, litter parameters or pup survival. Litter weights of the F_1 generation were reduced (approximately 10% at days 14 and 21) at 2000 ppm, probably leading to the finding of eye opening being delayed (< 0.5 day).

In the second mating, there were no treatment-related mortalities or clinical signs in parental animals. Body weights were approximately 15% lower than control values at 2000 ppm in both sexes from the start of the premating period onwards. Feed consumption was reduced at 2000 ppm in both sexes except in females during the lactation period. Parental animals had increased incidences of liver hypertrophy at 2000 ppm; hyperplasia of the pituitary basophilic cells was increased in top-dose males. There were no effects on mating, reproductive outcome, litter parameters or pup survival. Pup weights were reduced at 2000 ppm (8% at day 8, 7% at day 14 and 12% at day 21). Eye opening was delayed (by approximately 0.5 day) at 2000 ppm, probably secondary to lower body weights.

The NOAEL for parental toxicity was 200 ppm (equal to 14 mg/kg bw per day), based on reduced body weights and histopathological findings in the liver, spleen and pituitary at 2000 ppm (equal to 127 mg/kg bw per day). The minimal liver hypertrophy at 200 ppm is not considered to be adverse in isolation.

The NOAEL for reproductive toxicity was 2000 ppm (equal to 127 mg/kg bw per day), the highest dose tested.

The NOAEL for offspring toxicity was 200 ppm (equal to 14 mg/kg bw per day), based on reductions in body weight during lactation and a related delay in eye opening in both generations at 2000 ppm (equal to 127 mg/kg bw per day) (Fitzgerald, 1993).

(b) Developmental toxicity

Rats

Mated female Tif:RAIf (SPF) rats (24 per group) were given pymetrozine (batch no. P.102002; purity 98%) in an aqueous solution of carboxymethyl cellulose (0.5% weight per weight [w/w]) by gavage at a daily dose of 0, 30, 100 or 300 mg/kg bw per day from day 6 to day 15 of gestation. On day 21, females were euthanized. Ovaries were examined, and the number of corpora lutea was determined. The uterus was weighed, opened and examined for the number and distribution of fetuses and resorptions. All live fetuses were weighed, sexed and examined for external variations, anomalies and malformations. Approximately half of the fetuses from each litter were eviscerated and subsequently processed for skeletal examination. The remaining fetuses were examined for visceral abnormalities.

There were no treatment-related signs or mortalities. Feed consumption was reduced at 100 and 300 mg/kg bw per day during the treatment period. Body weights were reduced at 300 mg/kg bw per day on the first day of dosing (Table 20).

Table 20. Feed consumption and body weights in the rat developmental toxicity study of pymetrozine

Parameter / dose	0 mg/kg bw per day	30 mg/kg bw per day	100 mg/kg bw per day	300 mg/kg bw per day
Number of dams	23	22	23	22
Feed consumption (g/animal per day)				
Days 0–6	19.4	19.5	19.6	19.5
Days 6–11	23.5	23.0	21.9*	17.8**
Days 11–16	26.1	25.7	23.3**	21.6**
Days 16–21	26.7	26.2	26.3	26.9
Body weight (g)				
Day 0	199.8	199.6	198.9	199.1
Day 21	365.6	361.5	359.4	350.7
Body weight gain (g)				
Days 6–7	3.2	3.2	3.3	−1.0*
Days 6–8	7.2	7.2	7.8	0.8*
Days 6–11	24.5	23.9	22.2	14.9**
Days 0–21	165.8	161.9	160.4	151.7
Gravid uterine weight (g)	101.9	100.6	103.9	101.9
Carcass weight (g)	263.7	260.9	255.4	248.8
Net weight change from day 6[a] (g)	38.7	35.0	29.2*	22.8**

bw: body weight; *: $P < 0.05$; **: $P < 0.01$

[a] Carcass weight minus body weight on day 6.

Source: Fitzgerald (1992b)

One dam receiving 300 mg/kg bw per day had a total resorption of the litter. Overall post-implantation losses, number of live fetuses per litter and fetal weights were not affected by pymetrozine treatment. There were no effects on the number of live fetuses per litter or on fetal weights.

All treated groups had higher fetal incidence of bipartite cervical vertebral centres compared with controls. However, there was no dose–response relationship, and the litter incidences were within historical control limits in all groups. Displaced pubic bones (described as a malformation) were seen in four fetuses from the top-dose group. This finding has not been reported in the historical control data. The total number of skeletal anomalies was increased in the group dosed at 300 mg/kg bw per day (Table 21). There were also significant reductions in the level of ossification of the paws in the top-dose group. The incidence of dumbbell-shaped cervical vertebral centres was increased at 100 mg/kg bw per day, but there was no dose–response relationship for this finding, and it is not considered to be treatment related.

The NOAEL for maternal toxicity was 100 mg/kg bw per day, based on body weight loss from day 6 to day 7 and reduced body weight gain during the dosing period at 300 mg/kg bw per day; the small deficits in body weight seen at 100 mg/kg bw per day are not considered to be adverse. The NOAEL for developmental toxicity was 100 mg/kg bw per day, based on increases in a range of skeletal abnormalities (including malformations) at 300 mg/kg bw per day (Fitzgerald, 1992b).

Table 21. Skeletal abnormalities in the developmental toxicity study of pymetrozine in rats

Observation	No. of fetuses / no. of litters affected			
	0 mg/kg bw per day	30 mg/kg bw per day	100 mg/kg bw per day	300 mg/kg bw per day
No. of fetuses / litters evaluated	158 / 23	150 / 22	163 / 23	157 / 22
Total skeletal malformations	0 / 0	0 / 0	1 / 0	4** / 3
Displaced pubic bones	0 / 0	0 / 0	0 / 0	4** / 3
Total skeletal anomalies	3 / 3	4 / 4	5 / 5	17** / 12
Thickened ischium of pelvis	0 / 0	0 / 0	0 / 0	2 / 2
Asymmetrically shaped sternebra	1 / 1	0 / 0	0 / 0	5 / 5
Total skeletal variations	164 / 23	151 / 22	166 / 23	159 / 22
Shortened rib 13	13 / 10	11 / 8	16 / 11	40 / 17
Absent ossification of metatarsal-1	4 / 3	6 / 5	3 / 3	26** / 8
Absent ossification of proximal phalanges anterior digit-5	2 / 2	2 / 2	4 / 3	16** / 7
Absent ossification of posterior digit-2	17 / 9	9 / 6	20 / 10	45** / 13
Absent ossification of posterior digit-3	4 / 4	3 / 3	9 / 7	29** / 9
Absent ossification of posterior digit-4	5 / 4	5 / 3	12 / 9	25** / 9
Absent ossification of posterior digit-5	36 / 17	29 / 12	51 / 18	74** / 18
Dumbbell-shaped thoracic vertebral centres	0 / 0	1 / 1	3 / 3	6* / 6
Dumbbell-shaped cervical vertebral centres	6 / 4	2 / 2	17* / 12	6 / 5
Bipartite cervical vertebral centres	8 / 7	19* / 11	28** / 14	23** / 12

bw: body weight; *: $P < 0.05$; **: $P < 0.01$

Source: Fitzgerald (1992b)

Rabbits

Inseminated Chbb:HM rabbits received pymetrozine (batch no. P.102002; purity 98%) by gavage in 0.5% (w/w) aqueous sodium carboxymethyl cellulose. Dose levels were 0, 10, 75 and 125 mg/kg bw per day on days 7–19 of gestation. On day 29 of pregnancy, females were euthanized. Ovaries were examined, and the number of corpora lutea was determined. The uterus was weighed and examined for the number and distribution of fetuses and resorptions. All live fetuses were weighed, sexed and examined for external variations and malformations. The heads of approximately half of the fetuses from each litter were examined by Wilson's slicing technique. The viscera of each fetus were also examined. All fetal trunks and approximately half of the heads were subsequently processed for skeletal examination.

Two deaths at 125 mg/kg bw per day occurred between days 16 and 19. In the control group, one dam died on day 16. Feed consumption was dose-dependently reduced during the treatment period at 75 and 125 mg/kg bw per day (Table 22), with an associated body weight loss at the start of the dosing period (Table 22); the deficit was maintained until the end of the study.

Table 22. Feed intake and body weight data for dams in the developmental toxicity study of pymetrozine in rabbits

Parameter	0 mg/kg bw per day	10 mg/kg bw per day	75 mg/kg bw per day	125 mg/kg bw per day
Number of dams with viable fetuses	16	17	17	13
Feed consumption (g/animal per day)				
Days 0–4	88.8	87.5	81.1	95.3
Days 4–7	86.8	99.8	89.6	104.1
Days 7–12	90.0	104.1	75.5	58.3**
Days 12–16	92.7	99.3	73.9**	53.4**
Days 16–20	92.4	101.3	76.1	61.8
Body weight (g)				
Day 0	2 382	2 358	2 373	2 355
Day 29	2 633	2 647	2 562	2 501*
Body weight gain (g)				
Days 7–9	6	6	−11	−16*
Days 7–19	87	92	33*	−29**
Days 0–29	251	289	189	146
Mean gravid uterine weight (g)	348	345	334	282
Mean carcass weight (g)	2 284	2 302	2 228	2 219

bw: body weight; *: $P < 0.05$; **: $P < 0.01$

Source: Fitzgerland (1992a)

No treatment-related effects on gravid uterine or carcass weight or gross necropsy changes were seen. Early resorptions and post-implantation losses were dose-dependently increased in the 75 and 125 mg/kg bw per day groups. At 125 mg/kg bw per day, one abortion and three completely resorbed litters were recorded. Mean litter size was also reduced at 125 mg/kg bw per day.

Fetal weights were not affected by pymetrozine treatment. The incidence of positional anomalies of the forelimb was significantly higher in the 75 and 125 mg/kg bw per day groups and was above the historical control range (Table 23). Visceral findings appeared to be sporadic in nature (Table 23).

Skeletal malformations were absent in control and pymetrozine-treated groups. Skeletal anomalies and variations (fused sternebrae, extra ribs, reduced ossification of paws) were increased at 125 mg/kg bw per day (Table 24). Additional ribs and reduced pubis were also increased at 75 mg/kg bw per day.

The findings in this rabbit study show a similarity to those in the rat in terms of effects on the pubis, but the actual abnormalities are not identical in the two species.

The NOAEL for maternal toxicity was 10 mg/kg bw per day, based on initial body weight loss with reduced feed consumption at 75 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 10 mg/kg bw per day, based on an increase in 13th ribs and reduced pubis at 75 mg/kg bw per day (Fitzgerald, 1992a).

Table 23. Fetal data from the developmental toxicity study of pymetrozine in rabbits

Observation	No. of fetuses / no. of litters affected			
	0 mg/kg bw per day	10 mg/kg bw per day	75 mg/kg bw per day	125 mg/kg bw per day
No. of pregnant dams	16	17	18	16
Post-implantation losses	5	7	15	29
Mean litter size (per pregnant dam)	6.4	6.8	6.1	4.5
Fetuses evaluated / litters evaluated	103 / 16	116 / 17	110 / 17	72 / 13
Total external abnormalities	0 / 0	1 / 1	4 / 2	6** / 4
Flexure of the forepaw at the wrist (A)	0 / 0	1 / 1	4# / 2	6**# / 4
Total visceral malformations/abnormalities	0 / 0	3 / 2	1 / 1	2 / 2
Domed head (A)	0 / 0	1 / 1	0 / 0	0 / 0
External hydrocephalus (M)	0 / 0	2 / 1	0 / 0	0 / 0
Internal hydrocephalus (M)	0 / 0	1 / 1	0 / 0	0 / 0
Small gallbladder (A)	0 / 0	1 / 1	0 / 0	0 / 0
Small liver (A)	0 / 0	0 / 0	0 / 0	1 / 1
Renal aplasia (M)	0 / 0	0 / 0	1 / 1	1 / 1
Ureter aplasia (M)	0 / 0	0 / 0	1 / 1	1 / 1

A: abnormality; bw: body weight; M: malformation; *: $P < 0.05$; **: $P < 0.01$; #: also increased in comparison with historical controls

Source: Fitzgerland (1992a)

Table 24. Skeletal data from the developmental toxicity study of pymetrozine in rabbits

Observation	No. of fetuses / No. of litters affected			
	0 mg/kg bw per day	10 mg/kg bw per day	75 mg/kg bw per day	125 mg/kg bw per day
Fetuses evaluated / litters evaluated	103 / 16	116 / 17	110 / 17	72 / 13
Total malformations	0 / 0	0 / 0	0 / 0	0 / 0
Total mean anomalies	11 / 7	23 / 12	15 / 8	28*# / 11
Fused sternebrae 2–3	0 / 0	0 / 0	0 / 0	6**# / 2
Fused sternebrae 3–4	5 / 4	6 / 3	3 / 3	18**# / 8
Fused sternebrae 4–5	2 / 2	7 / 6	4 / 3	19**# / 8
Reduced pubis	0 / 0	0 / 0	2 / 2	3 / 2
Total variations	80 / 16	95 / 17	90 / 17	67 / 13
Additional caudal vertebral centres	14 / 10	17 / 10	21 / 13	31**/10
Additional rib 13	2 / 2	2 / 2	11*/ 6	21**# / 7
Poor ossification of metacarpal-1	1 / 1	2 / 2	2 / 2	10** / 5
Poor ossification of talus	1 / 1	2 / 1	3 / 2	8** / 4
Poor ossification of medial phalanx of anterior digit-5	9 / 6	19 / 11	14 / 9	22** / 9

bw: body weight; *: $P < 0.05$; **: $P < 0.01$; #: also increased in comparison with historical controls

Source: Fitzgerland (1992a)

2.6 Special studies

(a) Neurotoxicity

Acute study in rats

Groups of Crl:CD(SD)BR rats (10 of each sex per group) received pymetrozine (lot no. FL950247; purity 98.2%) at 0, 125, 500 or 2000 mg/kg bw by gavage in water. Animals were subjected to a functional observational battery and assessments of locomotor activity at 4–5 hours and at 8 and 15 days post-dosing. The functional observational battery included open field, reflex, neuromuscular and physiological assessments. At termination, animals were perfused with 2.5% buffered glutaraldehyde, a full postmortem was performed and samples of a range of nervous and muscle tissues were taken from five rats of each sex per group and preserved in either formalin or glutaraldehyde. Staining procedures included haematoxylin and eosin, luxol fast blue and cresyl fast violet.

Three top-dose males died or were euthanized by day 3. Body weight gain was reduced at 2000 mg/kg bw in both sexes (> 10%). Dose-related reductions in locomotor activity (figure 8 maze activity counts) were seen in all treated groups at 4–5 hours, but not subsequently. Marked variations in a number of parameters were also seen at 4–5 hours, occasionally persisting until day 8. There were no abnormal findings in the nervous or muscle tissue samples examined histologically.

The study did not show a NOAEL, with altered locomotor activity observed at the lowest dose tested (125 mg/kg bw per day) (Ferkany, 1997).

Subchronic study in rats

Groups of Crl:CD(SD)BR VAF/plus rats (10 of each sex per group) received pymetrozine (lot no. FL-950247; purity 98.2%) at 0, 500, 1000 or 3000 ppm in the diet. Mean intakes were 0, 35, 68 and 201 mg/kg bw per day for males and 0, 41, 81 and 204 mg/kg bw per day for females, respectively. Animals were subjected to a functional observational battery and assessments of locomotor activity at 4, 8 and 13 weeks. At termination, animals were perfused with 10% buffered formalin, a postmortem examination was performed, focusing on nervous and muscle tissues, and samples of a range of nervous tissues were taken from six rats of each sex per group in controls and the top-dose level and stained with haematoxylin and eosin or toluidine blue.

There were no deaths or substance-related clinical observations during the study. Body weight gain was reduced (15–25%) in both sexes at 3000 ppm, whereas feed consumption was reduced by 10%. There were no adverse findings in the microscopic examination of nervous or muscle tissues. Altered behaviours (continuous head movement in males; unusual gait in females) were seen at 3000 ppm.

The NOAEL for general toxicity and neurotoxicity was 1000 ppm (equal to 68 mg/kg bw per day), based on lower body weights and behavioural changes at 3000 ppm (equal to 201 mg/kg bw per day) (Weiler, 1997).

(b) Tumour mode of action

The sponsor submitted a number of mode of action studies on liver and thyroid. A specific mode of action has not been proposed, and human relevance has not been addressed, but the data support a non-genotoxic, threshold approach for liver tumours. (As there were no thyroid tumours, it is unclear why thyroid investigations were performed.)

Effects on liver enzyme activities in male mice

Groups of five or six male Tif: MAGf (SPF) mice were fed pymetrozine (batch no. P.102002; purity 98%) at a dietary concentration of 0, 10, 100, 500, 2000 or 5000 ppm (corresponding to mean dose levels of 0, 1.6, 15.4, 83.5, 325 and 899 mg/kg bw per day, respectively) for 14 days. Subgroups receiving 0 and 5000 ppm were kept on standard control diet for a recovery period of 28 days. In these subgroups, the mean dose during the treatment period corresponded to 954 mg/kg bw per day.

Before necropsy, the animals were fasted for 16 hours. Livers of all animals were quickly removed and weighed and processed for morphological (electron microscopy) and biochemical investigations:

- protein content
- microsomal cytochrome P450 (CYP) content
- microsomal 7-ethoxyresorufin *O*-deethylase (EROD) and 7-pentoxyresorufin *O*-dependentylase (PROD) activities
- microsomal lauric acid hydroxylation
- microsomal uridine diphosphate-glucuronosyltransferase (UDP-GT) activities
- cytosolic glutathione *S*-transferase (GST) activity
- cyanide-insensitive peroxisomal β-oxidation.

Immunoblot analyses were performed for CYP1A1, CYP1A2, CYP3A1 and CYP4A content.

During the first 2 days of treatment, feed consumption was slightly reduced in groups treated with 5000 ppm. Thereafter, the feed intake of the same groups was either similar to or slightly above the respective control values. Body weights were similar in control and treated groups. Absolute and relative liver weights were increased at 5000 ppm, up to 124% and 136% of control values, respectively. In addition, the relative liver weight of animals treated with 2000 ppm was increased to 124% of the respective control value in one subgroup.

Major treatment-related biochemical alterations are summarized in Table 25. There were no significant effects on the protein contents of 100 × *g* supernatants, peroxisomal fatty acid β-oxidation and the microsomal activities of PROD, lauric acid 12-hydroxylase or bilirubin UDP-GT. Animals treated at 5000 ppm showed proliferation of the smooth endoplasmic reticulum and increased glycogen deposition. There was evidence of partial recovery over 28 days (double the exposure period).

Table 25. Selected biochemical liver parameters following dietary administration of pymetrozine to male mice[a]

Dietary concentration (ppm)	Microsomal P450 (nmol/g liver)	EROD (nmol/min/g liver)	Cytosolic GST (mmol/min/ g liver)	Microsomal UDP-GT (nmol/min/g liver)	CYP3A-related protein (% control)	LA-11-OH (nmol/min/ g liver)
Treatment						
0	11.9	1.45	303	664	100	12.4
10	12.1	1.43	246	640	110	11.8
100	12.5	1.15	285	626	157	11.5
500	12.8	1.15	273	622	178	12.9
2 000	16.5**	2.36**	402	852**	289*	15.0
5 000	17.4**	2.90***	570***	864**	408*	15.9*
14-day treatment/ 28-day recovery						
0/0	10.5	1.19	355	550	100	9.6
5 000/0	13.6*	1.31	376	763***	180	10.3

CYP: cytochrome P450; EROD: 7-ethoxyresorufin *O*-deethylase; GST: glutathione *S*-transferase; LA-11-OH: lauric acid 11-hydroxylase; ppm: parts per million; UDP-GT: uridine diphosphate-glucuronosyltransferase; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$

[a] Values are means of six animals per group.

Source: Waechter & Persohn (1995)

These results show that pymetrozine in the diet at 2000 ppm and above is an inducer of xenobiotic metabolizing liver enzymes in the male mouse (Waechter & Persohn, 1995).

Effects on liver cell proliferation in male mice

Groups of five male Tif: MAGf (SPF) mice per group were kept on daily dietary exposure to pymetrozine for 4 days at 0 and 5000 ppm and for 14 and 42 days at 0, 10, 100, 500, 2000 and 5000 ppm. Subgroup animals receiving 5000 ppm for 14 days were subsequently kept on standard control diet for a recovery period of 28 days. Before necropsy, the animals were fasted for 16 hours and then killed. The livers of all animals were quickly removed and investigated for proliferating cell nuclear antigen (PCNA). Hepatocytes but not sinusoidal cells were evaluated for PCNA positive nuclei, and the labelling index was calculated.

No signs of toxicity were recorded throughout the treatment or recovery period. During the first 1 or 2 days of treatment, daily feed consumption was slightly reduced in groups treated with 5000 ppm, but body weights were similar in all groups. Absolute and relative liver weights were slightly increased after treatment with 5000 ppm for 4 days and moderately increased at the same dose level after 14 and 42 days. Slightly increased relative liver weights were observed at 2000 ppm after 14 and 42 days of treatment. Absolute and relative liver weights returned to control values in the 14-day treatment/28-day recovery animals.

The total number of hepatocyte nuclei per square millimetre was reduced after treatment with 5000 ppm for 14 and 42 days and after treatment with 2000 ppm for 42 days, indicating hypertrophy. Immunohistochemical staining of liver sections for PCNA revealed a moderate to strong increase in the fraction of DNA synthesizing hepatocytes in S-phase upon administration of 2000 and 5000 ppm at all investigated time points, indicating a proliferative response. The effects were reversible when a 14-day treatment period was followed by a 28-day recovery. There were no consistent effects at 500 ppm or below.

The results demonstrate that at 2000 and 5000 ppm, pymetrozine induced a sustained but reversible stimulation of hepatocytes (Persohn, 1995).

Liver and thyroid parameters in rats

Groups of five female Tif:RAIf (SPF) rats were fed pymetrozine (batch no. P.102002; purity 98%) at a dietary concentration of 0, 20, 100, 1000 or 3000 ppm daily for 14 or 42 days. Subgroups receiving 0 and 3000 ppm for 14 days were kept on standard control diet for a recovery period of 28 days. Additional groups received 0 or 3000 ppm for 4 days. Blood was sampled and plasma was prepared for the determination of the thyroid hormone status of all animals immediately prior to termination. The livers of all animals were quickly removed after termination, weighed and processed for morphological (electron microscopy) and biochemical investigations.

Livers of five animals per group (14-day treatment plus recovery groups) were investigated for:

- protein content of 100 × *g* supernatant and microsomal as well as cytosolic liver fractions
- microsomal cytochrome P450 content
- cyanide-insensitive peroxisomal β-oxidation
- microsomal EROD and PROD activities
- stereospecific hydroxylation of testosterone
- microsomal UDP-GT activity
- cytosolic GST activity.

Immunoblot analyses were performed for CYP1A1, CYP1A2, CYP2B1, CYP2B2, CYP3A and CYP4A. PCNA staining was performed, and for each animal a labelling index was calculated as the percentage of labelled nuclei over the total number of nuclei per unit area. Plasma concentrations of thyroid stimulating hormone (TSH) and the thyroid hormones thyroxine (T_4), triiodothyronine (T_3) and reverse T_3 (rT_3) were determined employing commercially available kits.

Relative liver weights were increased after 14 and 42 days of treatment with 3000 ppm (114% or 123% of control values, respectively). This effect was partially reversible (112% of control value). Biochemical liver parameters were investigated in the animals of the groups treated for 14 days and of the two recovery groups. The major treatment-related biochemical alterations were approximate 2- or 3-fold increases in EROD, PROD, UDP-GT and GST activities at 3000 ppm, with UDP-GT increased almost 2-fold at 1000 ppm.

Treatment had no effect on protein contents of $100 \times g$ supernatant or microsomal and cytosolic liver fractions, total cytochrome P450 content, site-specific hydroxylation of testosterone, peroxisomal fatty acid β-oxidation and protein content of the three cytochrome P450 isoenzymes of the CYP2B, CYP3A and CYP4A families, detected with isoenzyme-specific monoclonal antibodies. All treatment-related effects on biochemical liver parameters were reversible after the 28-day recovery period. TSH concentration was increased after 14 days of treatment, and T_3 was slightly increased (about 1.5- to 2-fold) after 42 days of treatment at 1000 and 3000 ppm, respectively.

Morphological investigation of the liver indicated a moderate reversible proliferation of smooth endoplasmic reticulum. Assessment of replicative DNA synthesis in hepatocytes gave no indication for a stimulation of liver cell proliferation.

These data indicate pymetrozine to be a weak to moderate inducer mainly of hepatic xenobiotic phase II metabolizing enzymes in the female rat. The dose of 100 ppm was a threshold. Pymetrozine did not stimulate hepatocyte cell proliferation (Beilstein, 1996a,b).

Groups of male F344 rats (16 per group), 6 weeks of age, received a single intraperitoneal injection of *N*-diethylnitrosamine at 100 mg/kg bw. The rats were then provided with water containing 0.1% dihydroxy-di-*N*-propylnitrosamine for 2 weeks. This was followed by 18 weeks of exposure to pymetrozine at a dietary level of 0, 25, 50, 100 or 1000 ppm (equal to 0, 1.3, 2.5, 5.0 and 52 mg/kg bw per day, respectively) or phenobarbital (500 ppm). The rats were terminated and their livers stained for placental GST positive foci. Thyroids were also investigated for evidence of follicular cell hyperplasia and tumours.

Relative liver weights were increased statistically significantly at 100 ppm (5%) and 1000 ppm (15%). Results for GST positive foci showed no effect of pymetrozine on the number of foci per square centimetre (0.3, 0.7, 0.5, 0.2 or 0.6, respectively) or area of foci. A clear effect was seen with phenobarbital (11.7 foci/cm^2). Increases in thyroid hyperplasia were seen at 1000 ppm, and follicular cell adenomas were increased at 100 ppm, but not 1000 ppm.

This study showed that pymetrozine at dose levels up to 1000 ppm does not have any liver tumour–promoting activity in male F344 rats (Tamano, 1996).

(c) Toxicity of metabolites

Twelve metabolites of pymetrozine were identified as being of potential relevance: 11 found in plants and one (CGA 313124) found in milk. No specific toxicological data were submitted on these metabolites, and therefore they were evaluated using the JMPR metabolite assessment scheme.[1] The details are summarized in Appendix 1.

CGA 313124 (6-hydroxymethyl-pymetrozine) is a major urinary metabolite of pymetrozine in rats, equating to approximately 30% (sum of CGA 313124 and its acid metabolite) of the administered dose. The toxicity of this metabolite has been addressed in studies on pymetrozine, and CGA 313124 would be covered by the reference doses for pymetrozine.

Nicotinic acid (vitamin B_3, niacin) and nicotinamide are natural compounds and are interconverted in the body. The recommended daily intake for vitamin B_3 is approximately 200 µg/kg

[1] http://www.who.int/entity/foodsafety/areas_work/chemical-risks/jmpr_Guidance_Document_FINAL.pdf.

bw, with approximately 2.5 mg in a serving of some breakfast cereals. Intakes arising from the use of pymetrozine are significantly below these values.

CGA 245342 and Ia7 have no alerts for genotoxicity and are in Cramer class III, with a threshold of toxicological concern (TTC) of 1.5 µg/kg bw per day.

Ia17 has a structural alert for genotoxicity, but has not been tested for genotoxicity. On the basis of the available information, the appropriate TTC for chronic exposure is 0.0025 µg/kg bw per day, and an appropriate TTC for a single exposure is 0.2 µg/kg bw, based on a modification of the approach of the European Medicines Agency (EMA).[2]

CGA 215525 has been proposed as a rat metabolite, but the data are inconsistent, and it is not possible to make use of the toxicological data on pymetrozine in evaluating this metabolite. CGA 215525 has a structural alert for genotoxicity, but there was no evidence of genotoxicity in an Ames test (Callander, 2005), and it is in Cramer class III. Therefore, the relevant chronic TTC value is 1.5 µg/kg bw per day. A single-exposure TTC for Cramer class III compounds of 5 µg/kg bw has been proposed by the European Food Safety Authority (EFSA, 2012) and is considered appropriate for use in assessing this metabolite.

CGA 96956 (trigonelline) is a natural component of a range of commodities, present at relatively high levels in common dietary constituents (e.g. up to 50 mg in a cup of coffee; Tice & Brevard, 1999).

CGA 23199 is a minor rat metabolite (< 3%), and its toxicity is considered not to have been adequately addressed by studies with pymetrozine. CGA 23199 has no structural alerts for genotoxicity and is in Cramer class III, with a TTC of 1.5 µg/kg bw per day, and a single-exposure TTC of 5 µg/kg bw based on the proposal from EFSA can be applied.

CGA 128632 (nicotinyl alcohol) has therapeutic uses as a vasodilator. The minimal therapeutic dose is approximately 1 mg/kg bw per day.

CGA 294849 has been proposed as a rat metabolite, but the data are inconsistent, and it is not possible to make use of the toxicological data on pymetrozine in evaluating this metabolite. CGA 294849 has a structural alert for genotoxicity, but has not been tested for genotoxicity. The appropriate TTC for chronic exposure is 0.0025 µg/kg bw per day and for a single exposure is 0.2 µg/kg bw, based on a modification of the approach of EMA.

CGA 300407 (pyridine carboxaldehyde) does not have a structural alert for genotoxicity, but the Meeting was made aware that in vitro and in vivo genotoxicity studies exist in which positive results were reported. These potentially adverse data were not submitted for evaluation. The appropriate TTC for chronic exposure is 0.0025 µg/kg bw per day and for a single exposure is 0.2 µg/kg bw, based on a modification of the approach of EMA.

3. Observations in humans

No adverse effects during medical examinations or incidents have been reported for workers in pymetrozine production or formulation plant employees (Geisler, 2014a).

Seven incidents involving exposures to users of pymetrozine products have been reported. The effects described were generic (e.g. nausea, headache, pain) and defined as mild (Geisler, 2014b).

[2] EMA (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500002907.pdf) set a TTC of 2 µg/kg bw (120 µg/person) for single exposures to genotoxic impurities in pharmaceuticals. The chronic TTC value used by EMA is 10-fold higher than that used by WHO for potentially genotoxic compounds. Therefore, the EMA single-exposure TTC value of 2 µg/kg bw (120 µg/person) was divided by 10 to give a single-exposure TTC value of 0.2 µg/kg bw, applicable to potentially genotoxic metabolites of pesticides.

Comments

Biochemical aspects

Absorption of pymetrozine (labelled with [6-^{14}C]triazine or [5-^{14}C]pyridine) administered to rats by gavage at 0.5 or 100 mg/kg bw was rapid, with maximal blood concentrations achieved at 15 minutes and 4 hours, respectively. The extent of oral absorption was high (> 80%) at both doses, based on urinary and biliary data. Pymetrozine was widely distributed in the body. High concentrations of both triazine- and pyridine-labelled material were found in the liver and kidney. The labelled material was rapidly excreted via urine (50–75% in 24 hours). There was a disproportionate increase in the AUC at 100 mg/kg bw, indicating a saturation of elimination.

Absorbed pymetrozine was extensively metabolized, with unmetabolized parent compound representing approximately 10% of the excreted radiolabel. Compounds containing both ring structures represented over 50% of the identified metabolites. The kinetics, excretion pattern, tissue distribution of radioactivity and metabolite profile were similar for both radiolabelled sites and both administered dose levels as well as when the administration of radiolabelled pymetrozine was preceded by 14 days of administration of the unlabelled material. A comparative study showed no notable differences in the metabolite profile in rats and mice.

Toxicological data

Pymetrozine was of low acute toxicity in rats via the oral route (LD_{50} = 5820 mg/kg bw) and dermal route (LD_{50} > 2000 mg/kg bw) and by inhalation exposure (LC_{50} > 1.8 mg/L air). Pymetrozine was not irritating to the skin of rabbits, but was transiently, mildly irritating to the eyes of rabbits. It was weakly sensitizing in the guinea-pig maximization test.

In all species, the liver was a target organ, with increases in weight, hepatocellular hypertrophy and necrosis. Investigations on liver enzyme activities in mice and rats revealed that pymetrozine administration resulted in significant reversible induction of the activities of some P450 enzymes and increased hepatocellular proliferation. Reduced thymus weight and thymic atrophy were also seen in all species, as were spleen and testicular effects. Reduced body weight gain, often associated with reductions in feed consumption, was also a consistent finding.

In a 90-day study of toxicity in mice, dietary pymetrozine concentrations were 0, 1000, 3000 and 7000 ppm (equal to 0, 143, 429 and 1000 mg/kg bw per day for males and 0, 252, 589 and 1240 mg/kg bw per day for females, respectively). No NOAEL was identified, as hepatocellular necrosis was observed at 1000 ppm (equal to 252 mg/kg bw per day) in female mice.

In a 28-day study of toxicity in rats, pymetrozine was administered by gavage at a dose of 0, 10, 100 or 600 mg/kg bw per day. The NOAEL was 10 mg/kg bw per day, based on thymic atrophy and hyperplasia of the splenic white pulp at 100 mg/kg bw per day.

In a 28-day study of toxicity in rats, dietary pymetrozine concentrations were 0, 100, 500, 2000 and 10 000 ppm (equal to 0, 10, 55, 203 and 691 mg/kg bw per day for males and 0, 10, 55, 212 and 699 mg/kg bw per day for females, respectively). The NOAEL was 2000 ppm (equal to 203 mg/kg bw per day), based on a range of effects on the liver, spleen, thymus, adrenals and testes at 10 000 ppm (equal to 691 mg/kg bw per day).

In a 90-day study of toxicity in rats, dietary pymetrozine concentrations were 0, 50, 500 and 5000 ppm (equal to 0, 3.4, 33 and 360 mg/kg bw per day for males and 0, 3.6, 34 and 370 mg/kg bw per day for females, respectively). The NOAEL was 500 ppm (equal to 33 mg/kg bw per day), on the basis of thymic atrophy, hepatocellular hypertrophy and renal calcification at 5000 ppm (equal to 360 mg/kg bw per day).

In a 28-day study in which dogs were administered pymetrozine in the diet at a concentration of 0, 100, 500 or 2500 ppm (equal to 0, 3.2, 15 and 55 mg/kg bw per day for males and 0, 2.8, 16 and 50 mg/kg bw per day for females, respectively), the NOAEL was 100 ppm (equal to 2.8 mg/kg bw per day), based on decreased thymus weights in females at 500 ppm (equal to 16 mg/kg bw per day).

In a 90-day study in which dogs received pymetrozine in the diet at a concentration of 0, 100, 500 or 2500 ppm (equal to 0, 3.1, 14 and 53 mg/kg bw per day for males and 0, 3.2, 15 and 60 mg/kg bw per day for females, respectively), the NOAEL was 100 ppm (equal to 3.1 mg/kg bw per day), based on thymic atrophy, testicular tubular atrophy, reduced spermatogenesis, hepatocellular necrosis and inflammatory changes in several organs at 500 ppm (equal to 14 mg/kg bw per day).

In a 1-year study in dogs in which pymetrozine was administered in the diet at a concentration of 0, 20, 200 or 1000 ppm (equal to 0, 0.57, 5.3 and 28 mg/kg bw per day for males and 0, 0.57, 5.0 and 27 mg/kg bw per day for females, respectively), the NOAEL was 20 ppm (equal to 0.57 mg/kg bw per day), on the basis of reduced testes weights, increased cholesterol and reduced haemoglobin at 200 ppm (equal to 5.0 mg/kg bw per day).

The pattern of findings in the 90-day and 1-year dog studies was similar, and the effects seen in the 1-year study at 200 and 1000 ppm were marginal. An overall NOAEL for the 90-day and 1-year studies of 100 ppm (equal to 3.1 mg/kg bw per day) was identified, with an overall lowest-observed-adverse-effect level (LOAEL) of 200 ppm (equal to 5.0 mg/kg bw per day).

In a 78-week toxicity and carcinogenicity study in mice, dietary concentrations were 0, 10, 100, 2000 and 5000 ppm (equal to 0, 1.2, 12, 254 and 678 mg/kg bw per day for males and 0, 1.2, 11, 243 and 673 mg/kg bw per day for females, respectively). The NOAEL for systemic toxicity was 100 ppm (equal to 11 mg/kg bw per day), based on liver hypertrophy, splenic haemosiderosis and haematopoiesis, and alterations in the weights of the kidneys, spleen and liver at 2000 ppm (equal to 243 mg/kg bw per day). Pymetrozine increased the incidences of hepatocellular adenomas and carcinomas in both sexes at 5000 ppm and of carcinomas in males at 2000 ppm; and of lung adenomas and carcinomas in females at 2000 and 5000 ppm. The NOAEL for carcinogenicity was 100 ppm (equal to 11 mg/kg bw per day), based on increased incidences of hepatocellular carcinomas in males receiving 2000 ppm (equal to 254 mg/kg bw per day) and lung adenomas and carcinomas in females receiving 2000 ppm (equal to 243 mg/kg bw per day).

In a 2-year toxicity and carcinogenicity study in rats, dietary concentrations were 0, 10, 100, 1000 and 3000 ppm (equal to 0, 0.4, 3.7, 39 and 128 mg/kg bw per day for males and 0, 0.4, 4.5, 47 and 154 mg/kg bw per day for females, respectively). The NOAEL for systemic toxicity was 100 ppm (equal to 3.7 mg/kg bw per day), on the basis of effects in both sexes (altered organ weights, foci of cellular change in the liver and thyroid hyperplasia) at 1000 ppm (equal to 39 mg/kg bw per day). Pymetrozine produced an increase in the incidence of hepatocellular adenoma in female rats given 3000 ppm in the diet. The NOAEL for carcinogenicity was 1000 ppm (equal to 47 mg/kg bw per day), based on an increase in the incidence of hepatocellular adenoma in females at 3000 ppm (equal to 154 mg/kg bw per day).

The Meeting concluded that pymetrozine is carcinogenic in male and female mice and in female but not male rats.

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

The Meeting concluded that pymetrozine is unlikely to be genotoxic.

Mechanistic studies were performed to investigate the mode of action of the liver tumour findings in mice and female rats. Male mice exposed to pymetrozine exhibited moderate increases in proliferation of hepatic smooth endoplasmic reticulum and in activities of P450 enzymes, a sustained stimulation of hepatocyte proliferation and increased hepatocellular hypertrophy. These effects were reversible. A dietary level of 500 ppm was a threshold for these effects in male mice. Female rats exposed to pymetrozine at 1000 ppm and above exhibited a weak and reversible induction of hepatic xenobiotic metabolizing enzymes, most prominently UDP-GT. There were no significant effects at 100 ppm, which is considered to be the threshold. Pymetrozine at up to 1000 ppm in the diet for 18 weeks exhibited no tumour-promoting potential in rats initiated with diethylnitrosamine and dihydroxy-di-*N*-propylnitrosamine.

No data were presented relating to the mode of action for the lung tumours observed in female mice exposed to pymetrozine, and it is noted that there are no preneoplastic lesions of the lungs in mice. However, there was no dose–response relationship, lung tumours are a common finding in mice and, generically, species-specific lung tumours in the mouse have been induced by a number of chemicals.

The available information and data do not permit the identification of the modes of action for the lung or liver tumours or the exclusion of their human relevance.

In view of the lack of genotoxicity and on the basis of other available toxicological information, the Meeting concluded that the modes of action for the liver tumours in mice and female rats and for the lung tumours in female mice are likely to involve a threshold. The Meeting concluded that pymetrozine 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, 20, 200 and 2000 ppm (equal to mean intakes of 0, 1.4, 14 and 127 mg/kg bw per day for males and 0, 1.6, 16 and 152 mg/kg bw per day for females, respectively). The NOAEL for reproductive effects was 2000 ppm (equal to 127 mg/kg bw per day), the highest dose tested. The NOAEL for parental toxicity was 200 ppm (equal to 14 mg/kg bw per day), based on reduced body weights and histopathological findings in the liver, spleen and pituitary at 2000 ppm (equal to 127 mg/kg bw per day). The NOAEL for effects on offspring was 200 ppm (equal to 14 mg/kg bw per day), based on reduced pup weight and a delay in eye opening at 2000 ppm (equal to 127 mg/kg bw per day).

In a study of developmental toxicity in rats dosed at 0, 30, 100 or 300 mg/kg bw per day, displaced pubic bones were seen in four fetuses at the top dose level; this malformation has not been recorded in contemporary historical control data. There were also increases in a number of skeletal variations at 300 mg/kg bw per day. The NOAEL for maternal toxicity was 100 mg/kg bw per day, on the basis of decreased body weight gain and initial body weight loss at 300 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 100 mg/kg bw per day, based on increases in skeletal abnormalities (including malformations) at 300 mg/kg bw per day.

In a study of developmental toxicity, rabbits were dosed at 0, 10, 75 or 125 mg/kg bw per day. Viable fetus numbers were reduced at 125 mg/kg bw per day. The NOAEL for maternal toxicity was 10 mg/kg bw per day, based on initial body weight loss with reduced feed consumption at 75 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 10 mg/kg bw per day, based on an increase in 13th ribs and reduced pubis at 75 mg/kg bw per day.

The Meeting concluded that pymetrozine is teratogenic in rats and possibly in rabbits.

The acute neurotoxicity of pymetrozine was investigated in rats at doses of 0, 125, 500 and 2000 mg/kg bw. Three males died at 2000 mg/kg bw. Dose-related reductions in locomotor activity were seen in all dose groups at 4–5 hours post-dosing, but not subsequently. There were no indications of neuropathy. No NOAEL was identified.

In a subchronic (90-day) neurotoxicity study in rats, dietary concentrations were 0, 500, 1000 and 3000 ppm (equal to 0, 35, 68 and 201 mg/kg bw per day for males and 0, 41, 81 and 204 mg/kg bw per day for females, respectively). The NOAEL for neurotoxicity and systemic toxicity was 1000 ppm (equal to 68 mg/kg bw per day), based on altered behaviours (continuous head movements and abnormal gait) and reduced body weights at 3000 ppm (equal to 201 mg/kg bw per day). There was no evidence of neuropathy.

Pymetrozine showed some evidence of reversible, clinical signs of neurotoxicity, but with no morphological correlates.

Toxicological data on metabolites and/or degradates

The Meeting considered information on 12 metabolites of pymetrozine: 11 found in plants and one (CGA 313124) found in milk. No specific toxicological data were submitted on these metabolites, and therefore they were evaluated using the JMPR metabolite assessment scheme.

CGA 313124 (6-hydroxymethyl-pymetrozine) is a major urinary metabolite of pymetrozine in rats, equating to approximately 30% (sum of CGA 313124 and its acid metabolite) of the administered dose. The Meeting concluded that the toxicity of CGA 313124 has been addressed in studies on pymetrozine and that CGA 313124 is covered by the reference doses for pymetrozine.

Nicotinic acid (vitamin B_3, niacin) and nicotinamide are natural compounds and are interconverted in the body. The recommended daily intake for vitamin B_3 is approximately 200 µg/kg bw, with approximately 2.5 mg in a serving of some breakfast cereals. Intakes arising from the use of pymetrozine are significantly below these values. Nicotinic acid and nicotinamide are considered not to be relevant metabolites of pymetrozine.

CGA 245342 and Ia7 have no alerts for genotoxicity and are in Cramer class III, with a chronic TTC of 1.5 µg/kg bw per day. For both compounds, the international estimated daily intakes (IEDIs) and international estimated short-term intakes (IESTIs) are below the chronic TTC. The Meeting concluded that CGA 245342 and Ia7 are not toxicologically significant plant metabolites of pymetrozine.

Ia17 has a structural alert for genotoxicity, but has not been tested for genotoxicity. On the basis of the available information, the appropriate TTC for chronic exposure is 0.0025 µg/kg bw per day. The IEDI is below 0.0025 µg/kg bw per day. For the acute exposure assessment, a single-exposure TTC of 0.2 µg/kg bw was considered appropriate by the Meeting.[3] The IESTI for Ia17 is below 0.2 µg/kg bw, and Ia17 is considered not to be a relevant plant metabolite of pymetrozine.

CGA 215525 has been proposed as a rat metabolite, but the data are inconsistent, and the Meeting was unable to make use of the toxicological data on pymetrozine in evaluating this metabolite. CGA 215525 has a structural alert for genotoxicity, but there was no evidence of genotoxicity in an Ames test, and it is in Cramer class III. Therefore, the relevant TTC is 1.5 µg/kg bw per day. The IEDI is below this value. A single-exposure TTC for Cramer class III compounds of 5 µg/kg bw has been proposed by EFSA, and the Meeting concluded that the use of this value would be conservative. The IESTI is below this value. The Meeting concluded that CGA 215525 is not a toxicologically significant plant metabolite of pymetrozine.

CGA 96956 (trigonelline) is a natural component of a range of commodities, and exposures from other sources are orders of magnitude greater than those from pymetrozine. The Meeting concluded that CGA 96956 is not a toxicologically significant plant metabolite of pymetrozine.

CGA 23199 is a minor rat metabolite (< 3%), and its toxicity is considered not to have been adequately addressed by studies with pymetrozine. CGA 23199 has no structural alerts for genotoxicity and is in Cramer class III, with a TTC of 1.5 µg/kg bw per day and a single-exposure TTC of 5 µg/kg bw. The IEDI and IESTI are below the applicable thresholds, and the Meeting concluded that CGA 23199 is not a toxicologically significant plant metabolite of pymetrozine.

CGA 128632 (nicotinyl alcohol) has therapeutic uses as a vasodilator. The minimal therapeutic dose is approximately 1 mg/kg bw per day. There is a margin between the minimal therapeutic dose of over 1000 relative to the IEDI and of over 50 relative to the IESTI. The Meeting concluded that CGA 128632 is not a toxicologically significant plant metabolite of pymetrozine.

CGA 294849 has been proposed as a rat metabolite, but the data are inconsistent, and the Meeting was unable to make use of the toxicological data on pymetrozine in evaluating this metabolite. CGA 294849 has a structural alert for genotoxicity, but has not been tested for genotoxicity. The IEDI and IESTI are above the applicable chronic TTC and single-exposure TTC values, respectively. The Meeting was unable to conclude on the toxicological significance of CGA 294849.

[3] This is based on the approach of EMA, which set a TTC of 2 µg/kg bw (120 µg/person) for single exposures to genotoxic impurities in pharmaceuticals. The chronic TTC value used by EMA is 10-fold higher than that used by WHO for potentially genotoxic compounds. Therefore, the EMA single-exposure TTC value of 2 µg/kg bw (120 µg/person) was divided by 10 to give a single-exposure TTC value of 0.2 µg/kg bw, applicable to potentially genotoxic metabolites of pesticides.

CGA 300407 does not have a structural alert for genotoxicity, but the Meeting was made aware that in vitro and in vivo genotoxicity studies exist in which positive results were reported. These data were not submitted, and therefore the Meeting was unable to conclude on the toxicological significance of CGA 300407.

Human data

No adverse effects have been reported during health surveillance of pymetrozine production and formulation plant workers, and no significant effects have been reported in exposed users of pymetrozine-based products.

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

Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) for pymetrozine of 0–0.03 mg/kg bw on the basis of the overall NOAEL of 3.1 mg/kg bw per day for effects on haematology, liver, thymus and testis from the 90-day and 1-year dog studies combined. A safety factor of 100 was applied. This is supported by the NOAEL of 3.7 mg/kg bw per day in the 2-year study of toxicity in rats. There is a margin of exposure of greater than 5000 between the upper bound of the ADI and the LOAEL of 154 mg/kg bw per day for tumours in female rats.

The Meeting established an acute reference dose (ARfD) for pymetrozine of 0.1 mg/kg bw, on the basis of the NOAEL of 10 mg/kg bw per day for developmental abnormalities and maternal body weight loss at the start of dosing in the developmental toxicity study in rabbits. A safety factor of 100 was applied.

Levels relevant to risk assessment of pymetrozine

Species	Study	Effect	NOAEL	LOAEL
Mouse	Eighteen-month study of toxicity and carcinogenicity[a]	Toxicity	100 ppm, equal to 11 mg/kg bw per day	2 000 ppm, equal to 243 mg/kg bw per day
		Carcinogenicity	100 ppm, equal to 11 mg/kg bw per day	2 000 ppm, equal to 243 mg/kg bw per day
Rat	Two-year study of toxicity and carcinogenicity[a]	Toxicity	100 ppm, equal to 3.7 mg/kg bw per day	1 000 ppm, equal to 39 mg/kg bw per day
		Carcinogenicity	1 000 ppm, equal to 47 mg/kg bw per day	3 000 ppm, equal to 154 mg/kg bw per day
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	2 000 ppm, equal to 127 mg/kg bw per day[b]	–
		Parental toxicity	200 ppm, equal to 14 mg/kg bw per day	2 000 ppm, equal to 127 mg/kg bw per day
		Offspring toxicity	200 ppm, equal to 14 mg/kg bw per day	2 000 ppm, equal to 127 mg/kg bw per day
	Developmental toxicity study[c]	Maternal toxicity	100 mg/kg bw per day	300 mg/kg bw per day
		Embryo and fetal toxicity	100 mg/kg bw per day	300 mg/kg bw per day
Rabbit	Developmental	Maternal toxicity	10 mg/kg bw per day	75 mg/kg bw per day

Species	Study	Effect	NOAEL	LOAEL
	toxicity study[c]	Embryo and fetal toxicity	10 mg/kg bw per day	75 mg/kg bw per day
Dog	Ninety-day and 1-year studies of toxicity[a,d]	Toxicity	100 ppm, equal to 3.1 mg/kg bw per day	200 ppm, equal to 5.0 mg/kg bw per day

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

Estimate of acceptable daily intake (ADI)

0–0.03 mg/kg bw

Estimate of acute reference dose (ARfD)

0.1 mg/kg bw

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

Results from epidemiological, occupational health and other such observational studies of human exposure; all information on the toxicity of plant and animal metabolites

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

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid, blood T_{max} < 4 hours; > 80%
Dermal absorption	No study submitted
Distribution	Widely distributed; highest concentrations in liver and kidney
Potential for accumulation	No evidence of accumulation
Rate and extent of excretion	Largely cleared within 24 hours; primarily via urine (> 50%), bile (10–30%) and faeces (15–30%); evidence of saturation at 100 mg/kg bw
Metabolism in animals	Extensive; mainly by oxidation reactions; cleavage between the two rings is not extensive
Toxicologically significant compounds in animals and plants	Pymetrozine and CGA 313124 [CGA 294849 and CGA 300407][4]
Acute toxicity	
Rat, LD_{50}, oral	5 820 mg/kg bw
Rat, LD_{50}, dermal	> 2 000 mg/kg bw
Rat, LC_{50}, inhalation	> 1.8 mg/L
Rabbit, dermal irritation	Not irritating
Rabbit, ocular irritation	Transiently, mildly irritating
Guinea-pig, dermal sensitization	Weakly sensitizing (maximization test)

[4] Toxicological significance cannot be determined on the basis of the available information.

Short-term studies of toxicity	
Target/critical effect	Haematology; liver lesions, thymus weight/atrophy and testes lesions
Lowest relevant oral NOAEL	3.1 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 studies of toxicity and carcinogenicity	
Target/critical effect	Liver lesions, thyroid hyperplasia, liver and spleen weights
Lowest relevant NOAEL	3.7 mg/kg bw per day (rat)
Carcinogenicity	Liver tumours (rat and mouse); lung tumours (mouse) Unlikely to pose a carcinogenic risk to humans from the diet
Genotoxicity	
	Unlikely to be genotoxic
Reproductive toxicity	
Target/critical effect	Lower pup weight at parentally toxic dose
Lowest relevant parental NOAEL	14 mg/kg bw per day
Lowest relevant offspring NOAEL	14 mg/kg bw per day
Lowest relevant reproductive NOAEL	127 mg/kg bw per day, highest dose tested
Developmental toxicity	
Target/critical effect	Skeletal malformations (rat) and abnormalities (rabbit)
Lowest relevant maternal NOAEL	10 mg/kg bw per day (rabbit)
Lowest relevant embryo/fetal NOAEL	10 mg/kg bw per day (rabbit)
Neurotoxicity	
Acute neurotoxicity NOAEL	< 125 mg/kg bw per day, lowest dose tested (rat)
Subchronic neurotoxicity NOAEL	68 mg/kg bw per day (rat)
Developmental neurotoxicity NOAEL	No data
Other toxicological studies	
Immunotoxicity	No specific studies
Mechanistic data	Hepatocyte proliferation (mice); induction of enzymatic activities (rats and mice)
Studies on metabolites	No in vivo data submitted on individual metabolites CGA 313124 – significant rat metabolite, addressed by studies with pymetrozine Nicotinic acid, nicotinamide, CGA 245342, CGA 215525, CGA 96956, CGA 23199, CGA 128632, Ia7 and Ia17 were considered to be not toxicologically significant plant metabolites based on comparisons of intakes from pymetrozine uses with other types of exposure or the appropriate TTC values It was not possible to conclude on the toxicological significance of CGA 294849 or CGA 300407

Medical data	
	No notable adverse effects reported

Summary

	Value	Study	Safety factor
ADI	0–0.03 mg/kg bw	Ninety-day and 1-year studies of toxicity (dog)	100
ARfD	0.1 mg/kg bw	Developmental toxicity study (rabbit)	100

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Appendix 1. Summary of information on pymetrozine metabolites

	Pymetrozine	**Nicotinic acid + nicotinamide, vitamin B_3**	**CGA 245342**	**Ia7**	**Ia17**	**CGA 300407**	**CGA 215525**	**CGA 294849**	**CGA 96956 (trigonelline)**	**GS 23199**	**Nicotinyl alcohol, CGA 128632**
% in rat metabolism	-	0	0	0	0	0	< 1–17%	< 1–9%	< 1–14%	1–3%	0
Genotoxicity alert	No	-	No	No	Yes	No	Yes	Yes	-	No	-
Genotoxicity data	−ve	-	No	No	No	+ve	Ames −ve	No	-	No	-
Cramer class	-	-	III	III	-	-	III	-	-	III	-
TTC (µg/kg bw per day)	-	-	1.5	1.5	0.002 5	0.002 5	1.5	0.002 5	-	1.5	-
Acute TTC (µg/kg bw)	-	-	5	5	0.2	0.2	5	0.2	-	5	-
Other data	ADI 0–0.03 mg/kg bw	RDA 200 µg/kg bw per day; 2 mg/cereal serving	None	None	None	None	None	None	Natural alkaloid in range of foods (50 mg/cup coffee)	None	Minimal therapeutic dose as vasodilator = 1 mg/kg bw per day

-: not considered, as alternative information available; −ve: negative; +ve: positive; RDA: recommended dietary allowance; TTC: threshold of toxicological concern

TRIFORINE

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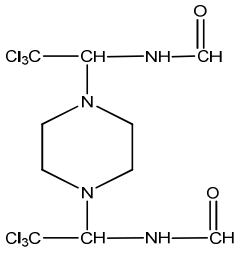
Explanation

Triforine (Fig. 1) is the International Organization for Standardization (ISO)–approved common name for *N*,*N*′-[piperazine-1,4-diylbis[(trichloromethyl)methylene)] diformamide (International Union of Pure and Applied Chemistry), with Chemical Abstracts Service number 37273-84-0. Triforine is a systemic fungicide that acts by inhibition of sterol biosynthesis in the membranes of fungi (black spot, rust, powdery mildew).

Fig. 1. Chemical structure of triforine



Triforine was first evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 1977, when no acceptable daily intake (ADI) was established. When more toxicological data were made available to the Meeting for review in 1978, an ADI of 0–0.02 mg/kg body weight (bw) was

established. The Meeting reviewed triforine in 1997 within the periodic review programme of the Codex Committee on Pesticide Residues (CCPR) and reaffirmed the ADI of 0–0.02 mg/kg bw. No acute reference dose (ARfD) was established, because the establishment of ARfDs by JMPR was not common practice in 1997.

Triforine was reviewed by the present Meeting as part of the periodic review programme of CCPR. New information and studies were submitted for evaluation, including pharmacokinetics, distribution and excretion studies, a combined 90-day toxicity/neurotoxicity study in rats, genotoxicity studies, a teratogenicity study in rabbits and immunotoxicity studies in mice and rats.

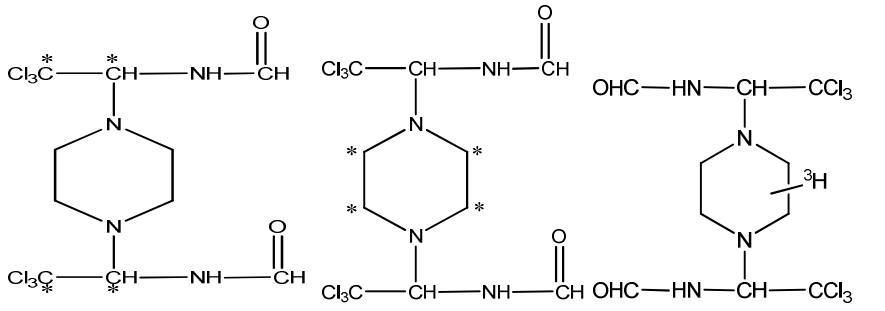
Some of the critical studies do not comply with good laboratory practice (GLP), as the data were generated before the implementation of GLP regulations. Overall, however, the Meeting considered that the database was adequate for the risk assessment.

Evaluation for acceptable daily intake

1. Biochemical aspects

For study of the absorption, distribution, metabolism and excretion (ADME) of triforine in mammals, three radiolabelled forms of triforine were used by various laboratories: ^{14}C-(side-chain)-triforine; ^{14}C-(ring)-triforine; and, in older studies, ^{3}H-(ring)-triforine. In Fig. 2, the label positions are indicated with an asterisk (*) or ^{3}H.

Fig. 2. Radiolabelled forms of triforine used in ADME studies



^{14}C-(side-chain)-triforine ^{14}C-(ring)-triforine ^{3}H-(ring)-triforine

1.1 Absorption, distribution and excretion

Rats

The absorption and distribution of ^{14}C-labelled triforine were studied in Sprague-Dawley rats after oral administration of a dose of 10 mg/kg bw (both as a single dose and after pretreatment with non-radiolabelled triflorine for 14 days) or 1000 mg/kg bw (single dose only). Intravenous dose studies were not performed, because of the limited aqueous solubility of triforine.

For the main studies, Sprague-Dawley (CD) rats (aged 6–8 weeks [males], 8–10 weeks [females]; weights 200–250 g [males], 175–225 g [females]) were dosed with triforine ^{14}C-labelled in the aliphatic side-chain (^{14}C-(side-chain)-triforine) (lot/batch no. 91056; purity 95–96%); additionally, some rats were administered triforine ^{14}C-labelled in the piperazine ring (^{14}C-(ring)-triforine) (lot/batch no. DJL 1/84; purity 74%) at the 10 mg/kg bw dose only.

After the administration of single doses of ^{14}C-(side-chain)-triforine to rats at 10 mg/kg bw, radioactivity was well absorbed (> 80% of the dose) and rapidly excreted, mainly in the urine. Means of 78.3% (male) and 79.0% (female) of the dose were excreted in the urine over 7 days, mainly within the first 24 hours. An additional 5.2% (male) and 6.0% (female) of the dose were excreted (as $^{14}CO_2$) in the expired air. Most of the remaining dose was excreted in the faeces (means: 12.3% males, 14.3%

females); however, a small amount of the dose (2.5% males, 1.8% females; excluding designated sampled tissues) was retained in the carcass at 7 days after dosing. Recovery of the dose was essentially complete (overall mean 99.8%; excluding designated sampled tissues). There were no apparent sex differences in the rates or the routes of excretion of radioactivity.

After the administration of single oral doses of ^{14}C-(ring)-triforine to rats at 10 mg/kg bw, the rates and routes of excretion were generally similar to those measured after the administration of ^{14}C-(side-chain)-triforine. Over a 5-day period, overall means of 79.1%, 2.4% and 18.2% were excreted in the urine, expired air and faeces, respectively. The mean overall recovery was 102% of the administered dose.

After the administration of single oral doses of ^{14}C-labelled triforine to rats at a nominal dose of 1000 mg/kg bw, radioactivity was less well absorbed (in total, approximately 10% of the dose in males and 20% in females, based on the extent of excretion of the dose in the urine) than after dosing at 10 mg/kg bw. Urinary excretion accounted for only 10.7% of the dose (males) and 19.1% of the dose (females) after 7 days, and most of this was excreted 6–48 hours after dosing. The delayed urinary excretion of this dose probably reflects the dissolution rate, owing to a limited aqueous solubility at this relatively high dose. At this dose, most of the radioactivity (means: 84.5% males, 77.2% females) was excreted in the faeces and probably represents mainly unabsorbed dose. Overall recovery of the dose was essentially complete (overall mean 97.6%, excluding designated sampled tissues).

After the administration of 14 consecutive daily oral doses of non-radiolabelled triforine to rats at 10 mg/kg bw, followed (on day 15) by a single oral dose of ^{14}C-labelled triforine at the same dose, the rates and routes of excretion were very similar to those found after the administration of a single dose of ^{14}C-labelled triforine at 10 mg/kg bw. Recovery accounted for an overall mean of 97.7% of the dose (excluding designated sampled tissues). These data indicate that repeated dosing at 10 mg/kg bw per day had no apparent effect on the disposition of ^{14}C-labelled triforine.

The concentration of radioactivity in tissues was measured at 7 days after dosing (for each dosing regimen). Radioactivity was found at low levels in all tissues, and in most cases the concentrations were higher than that found in the plasma at 7 days. The highest concentration of radioactivity was generally found in whole blood, liver, kidney and skin. A mean total of 2.8% of the dose was found in tissues and organs of rats dosed at 10 mg/kg bw. These data indicate a slow clearance of a relatively small proportion of the dose from the tissues.

The study was GLP compliant, and a quality assurance (QA) statement was attached (Hawkins et al., 1992).

In another study, the pharmacokinetics, biliary excretion and tissue distribution of triforine were investigated in male and female Sprague-Dawley (Crl: CD BR,CVF for biliary excretion) rats (7–10 weeks old) after the administration of a single oral dose of ^{14}C-(ring)-triforine (lot/batch no. AC8904-25; purity 98.03% [high-performance liquid chromatography]; specific activity: 346 kBq/mg) at 0 mg/kg bw (vehicle control), 10 mg/kg bw (low dose) or 1000 mg/kg bw (high dose).

Blood samples from two or four rats of each sex were collected at 0.25, 0.5, 1, 2, 4, 8, 12 and 24 hours after dosing and daily thereafter for 7 days. Total radioactive residue (TRR) was determined in plasma and red blood cells, and concentration versus time data were used for pharmacokinetic parameter determination. Tissue distribution was examined at the time of peak concentration in plasma (T_{max}; 2–6 hours) and at 24 and 168 hours post-dosing in male and female rats after low- and high-dose administration. In the treated groups, three male and three female rats were killed at each time point, and the same number of control rats were killed at 168 hours post-dosing. Radioactive residues in plasma, red blood cells, adrenals, bone, bone marrow, brain, fat, gastrointestinal tract and contents, heart, kidneys, liver, lungs, muscle, ovaries, pancreas, pituitary, spleen, testes, thymus, thyroid, uterus and residual carcass were determined. Biliary extraction was studied using bile duct–cannulated rats. Two rats of each sex (control) or four rats of each sex (treatment groups) were used. Bile was collected at 0–2, 2–4, 4–24 and 24–48 hours post-dosing, and faeces were collected at 0–24

and 24–48 hours post-dosing. Gastrointestinal tract and contents including wash were collected at 48 hours post-dosing when the animals were killed.

The pharmacokinetic parameters are given in Table 1. Triforine at 10 mg/kg bw was rapidly absorbed, with mean T_{max} values of 3.5 and 2.0 hours for males and females, respectively. The mean peak concentration (C_{max}) values of total radioactivity in male and female rats were 0.78 and 0.67 µg/g, respectively. The terminal elimination half-lives ($t_{½}$) were 124.97 hours in males and 95.70 hours in females. The areas under the concentration–time curve from time 0 to time *t* and from time 0 to infinity ($AUC_{0–t}$ and $AUC_{0–\infty}$) in males were 34.56 µg·h/g and 52.79 µg·h/g, respectively, and those of females were 13.52 µg·h/g and 17.66 µg·h/g, respectively. Male rats exhibited significantly longer T_{max} ($P < 0.05$) and $t_{½}$ (not significant) and higher AUC values ($P < 0.01$) compared with female rats. At 1000 mg/kg bw, mean T_{max} values of 5.5 hours, which were longer than those observed in the low-dose animals, were obtained for both males and females. The C_{max} values in male and female rats were 8.10 and 7.62 µg/g, respectively. There were no significant differences between the $t_{½}$ values obtained in males (98.83 hours) and females (111.54 hours). The $AUC_{0–t}$ and $AUC_{0–\infty}$ values were 419.21 and 583.18 µg·h/g, respectively, in males and 226.94 and 299.23 µg·h/g, respectively, in females. Exposures in males appeared to be greater than those in females, but the difference was not statistically significant.

Table 1. Pharmacokinetic parameters calculated for [^{14}C]triforine

Sex	Dose (mg/kg bw)	Mean values ± standard deviation				
		T_{max} (h)	C_{max} (µg/g)	$t_{½}$ (h)	$AUC_{0–t}$ (µg·h/g)	$AUC_{0–\infty}$ (µg·h/g)
Male	10	3.5 ± 1.0*	0.78 ± 0.13	124.97 ± 33.84	34.56 ± 5.27**	52.79 ± 9.89**
	1 000	5.5 ± 3.0	8.10 ± 1.46	98.83 ± 17.23	419.21 ± 171.48	583.18 ± 270.47
Female	10	2.0 ± 0.0	0.67 ± 0.03	95.70 ± 7.60	13.52 ± 1.35	17.66 ± 1.21
	1 000	5.5 ± 3.0	7.62 ± 1.63	111.54 ± 15.25	226.94 ± 51.97	299.23 ± 83.89

$AUC_{0–t}$: area under the concentration–time curve from time 0 to time *t*; $AUC_{0–\infty}$: area under the concentration–time curve from time 0 to infinity; bw: body weight; C_{max}: peak concentration; $t_{½}$: terminal elimination half-life; T_{max}: time to reach C_{max}; *: $P < 0.05$; **: $P < 0.01$ (compared between males and females)

Source: Wu (1996)

The TRR values in red blood cells were higher than those in plasma, which were below or near the minimum quantifiable level in male and female rats of both dose groups. The mean residual level in red blood cells in the low-dose group was 0.169 part per million (ppm) in males and 0.049 ppm in females, and that in the high-dose group was 1.938 ppm in males and 0.794 ppm in females. The red blood cell TRR levels in the low- and high-dose groups were higher in males than in females. The TRR data in plasma and in red blood cells 168 hours after dosing are presented in Table 2.

Table 2. Total radioactive residues in plasma and red blood cells at 168 hours

Sex	Dose (mg/kg bw)	Mean TRR ± standard deviation (ppm)	
		Plasma	Red blood cells
Male	10	0.026 ± 0.006	0.169 ± 0.036
	1 000	< MQL[a]	1.938 ± 0.900
Female	10	< MQL[b]	0.049 ± 0.003
	1 000	< MQL[a]	0.794 ± 0.199

bw: body weight; MQL: minimum quantifiable level; ppm: parts per million; TRR: total radioactive residues

[a] 0.02 ppm.

[b] 0.4 ppm.

Source: Wu (1996)

The TRR data from both dose groups indicate that initially the gastrointestinal tract and contents had the highest ^{14}C residues, which is to be expected, because this area was the point of entry. The high ^{14}C residue content in liver at the T_{max} indicates that this organ was actively involved in the metabolism of the compound. Other organs and tissues in the low-dose group that initially had a high uptake (> 1 ppm) of the compound were kidneys, pancreas, lungs, adrenals, pituitary, thyroid, spleen and bone marrow. In females, plasma, fat, heart, muscle, ovaries, thymus, uterus and residual carcass also had greater than 1 ppm residue. At 24 hours post-dosing, residue levels in tissues decreased significantly. Only the liver in males contained more than 1 ppm of ^{14}C residue. At 168 hours, ^{14}C residues were further depleted, with liver containing the highest level of radioactivity. The TRR data from the high-dose group indicate that the relative distribution was similar in this group, but for the most part was 5- to 50-fold higher than in the low-dose group, which was to be expected because of a 100-fold difference in dose. Initially, tissues in females generally contained higher TRR levels than tissues in males, but at 168 hours post-dosing, males had higher TRR levels than females (Table 3).

Results of biliary excretion experiments indicated that in the low-dose group, 8.69–13.35% of the dose was excreted from bile, 14.00–19.57% was excreted via faeces and 1.19–7.36% remained in the gastrointestinal tract and contents. These results indicated that an average of about 79% of the dose was absorbed and that 11–17% of the absorbed dose was excreted from bile. In the high-dose group, 2.28–3.73% of the dose was excreted from bile, 30.94–55.85% was excreted via faeces and 19.85–40.38% remained in the gastrointestinal tract and contents (Table 4). This suggested that only an average of 24–29% of the high dose was absorbed and that 9–13% of the absorbed dose was excreted from the bile.

The study was not GLP compliant (Wu, 1996).

In another two sets of experiments (one by oral administration and the other by intraperitoneal injection), three or 10 male FM 49 rats, respectively, were treated with ^{3}H-(ring)-triforine at a volume of 0.4 mL (2.3 mg triforine and 1.59 MBq radioactivity). After dosing, blood levels, excretion in urine, faeces and bile and tissue distribution were examined.

A maximum concentration of the orally administered radioactivity was observed in the blood after 4 hours, this being predominantly excreted in urine. Nineteen per cent of the applied dose was excreted in bile with a half-life of 4 hours and appeared in faeces.

Triforine showed a slower excretion rate after intraperitoneal injection than after oral administration.

Triforine was completely absorbed after oral administration. Ninety-one per cent of the administered dose was excreted in urine and faeces within 24 hours. Accumulation did not occur.

The studies were not GLP compliant (Darda, 1971, 1977).

Another study was performed as a continuation and expansion of a previous study (Darda, 1971). Two to 10 male FM 49 rats in each experiment were treated with ^{14}C-labelled triforine (labelled side-chains) suspended in a tylose or glycerol formal solution and ^{3}H-labelled triforine (labelled piperazine ring) according to the experimental design presented in Table 5.

Blood levels and excretion in urine, faeces and bile were determined.

After oral administration of ^{14}C-labelled triforine suspended in tylose, 55% of the administered dose was excreted renally within 3 days, most of this during the first 24 hours (52% of the administered dose), whereas 40% was found in the faeces within 30 hours. The highest levels in blood occurred 4 hours after oral administration in rats treated with ^{3}H-labelled triforine, as in rats treated with ^{14}C-labelled triforine. However, the maximum concentration of ^{14}C-labelled triforine in blood (2.5%) was almost double that of ^{3}H-labelled triforine (1.3%). Renal excretion was examined

Table 3. Tissue concentrations of [^{14}C]triforine

	Concentration of [^{14}C]triforine (ppm)[a]											
	Males						Females					
	10 mg/kg bw			1 000 mg/kg bw			10 mg/kg bw			1 000 mg/kg bw		
Sample	T_{max}[b]	**24 h**	**168 h**	T_{max}[c]	**24 h**	**168 h**	T_{max}[d]	**24 h**	**168 h**	T_{max}[b]	**24 h**	**168 h**
Plasma	0.575	0.177	< MQL	7.861	2.532	< MQL	1.078	0.065	< MQL	10.580	4.016	< MQL
RBC	0.586	0.329	0.213	7.691	5.305	3.168	0.957	0.102	0.059	7.574	4.163	0.808
Adrenals	1.697	0.361	0.085	20.453	4.716	1.333	9.734	0.157	0.036	39.170	19.882	0.490
Bone	0.498	0.192	0.085	12.950	2.586	0.921	0.804	0.060	0.028	20.548	2.774	< MQL
Bone marrow	1.064	0.388	0.052	16.623	3.038	0.900	2.112	0.165	0.021	21.493	7.649	< MQL
Brain	0.218	0.052	0.029	3.202	0.752	< MQL	0.604	0.023	< MQL	3.741	1.573	< MQL
Fat	0.323	0.066	0.040	3.220	1.165	1.014	1.050	< MQL	< MQL	6.795	2.856	< MQL
GI tract	4.966	0.288	0.040	237.121	9.757	0.791	11.452	0.172	0.020	326.951	70.193	< MQL
GI tract contents	27.809	0.289	< MQL	2301.691	38.537	< MQL	31.200	0.360	< MQL	4100.395	779.035	< MQL
Heart	0.716	0.214	0.086	8.016	3.144	1.147	1.940	0.082	0.030	12.422	5.281	0.506
Kidneys	3.671	0.668	0.144	20.923	8.652	1.886	3.727	0.205	0047	33.721	12.283	0.962
Liver	7.216	1.701	0.229	38.516	14.380	2.777	9.460	0.507	0.084	26.750	21.213	1.097
Lungs	2.361	0.389	0.106	21.231	5.221	1.288	7.315	0.183	0.039	32.038	18.412	0.512
Muscle	0.603	0.122	0.055	7.007	1.627	0.909	1.416	0.062	0.020	8.556	5.107	< MQL
Ovaries	NA	NA	NA	NA	NA	NA	2.263	0.95	0.022	12.621	6.605	< MQL
Pancreas	2.080	0.269	0.094	15.266	3.859	1.175	3.237	0.103	0.068	23.707	10.694	< MQL
Pituitary	1.649	0.421	0.168	14.487	7.079	1.976	3.250	0.176	0.034	18.457	10.181	0.983
Spleen	1.258	0.322	0.086	11.080	3.778	1.106	2.454	0.119	0.031	12.959	8.004	0.462
Testes	0.747	0.227	0.041	9.149	3.230	0.471	NA	NA	NA	NA	NA	NA
Thymus	0.693	0.174	0.048	6.698	2.153	0.786	1.578	0.063	0.025	8.037	5.154	< MQL

	Concentration of [^{14}C]triforine (ppm)[a]											
	Males						Females					
	10 mg/kg bw			1 000 mg/kg bw			10 mg/kg bw			1 000 mg/kg bw		
Sample	T_{max}[b]	**24 h**	**168 h**	T_{max}[c]	**24 h**	**168 h**	T_{max}[d]	**24 h**	**168 h**	T_{max}[b]	**24 h**	**168 h**
Thyroid	1.671	0.428	0.175	18.558	6.050	2.977	2.073	0.383	0.079	19.460	9.932	0.703
Uterus	NA	NA	NA	NA	NA	NA	1.345	0.079	0.023	6.685	5.568	< MQL
Residual carcass	0.728	0.176	0.091	21.509	2.544	1.177	1.624	0.087	0.038	108.368	5.762	0.618

bw: body weight; GI: gastrointestinal; MQL: minimum quantifiable level; NA: not applicable; ppm: parts per million; RBC: red blood cells; T_{max}: time to reach peak concentration

[a] MQL = 0.4 ppm for all tissues except fat and pancreas (0.8 ppm).
[b] T_{max}= 4.0 hours.
[c] T_{max}= 6.0 hours.
[d] T_{max}= 2.0 hours.

Source: Wu (1996)

Table 4. Summary of total radioactivity recovered from bile, faeces, gastrointestinal tract and gastrointestinal tract contents 48 hours after dosing

		% of dose administered			
Sex	**Dose (mg/kg bw)**	**Bile**	**Faeces**	**Gastrointestinal tract and contents**	**Total**
Male	10	13.35	19.57	1.19	34.11
	1 000	3.73	30.94	40.38	75.05
Female	10	8.69	14.00	7.36	30.05
	1 000	2.28	55.85	19.85	77.98

bw: body weight

Source: Wu (1996)

Table 5. Experimental design

Experiment	**Test substance**	**Route of administration**	**No. of animals**	**Dose (mg/kg bw)**	**Solvent**	**Samples collected; collection schedule**
Blood level	^{14}C-labelled triforine	Oral	3	25	Tylose	Blood; 5, 10, 15, 30 and 60 min and 2, 4, 6, 8, 24, 30 and 48 h after administration
	^{3}H-labelled triforine	Oral	2	50	Glycerol formal	Blood; 5, 10, 15, 30 and 60 min and 2, 3, 4 and 6 h after administration
	^{3}H-labelled triforine	Intraperitoneal	2	50	Glycerol formal	Blood; 5, 10, 15, 30 and 60 min and 2, 3, 4 and 6 h after administration
Excretion[a]	^{14}C-labelled triforine	Oral	10	15	Tylose	Urine; 6, 24, 30, 48 and 72 h after administration Faeces; 24 and 30 h
	^{3}H-labelled triforine	Oral	2	25	Tylose	Urine; 24 and 48 h
			2	50	Tylose	Urine; 24 and 48 h
			2	100	Tylose	Urine; 24 and 48 h
			2	200	Tylose	Urine; 24 and 48 h
	^{14}C-labelled triforine	Oral	2	50	Tylose	Urine; 24 and 48 h
			2	100	Tylose	Urine; 24 and 48 h
Biliary excretion and metabolite pattern	^{14}C-labelled triforine	Oral	2	15	Tylose	Biliary excretion and collection; 8 and 24 h

bw: body weight

[a] Urine and faeces were used for determining metabolite pattern (see section 1.2).

Source: Darda (1974)

for increasing doses of triforine up to 200 mg/kg bw. The relative excretion rates were not affected by the dose levels.

When triforine was dissolved in 80% glycerol formal and administered orally, it was completely and more rapidly absorbed than after oral administration in a tylose suspension.

^{14}C-labelled triforine was completely absorbed and then excreted in urine and faeces within 24 hours.

The study was not GLP compliant (Darda, 1974).

1.2 Biotransformation

In the study in rats described in the previous section (Hawkins et al., 1992), absorbed triforine was essentially completely metabolized and excreted in urine mainly as the metabolite *N*-[2,2,2-trichloro-1-(piperazin-1-yl)ethyl]formamide, formed by cleavage of one of the two identical side-chains in the triforine molecule. The cleaved side-chain was further metabolized prior to excretion, mainly to trichloroethanol, which was excreted in urine primarily as the glucuronic acid conjugate. During 24 hours after dosing (at 10 mg/kg bw), the metabolite *N*-[2,2,2-trichloro-1-(piperazin-1-yl)ethyl]formamide accounted for approximately 50% of the ^{14}C-(ring)-triforine radioactive dose and for almost 30% of the ^{14}C-(side-chain)-triforine radioactive dose (single or repeated dose). Trichloroethanol (mainly as its glucuronic acid conjugate) accounted for approximately 20% of the ^{14}C-(side-chain)-triforine radioactive dose at 10 mg/kg bw. A further urinary metabolite of triforine (accounting for up to approximately 15% of the dose) was identified as the *N*-acetylcysteine conjugate of trichloroethylamine.

Radioactivity in faeces, after dosing at 10 mg/kg bw, was mainly associated with the metabolite *N*-[2,2,2-trichloro-1-(piperazin-1-yl)ethyl]formamide and an unidentified, chromatographically very polar component. Unchanged triforine accounted for a very small proportion (0–1% of the dose) of radioactivity in faeces at the 10 mg/kg bw dose level, but was the major component in faeces after dosing at 1000 mg/kg bw. At the higher dose level, unchanged triforine accounted for approximately 70–80% of the dose in faeces, presumably corresponding to unabsorbed compound. This result suggests that absorption of triforine is a saturable process, resulting in excretion of a large amount of the unchanged form (Hawkins et al., 1992).

In the studies by Darda (1971, 1977) described above (section 1.1), the main components in bile (0–8 hours) were the components M1 and M2, at 48% and 37% of the radioactivity, respectively. In urine (0–24 hours), M2 was the main component, amounting to about 90% of the activity. The more hydrophilic metabolite M1 was rather more rapidly excreted than M2. The M1:M2 ratios in the urine of the 0- to 10-hour interval and in the total urine during the 0- to 24-hour period were 56:37 and 32:68, respectively. Considerable amounts of tritium activity in M1 and M2 indicated that parts of the piperazine ring were present.

The isolation and identification of M2 by mass spectrometry were performed, and M2 was confirmed as *N*-[2,2,2-trichloro-l-yl]ethyl]formamide.

In the study by Darda (1974) described previously (section 1.1), triforine was excreted in bile and most probably reabsorbed. Most of the radioactivity was excreted in a metabolized form via the kidneys, and unchanged triforine was detected in the faeces (maximum of 15–20% of the administered dose).

The primary metabolite in urine was *N*-[2,2,2-trichloro-l-yl]ethyl]formamide, formed via the splitting off of one of the two identical side-chains of triforine. This triforine metabolite was considered to be a true end product of metabolism, as it was completely absorbed and excreted renally in an unchanged form after enterohepatic circulation. Only unchanged triforine was detected in the faeces.

In summary, triforine is rapidly metabolized and excreted in rats; unchanged compound accounts for only 0–8% of the dose (Hawkins et al., 1992). Substantial quantities of unchanged triforine were recovered only from faeces (Darda, 1974).

The first metabolite to be identified was *N*-[2,2,2-trichloro-1-(piperazin-1-yl)ethyl]-formamide, which is formed by the cleavage of an entire side-chain (Darda, 1977). In later metabolic studies with ^{14}C labelling in the piperazine ring and aliphatic side-chain (Hawkins et al., 1992), triforine underwent virtually complete metabolism after administration as a single oral dose of 10 mg/kg bw. *N*-[2,2,2-Trichloro-1-(piperazin-1-yl)ethyl]formamide, the major radiolabelled urinary component in rats receiving piperazine ring–labelled ^{14}C-triforine, accounted for 46–53% of the dose over 0–24 hours; however, in rats receiving side-chain-labelled ^{14}C-triforine, the proportion was reduced to 24–27% after a single 10 mg/kg bw dose and to 21–24% after repeated doses. It was excreted in urine as the glucuronide.

The side-chain metabolite trichloroethanol and its glucuronide represented 18–21% of the dose. Another side-chain metabolite occurring in the urine was the *N*-acetylcysteine conjugate of 2,2,2-trichloroethylamine, which represented 13–15% of the administered dose. In faeces collected from female rats over 0–48 hours, 3.6% of the single 10 mg/kg bw dose and 3.4% of the repeated doses were present as *N*-[2,2,2-trichloro-1-(piperazin-1-yl)ethyl]formamide. The *N*-acetylcysteine conjugate of 2,2,2-trichloroethylamine is a final metabolite that has probably been formed by the degradation of a preceding glutathione conjugate of 2,2,2-trichloroethylamine. This metabolite was not detected in the faeces of rats receiving 1000 mg/kg bw. Very little unchanged triforine (0–1%) was detected in the faeces of rats given the low dose, whereas it represented 70–80% of the dose in rats given 1000 mg/kg bw (Hawkins et al., 1992). This result suggests that absorption of triforine is a saturable process, resulting in excretion of a large amount of the unchanged form.

A scheme of the metabolic pathways of triforine (Darda, 1974; Hawkins et al., 1992) is presented in Fig. 3

2. Toxicological studies

2.1 Acute toxicity

The results of acute toxicity studies with triforine are summarized in Table 6. All these studies were conducted according to GLP and/or a QA statement was attached.

2.2 Short-term studies of toxicity

(a) Oral administration

Mice

Four groups of five male and five female mice (KFM-Han, NMRI (SPF)) were given technical-grade triforine (batch no. 2764; purity 98.1 ± 0.9%) in the diet at a concentration of 0, 200, 1000 or 5000 ppm (equal to 0, 39.0, 195.8 and 982.1 mg/kg bw per day for males and 0, 45.2, 237.0 and 1284.3 mg/kg bw per day for females, respectively) for 4 weeks. The animals were 5 weeks of age and weighed about 10–20 g (males) or 16–18 g (females) at receipt. Mortality was observed twice daily. Clinical signs were recorded at least once daily. Body weights and feed consumption were recorded weekly, and haematology was performed in week 4. All animals were subjected to necropsy, postmortem examination, organ weight recording and tissue preservation. Major organs and tissues from all animals were processed and examined by light microscopy. Data were analysed statistically where appropriate.

No deaths or clinical signs of toxicity were recorded, and there were no effects on feed consumption; the apparent increased feed consumption by females at 5000 ppm was possibly due to greater spillage. At the end of the study, males at 5000 ppm had 8% less body weight gain than controls. Haematological changes (Table 7) included decreased erythrocyte count (by 8–11%; $P < 0.05$), haemoglobin concentration (by 7–9%; $P < 0.05$) and haematocrit value (by 6–8%; $P < 0.05$) and increased polychromatic erythrocytes in animals of both sexes at 5000 ppm. Males at this dose

also had moderately increased reticulocyte counts and decreased leukocyte counts. A significant decrease in mean erythrocyte count was also observed in males at 1000 ppm, although most of the values fell within the range for the concurrent control group.

Fig. .

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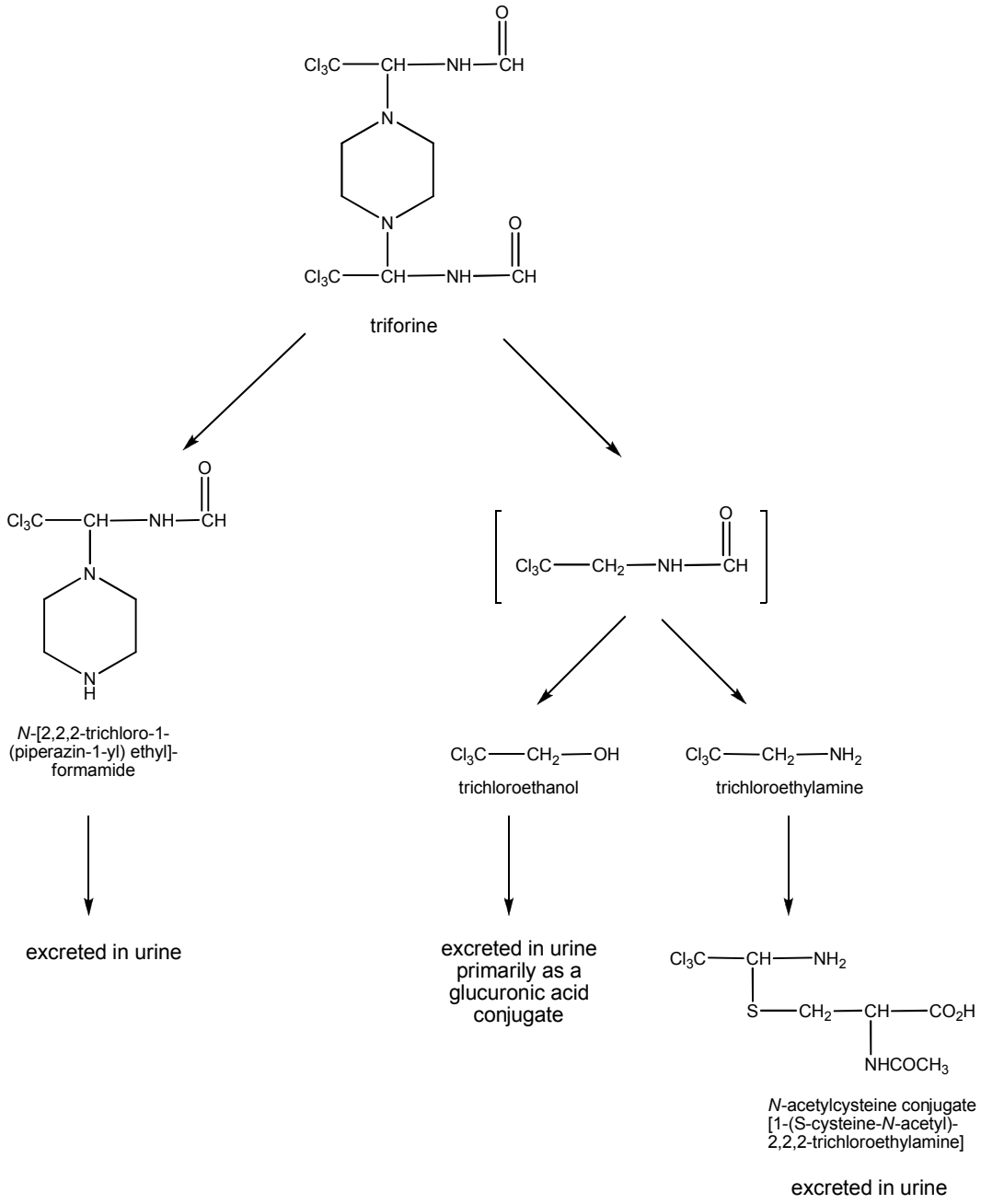
Fig. 3. Proposed metabolic pathway of triforine in the Sprague-Dawley (CD) rat



Source: Darda (1974); Hawkins et al. (1992)

Table 6. Summary of acute toxicity studies with triforine

Species	Strain	Sex	Route	Batch no. and purity	LD_{50}/LC_{50}	Reference[a]
Mouse	KFM-NMRI (SPF)	M + F	Oral	2764 98.1 ± 0.9%	> 5 000 mg/kg bw	Ullmann et al. (1986a)
Rat	KFM-Han Wistar (SPF)	M + F	Oral	2764 98.1 ± 0.9%	> 5 000[b] mg/kg bw	Ullmann et al. (1986b)
Rat	Wistar - AF/HAN-EMD-SPF	M + F	Oral	6/70 Not specified	> 16 000 mg/kg bw	Frohberg, von Eberstein & Weisse (1973)
Rat	Wistar – AF/HAN-EMD-SPF	M + F	Dermal	6/70 Not specified	> 10 000 mg/kg bw	Frohberg, von Eberstein & Weisse (1973)
Rat	HanIbm:WIST (SPF)	M + F	Dermal	2764 99.1%	> 2 000 mg/kg bw	Ullmann et al. (1990)
Rat	Sprague- Dawley	M + F	Inhalation (4 h) MMAD: 2.7 µm	TRF 1002027 100.94%	> 0.51 mg/L	Doig (2011)
Rat	KFM-HAN Wistar (SPF)	M + F	Inhalation (4 h nose only)	2764 Not specified	> 5.12 mg/L	Ullmann et al. (1986c)
Rat	Sprague-Dawley	M + F	Inhalation (1 h nose only)	SX 760 Not specified	> 4.5 mg/L	Bullock & Narcisse (1973)*
Rabbit	New Zealand White KFM (SPF)	M + F	Skin irritation	2764 98.1 ± 0.9%	Not irritating	Ullmann & Porricello (1988a)
Rabbit	TH/THOM-SPF	M + F	Skin irritation	1/75 Not specified	Not irritating	Von Eberstein (1976)*
Rabbit	New Zealand White EMD	M	Eye irritation	6/70 Not specified	Not irritating	Frohberg, von Eberstein & Weisse (1973)
Rabbit	New Zealand White KFM (SPF)	M + F	Eye irritation	2764 98.1 ± 0.9%	Not irritating	Ullmann & Porricello (1988b)
Guinea-pig	Dunkin-Hartley (DUHA KFM), albino	M + F	Skin sensitization (Maurer optimization test)	2230 99.9%	Not sensitizing	Ullmann & Surer (1984)

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

[a] The studies marked with an asterisk (*) are not compliant with GLP.

[b] The highest dose tested. Sedation or ruffled fur was observed in all animals at 1 hour or 3 hours after treatment, respectively, but all rats had recovered within 2 days after treatment.

Table 7. Haematological changes in mice administered triforine in the diet

Sex	Dietary concentration (ppm)	Group mean value								
		RBC (× 10^{12}/L)	Hb (mmol/L)	Hct (L/L)	MCV (fL)	MCH (fmol)	MCHC (mmol/L)	Retic (L)	Poly score 0/3	WBC (g/L)
Males	0	10.8	10.8	0.58	53.4	1.00	18.7	0.024	0	8.6
	200	10.4	10.5	0.56	53.9	1.01	18.7	0.027	0	7.1
	1 000	10.1*	10.3	0.55	55.0	1.02	18.6	0.025	0	5.9
	5 000	9.6*	9.8*	0.53*	55.3	1.02	18.5	0.036*	1	5.1*
Females	0	10.2	10.5	0.54	53.3	1.03	19.3	0.030	0	5.6
	200	10.0	10.2	0.54	53.9	1.02	18.9	0.026	0	5.0
	1 000	10.0	10.3	0.54	54.3	1.03	19.0	0.025	0	4.8
	5 000	9.4*	9.7*	0.51*	54.4	1.03	19.0	0.042	1	4.1

Hb: haemoglobin; Hct: haematocrit; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; MCV: mean corpuscular volume; Poly: polychromatic erythrocytes; ppm: parts per million; RBC: red blood cells; Retic: reticulocytes; WBC: white blood cells; *: $P < 0.05$ (Dunnett test)

Source: Tennekes et al. (1988)

Males and females at 5000 ppm showed increased absolute and relative spleen weights, and females had increased relative liver weights (by about 16%; $P < 0.01$). There were no gross or microscopic changes.

The no-observed-adverse-effect level (NOAEL) was 1000 ppm (equal to 195.8 mg/kg bw per day), on the basis of haematological changes in animals of both sexes, slightly reduced body weight gain in males and increased relative liver weight in females at 5000 ppm (equal to 982.1 mg/kg bw per day).

The study was compliant with GLP, and a QA statement was attached (Tennekes et al., 1988).

In another study, which was designed to provide information on the maximum tolerated dose (MTD) of triforine in mice over a 13-week period for a long-term toxicity and carcinogenicity study, groups of 10 CD-1 mice of each sex were given technical-grade triforine (batch no. 2764; purity 99.1%) in the diet at a concentration of 0 or 7000 ppm (equal to 1354 mg/kg bw per day for males and 2239 mg/kg bw per day for females) for 13 weeks. The achieved dose was not available in the report but was calculated by Sumitomo Corporation for submission in Japan (to the Japanese Ministry of Agriculture, Forestry, and Fisheries [JMAFF]). The animals were 5 weeks of age and weighed about 23–28 g (males) and 19–23 g (females) at delivery. Mortality was observed twice daily. Clinical signs were recorded daily, and detailed clinical examinations were conducted once a week. Body weights and feed consumption were recorded weekly before and during the treatment period. Water consumption was monitored by visual inspection throughout the study. Blood samples were taken from all animals during week 13 of dosing. All animals were subjected to necropsy, postmortem examination, organ weight recording and tissue preservation. No histopathological examination was carried out. Data were analysed statistically where appropriate.

No deaths or clinical signs of toxicity were observed, and there were no treatment-related effects on feed consumption, water consumption or body weight gain. The haematological changes included a slight reduction in erythrocyte numbers, haemoglobin concentration and haematocrit in animals of both sexes. The absolute weights of the spleen were increased by 38% in males and 56% in females, and that of the liver was increased by 17% in males. The relative liver weights were increased in males and females (17% and 13%, respectively), as were the relative spleen weights in males (40%) (all $P < 0.01$). There were no gross pathological findings; tissues were not examined

microscopically, as the study was designed to select concentrations for use in a long-term study of toxicity and carcinogenicity.

In view of the above, it can be concluded that mice treated with 7000 ppm triforine for 13 weeks showed slight disturbances in red blood cell parameters and increased spleen and liver weights, and 7000 ppm was considered to be the MTD. A NOAEL was not established in the present study.

The study was not GLP compliant, but a QA statement was attached (Atkinson, Perry & Hudson, 1991a).

Rats

Groups of five male and five female Wistar (KFM-Han,Wistar (SPF)) rats were given technical-grade triforine (batch no. 2764; purity 98.1 ± 0.9%) in the diet at a concentration of 0, 500, 2500 or 12 500 ppm (equal to 0, 49.7, 238.2 and 1233.7 mg/kg bw per day for males and 0, 48.5, 233.2 and 1180.8 mg/kg bw per day for females, respectively) for 4 weeks. The animals were 5 weeks of age and weighed about 65–88 g (males) or 53–68 g (females) at delivery. Mortality was observed twice a day. Clinical signs were recorded at least once daily. Body weights and feed consumption were recorded weekly, and haematology, clinical chemistry and urine analyses were performed in week 4. All animals were subjected to necropsy, postmortem examination, organ weight recording and tissue preservation. Major organs and tissues from all animals were processed and examined by light microscopy. Data were analysed statistically where appropriate.

No deaths or clinical signs of toxicity were recorded, and there were no effects on feed consumption. The terminal body weight was reduced by about 11% in males at the high dose ($P < 0.05$), whereas there were no consistent, treatment-related effects in females. Haematological changes indicative of slight anaemia were observed. In rats at 12 500 ppm, slight decreases in mean corpuscular haemoglobin concentration (MCHC) ($P < 0.01$) and mean corpuscular volume (MCV) were seen in females ($P < 0.05$), and increases in reticulocyte ($P < 0.01$) and polychromatic erythrocyte counts were seen in animals of both sexes. Increased proportions of circulating immature cells were also seen in rats at 2500 ppm, and the increase was significant in males ($P < 0.01$). Prothrombin time was shortened in females at 12 500 ppm. At this dose, a slight increase in total protein (by about 6%; $P < 0.05$) was seen in animals of both sexes, and a slight increase in cholesterol content (57%; $P < 0.01$) was seen in females. Urine volume was increased in females at this dose. Changes in organ weights were limited to rats at 12 500 ppm. Absolute spleen weights were increased in animals of both sexes ($P < 0.05$), as were the absolute weights of the liver, thyroid and kidney in females. Increased relative weights were observed for liver ($P < 0.01$) and spleen ($P < 0.05$) in animals of both sexes and for thyroid in males ($P < 0.05$). There were no treatment-related gross pathological findings. The treatment-related microscopic changes were slight or moderate haemosiderin deposition in the spleens of males and females at 2500 and 12 500 ppm and in females also at 500 ppm (Table 8).

Table 8. Histopathology: Iron pigment in spleen

	Males				**Females**			
Findings	**0 ppm**	**500 ppm**	**2 500 ppm**	**12 500 ppm**	**0 ppm**	**500 ppm**	**2 500 ppm**	**12 500 ppm**
Iron-PAS staining								
No. of animals examined	*5*	*5*	*5*	*5*	*5*	*5*	*5*	*5*
Haemosiderin pigment +1	0	0	4	0	4	3	1	0
Haemosiderin pigment +2	0	0	1	5	0	2	4	1
Haemosiderin pigment +3	0	0	0	0	0	0	0	4
Haemosiderin pigment total	0	0	5	5	4	5	5	5

PAS: periodic acid–Schiff; ppm: parts per million

Source: Tennekes et al. (1989)

A NOAEL was not established in the present study because the iron-positive pigment in the spleen was recorded in females of the lowest dose group.

The study was GLP compliant, and a QA statement was attached (Tennekes et al., 1989).

A study was designed to give MTD information on triforine in rats over a 13-week period for a carcinogenicity study. Groups of 10 male and 10 female Sprague-Dawley rats (aged about 5–6 weeks [males and females], weighing 98–122 g [males] or 72–95 g [females]) were given technical-grade triforine (batch no. 2764; purity 99.1%) in the diet for 13 weeks at a concentration of 0 or 20 000 ppm, equal to mean achieved doses of 1148–2630 (mean 1630) mg/kg bw per day for males and 1475–2826 (mean 1945) mg/kg bw per day for females and a mean achieved dose of 1300 mg/kg bw per day for males and females considered together. The group mean achieved dose was not available in the report but was calculated by Sumitomo Corporation for submission in Japan (to JMAFF). Mortality was observed twice daily. Clinical signs were recorded daily, and detailed clinical examinations were conducted once each week. Body weights and feed consumption were measured weekly before and during the treatment period. Water consumption was monitored by visual inspection throughout the study. Blood samples were taken from all animals during week 13 of dosing. All animals were subjected to necropsy, postmortem examination, organ weight recording and tissue preservation. No histopathological examination was carried out. Data were analysed statistically where appropriate.

No deaths or clinical signs of toxicity were observed, and there were no effects on feed consumption, water consumption or body weight gain. The haematological changes included statistically significant but slight reductions in MCHC of males (1%; $P < 0.01$) and females (2%; $P < 0.05$), reductions in red blood cell counts of females (5%; $P < 0.05$), as well as an increase in MCV of females (4%; $P < 0.05$). Increases in the absolute and relative weights of the liver and spleen were observed in animals of both sexes. There were no gross pathological findings. The tissues were not examined microscopically.

In conclusion, rats treated with 20 000 ppm triforine for 13 weeks showed slight disturbances in red blood cell parameters and increased spleen and liver weights, and 20 000 ppm was considered to be the MTD for a 104-week carcinogenicity study. A NOAEL was not established in the present study.

The study was not GLP compliant, but a QA statement was attached (Atkinson, Perry & Hudson, 1991b).

In a 3-month study, groups of 10 male and 10 female Wistar rats (aged 5 weeks [males and females] and weighing about 130.7 ± 6.2 g [males] or 112.5 ± 5.3 g [females]) were treated orally, by dietary administration, with triforine (batch no. 1/75; purity 99.2%) at a nominal dose level of 0, 10, 100 or 1000 mg/kg bw per day. Mortality and clinical signs were recorded at least once daily. Body weights and feed consumption were recorded weekly. Haematology and clinical chemistry were performed at the end of the treatment period. Urine analysis was performed the day before necropsy. All animals were subjected to necropsy, postmortem examination, organ weight recording and histopathological examination. Data were analysed statistically where appropriate.

One male animal in the control group died due to a congenital renal disorder. No adverse clinical signs occurred in any treatment group. There were no marked differences in body weight, feed consumption or urine analysis between control rats and rats treated with triforine. There were no toxicologically significant changes in clinical chemistry parameters at any dose level. There were no treatment-related effects on macroscopic or microscopic examination at any dose level.

Slight anaemia occurred in both sexes at 100 or 1000 mg/kg bw per day. Although reductions in neutrophils and monocytes and increased lymphocytes in differential white blood cell count were noted in all treated groups, similar changes were not detected on myelography. Therefore, the changes

in differential white blood cell count were considered not to be toxicologically significant. Absolute and relative liver weights were increased in both sexes at 100 and 1000 mg/kg bw per day (Table 9).

Table 9. Group mean values for selected haematological data and liver weights

Sex	Dose (mg/kg bw per day)	RBC ($\times 10^4$/mm^3)	Hb (g/dL)	Hct (%)	Absolute (g)		Relative (%)	
					BW (g)	Liver	BW (g)	Liver
Males	0	827.0	16.68	49.4	450.1	13.72	450.1	3.069
	10	816.5	16.18	48.0	468.0	15.16	468.0	3.271
	100	839.9	16.03*	47.8	451.3	16.46*	451.3	3.636*
	1 000	789.9	15.69*	47.3*	442.1	18.92**	442.1	4.283**
Females	0	750.7	15.21	43.1	257.8	8.49	257.8	3.295
	10	741.9	15.16	42.7	261.0	8.45	261.0	3.235
	100	706.5**	14.82	43.5	248.0	9.57*	248.0	3.870**
	1 000	697.7*	14.48**	41.7	251.4	10.77**	251.4	4.291**

bw/BW: body weight; Hb: haemoglobin; Hct: haematocrit; RBC: red blood cells; *: $P < 0.05$; **: $P < 0.01$

Source: Ito & Kajiwara (1979)

In view of the above, the NOAEL in this study was 10 mg/kg bw per day for males and females, based on decreased red blood cells, haemoglobin and haematocrit and increased liver weights at 100 and 1000 mg/kg bw per day.

The study was not GLP compliant, and no QA statement was attached (Ito & Kajiwara, 1979).

In another dietary administration study, groups of 15 male and 15 female rats (FW 49 Kirchborchen, Biberach; age 62 days, weighing about 245 g [males] or 190 g [females]) were treated orally with triforine (batch no. III; purity 97.1%) at a nominal concentration of 0, 2500, 7000 or 20 000 ppm (equal to 0, 162.7, 453.6 and 1315.3 mg/kg bw per day for males and 0, 174.1, 491.4 and 1451.4 mg/kg bw per day for females, respectively) for 13 weeks. The group mean achieved doses were not available in the report but were calculated by Sumitomo Corporation for submission in Japan (to JMAFF). Additionally, 10 male and 10 female rats were used for a recovery group. They were treated at 20 000 ppm for 13 weeks and subsequently observed for 6 weeks after the end of the treatment. Mortality and clinical signs were recorded at least once daily. Body weights and feed consumption were recorded weekly, and water consumption was determined in week 12. Haematology and clinical chemistry were performed on 10 males and 10 females in weeks 0, 6, 13 and 18 or 19, and urine analysis was performed on 10 males and 10 females in the control and high-dose groups in weeks 0 and 13. All animals were subjected to necropsy, postmortem examination, organ weight recording and tissue preservation. A full range of tissues from 10 males and 10 females in each group was stained with haematoxylin and eosin and examined microscopically. The incidence and degree of histopathological findings were reanalysed statistically according to JMAFF requirements (Hagiwara, 2011a).

One male animal in the 20 000 ppm group died spontaneously on day 47, but the cause of death could not be clarified due to severe autolysis. No adverse clinical signs occurred in any treatment group. There were no marked differences in body weights or feed consumption between control rats and rats treated with triforine. The haematological investigations revealed slight decreases in erythrocytes, haematocrit and haemoglobin values from 2500 ppm onwards, the effects being greater in females than in males. There was an increase in the number of reticulocytes in all the dose

groups, suggesting mild haemolytic anaemia. These findings were reversible after 6 weeks off-dose. The clinical chemistry indicated a reduction in alkaline phosphatase in females from 2500 ppm onwards, but this finding was considered not to be toxicologically relevant.

The cholesterol level in serum was significantly increased in female rats treated with 7000 or 20 000 ppm triforine. These changes were reversible. There were no treatment-related changes in urine analysis or macroscopic pathology. A slight, dose-dependent increase in liver weights and a slight increase in thyroid gland weights in male and female animals at 20 000 ppm were observed. These changes were reversible. Histopathological investigations showed a dose-dependent increase in siderosis of liver and spleen from 2500 ppm. Iron deposits were also seen in the kidneys and, in some animals, in the myocardial fibres and in the lungs from 7000 ppm onwards. The iron deposits were observed in the recovery animals (Table 10).

Table 10. Selected histopathological findings

Dietary concentration (ppm)	No. of animals examined	Haemosiderin deposits							
			Spleen						
		Liver	Slight	Moderate	Marked	Total	Kidney	Lung	Heart
Week 13									
Males									
0	10	1	2	7	1	10	0	0	0
2 500	10	1	5	3	2	10	0	0	0
7 000	10	0	4	4	1	9	1	0	0
20 000	10	7*	1	2	7*	10	3	0	0
Females									
0	10	3	2	6	2	10	2	1	0
2 500	10	3	0	3	7*	10	3	0	0
7 000	10	10**	0	0	10**	10	6	1[a]	1
20 000	10	7	0	1	9**	10	6	2	0
Week 19 (recovery group)									
Males									
20 000	10	8	0	2	8	10	8	7	5*
Females									
20 000	10	6	0	2	8	10	6	0	0

F: female; M: male; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$

[a] One animal lung was not examined.

Source: Stötzer et al. (1971a)

A NOAEL was not established in the present study, because haemolytic anaemia correlating with haemosiderin deposits in the spleen was observed at 2500 ppm, the lowest dose tested.

The study was not GLP compliant, and no QA statement was attached (Stötzer et al., 1971a).

As a NOAEL could not be established in the above-described 13-week dietary study (Stötzer et al., 1971a), another study was performed to establish a NOAEL using lower dose levels. Three groups of 15 male and 15 female rats (FW 49 Kirchborchen, Biberach; age 73 days, weighing about 275 g [males] or 200 g [females]) were treated orally, by dietary administration, with triforine (batch no. III; purity 97.1%) at a nominal dose of 0, 100 or 500 ppm (equal to 0, 6.0 and 30.4 mg/kg bw per day for males and 0, 6.9 and 34.0 mg/kg bw per day for females, respectively) for 14 weeks. Group mean achieved doses were not available in the report but were calculated by Sumitomo Corporation for submission in Japan (to JMAFF). Mortality and clinical signs were recorded at least once daily. Body weights and feed consumption were recorded weekly, and water consumption was measured at week 13. Haematology and clinical chemistry of 10 males and 10 females were performed in weeks 0, 6 and 13. All animals were subjected to necropsy, postmortem examination, organ weight recording and tissue preservation. A full range of tissues from 10 males and 10 females in each group was stained with haematoxylin and eosin and examined microscopically.

There were no adverse effects on survival, clinical signs, body weights, feed consumption, water consumption, haematology, organ weights, or microscopic and macroscopic pathological examinations. The reduction in the serum glucose level in the highest-dose group was not considered to be treatment related, because such changes were not observed in the previous study using doses up to 20 000 ppm. A reduction in alkaline phosphatase was considered to be an incidental finding, as there were no corresponding histopathological findings.

The NOAEL was identified as 500 ppm (equal to 30.4 mg/kg bw per day), as no effects were observed up to that level, the highest dose tested.

The study was not GLP compliant, and a QA statement was not attached (Stötzer et al., 1971b).

Another 90-day repeated-dose dietary study was performed to assess the systemic toxicity and neurotoxicity of triforine. Four groups of 15 male and 15 female Crl:CD(SD) strain rats (aged 5 weeks and weighing about 134–161 g [males] or 115–145 g [females]) were treated orally, by diet admixture, with triforine (batch no. 1184; purity 99.7%) at a dose level of 0, 200, 2000 or 20 000 ppm (equal to 0, 13, 133 and 1344 mg/kg bw per day for males and 0, 15, 150 and 1540 mg/kg bw per day for females, respectively) for at least 90 days. Five of 15 test animals of each sex were assigned to a perfusion fixation group and designated for the neuropathological examination. All animals were observed twice daily for mortality and clinical observations. The detailed clinical observations were scored before treatment and 2, 4, 8 and 13 weeks after treatment. Body weights and feed consumption were recorded on days 1, 4, 8, 15, 22, 29, 36, 43, 50, 57, 64, 71, 78, 85 and 91 of the treatment period. Ophthalmological examination was conducted for the control and high-dose groups in week 13. Urine analysis was conducted in seven males and seven females per group in week 13, and haematological analysis and clinical chemistry were conducted in the fasted animals (10 males and 10 females per group) at necropsy. A functional observational battery was performed on all animals before treatment and in weeks 2, 4, 8 and 13 of the treatment period, including the measurements of several reactivities, righting reflex, grip strength, hind limb splay and motor activity. In the perfusion fixation groups, five males and five females per group were subjected to perfusion fixation on day 94. The centre of the cerebrum, including forebrain and hippocampus, midbrain, cerebellum, pons, medulla oblongata, eyeballs with optic nerve and retina, spinal cord at cervical and lumbar swellings, dorsal root ganglia, dorsal and ventral root fibres, proximal sciatic nerve, proximal tibia nerve (at the knee) and tibia nerve calf muscle branches and skeletal muscle (calf muscle) were removed, stained with toluidine blue or haematoxylin and eosin and examined microscopically for the control and the 20 000 ppm groups. The other 10 males and 10 females per group were killed after 91 days of treatment and subjected to necropsy and postmortem examination. After organ weight measurement, major organs and tissues were examined histopathologically for the control and the 20 000 ppm groups except for liver and kidney, which were examined for all groups. Data were analysed statistically where appropriate.

With regard to the neurotoxicity part of the study, the histopathological examination of the nervous systems showed that there were no abnormal findings in the central or peripheral nerve system, including the optic nerve, in males or females in the 20 000 ppm group. There were no remarkable changes in clinical signs or detailed clinical observations, functional observational battery, ophthalmological or necropsy findings.

With respect to systemic toxicity, there were no remarkable changes in clinical signs or detailed clinical observations, ophthalmological and necropsy findings. Treatment-related decreased body weight and feed consumption occurred only in males treated at 20 000 ppm. In urine, treatment-related statistically significantly decreased specific gravity was detected in males in the 2000 ppm group. There was a tendency for anaemia, as red blood cell count, haematocrit and haemoglobin concentration were statistically significantly decreased or tended to decrease in males and females in the 2000 ppm group and above. Total cholesterol level in males and total protein level in females were statistically significantly increased in the 2000 ppm group. In the 20 000 ppm group, total protein level and gamma-glutamyltranspeptidase were statistically significantly increased in males and females, whereas urea nitrogen, albumin and total cholesterol levels were statistically significantly increased in males. In that group, triglyceride level was statistically significantly decreased, and α_1-globulin ratio of the protein fraction was statistically significantly increased in females.

Absolute and/or relative liver weights were statistically significantly increased in males and females at 2000 ppm and above. Incidences of hyaline droplets and eosinophilic bodies in the proximal tubular epithelium of the kidneys were statistically significantly increased in males at 2000 ppm and above, and the severity was also increased in males in the 20 000 ppm group.

It was concluded that triforine did not elicit functional or morphological evidence of neurotoxicity up to 20 000 ppm. Therefore, the NOAEL for neurotoxicity was established as 20 000 ppm (equal to 1344 mg/kg bw per day), the highest dose tested.

The NOAEL for all other effects was identified as 200 ppm (equal to 13 mg/kg bw per day), based on the occurrence of anaemia and the effects on kidney and liver that were observed in histopathology and clinical laboratory examinations at 2000 ppm (equal to 133 mg/kg bw per day) and above.

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

Dogs

In a 13-week dietary study, four groups of four male and four female Beagle dogs (about 8 months of age and weighing around 10.6 kg [males] or about 10.0 kg [females]) were treated orally with triforine (batch no. III; purity 97.1%), by dietary administration, at a nominal dose of 0, 3500, 10 000 or 30 000 ppm (equal to 0, 83, 230 and 690 mg/kg bw per day for males and 0, 85, 240 and 730 mg/kg bw per day for females, respectively) for 13 weeks. Additionally, recovery animals comprised four males and four females that had been administered 30 000 ppm (equal to 710 and 720 mg/kg bw per day for males and females, respectively) for 13 weeks and then observed without treatment for a further 6 weeks to determine the reversibility of effects.

The animals were observed daily for viability, physical condition and behaviour. Body weights were determined once a week, and feed consumption was measured every day. Haematology, clinical chemistry, urine analysis and ophthalmic examinations were conducted prior to the treatment and after 6, 13 and 19 (recovery animals only) weeks. All animals were subjected to necropsy, postmortem examination, organ weight recording and tissue preservation. The major organs and tissues were preserved, embedded and stained with haematoxylin and eosin. The heart was additionally stained with Masson trichrome. The iron content of the heart, lungs, liver, kidney and spleen was determined by staining with Berlin blue. Fat staining of fixed frozen sections of heart, liver, kidney and adrenal with Fat Red 7B and periodic acid–Schiff (PAS) staining of liver and kidney according to McManus were also conducted.

Signs of haemolytic anaemia, manifested as reductions in erythrocyte counts and haemoglobin concentration and occasionally also in haematocrit, were observed at doses of 10 000 ppm and higher in weeks 6 and 13. Reduced haemoglobin concentration in week 6 and reduced red blood cells at 13 weeks also occurred at 3500 ppm. These effects were accompanied by a consistent increase in the number of reticulocytes in dogs at 10 000 and 30 000 ppm. All values returned to normal in the recovery group at 30 000 ppm after a 6-week non-treatment period. Serum chemistry in weeks 6 and 13 showed slight increases in alkaline phosphatase activity and bilirubin and cholesterol concentrations at 10 000 and 30 000 ppm, but these values also returned to normal in dogs at 30 000 ppm within 6 weeks after treatment (Table 11). Urine analysis, ophthalmoscopy and macroscopic examinations showed no differences between the groups. Fine, drop-like fatty infiltration of the myocardium was seen in 0/8, 5/8, 1/8, 5/8 and 1/8 dogs in the 0, 3500, 10 000, 30 000 and 30 000 (recovery) ppm test groups, respectively, and fatty accumulation in the liver in 0/8, 3/8, 0/8, 4/8 and 0/8 dogs in the same groups, respectively. The fatty infiltration appeared to be reversible, as it was no longer detected in the dogs of the recovery group after 6 weeks.

Table 11. Group mean values for selected haematological and clinical chemistry data

Dietary concentration (ppm)	RBC ($\times 10^3/mm^3$)	Hct (%)	Hb (g/100 mL)	Retic (‰)	ALP (King-Armstrong unit)	Bil (mg/dL)	Chol (mg/dL)	Alb (g/dL)
Week 6								
Males and females								
0	6.76	49.50	15.31	10.37	9.93	0.08	131.62	3.096
3 500	6.13	45.37	14.06*	13.15	11.92	0.08	153.87	2.885
10 000	5.15**	45.62	13.87*	17.25***	15.46	0.10	164.37	2.878
30 000	5.72**	45.75	14.12*	21.00***	15.17	0.10	191.37	2.887
30 000[a]	5.60**	45.25	13.43*	20.50***	16.01	0.11	182.00	2.852
Week 13								
Males and females								
0	6.64	48.37	15.00	11.87	9.96	0.08	126.12	3.028
3 500	5.93*	45.75	14.12	12.00	11.71	0.09	165.00	2.872
10 000	5.74*	45.75	13.68*	10.25	16.42	0.12	168.25	2.896
30 000	5.82*	46.62	13.31*	25.50	18.29	0.14	214.12	3.515
30 000[a]	5.72*	46.62	13.56*	14.50	20.83	0.16	223.75	3.441
Week 19 (recovery group)								
Males and females								
30 000[a]	6.66	48.37	15.25	9.50	9.42	0.06	150.25	3.225

Alb: albumin; ALP: alkaline phosphatase; Bil: bilirubin; Chol: cholesterol; Hb: haemoglobin; Hct: haematocrit; ppm: parts per million; RBC: red blood cells; Retic: reticulocytes; *: $P < 0.05$; **: $P < 0.025$; ***: $P < 0.01$

[a] Recovery animals.

Source: Stötzer et al. (1971c)

Treatment-related siderosis in Kupffer cells in the liver showed a clear tendency towards reversibility in the animals allowed to recover.

A NOAEL was not identified in this study, because signs of anaemia were observed at 3500 ppm (equal to 83 mg/kg bw per day), the lowest dose tested. Hence, further tests were recommended using lower doses.

The study was not GLP compliant (Stötzer et al., 1971c).

In another 13-week study, four groups of four male and four female Beagle dogs (aged 9–10 months [males] or 8–9 months [females]; weighing around 10.7–12.1 kg [males] or 8.0–9.3 kg [females]) were treated orally, by dietary administration, with triforine (batch no. T-3/70; purity 96.6%) at a nominal dose of 0, 100, 600 or 3500 ppm (equal to 0, 3.6, 22.6 and 121.0 mg/kg bw per day for males and 0, 3.4, 21.3 and 120.7 mg/kg bw per day for females, respectively). The animals were observed daily for viability, behaviour, appearance, feed consumption, water consumption and appearance of faeces. Body weights were measured once a week. Haematology (including osmotic resistance of erythrocytes), clinical chemistry, urine analysis, ophthalmic, hearing and teeth examinations were conducted prior to the first administration and after 4, 8 and 13 weeks of treatment in all animals. All animals were subjected to necropsy, postmortem examination, organ weight recording and tissue preservation. The liver, kidney, spleen and bone marrow were prepared and, after staining for iron (Berlin blue reaction), investigated histologically. A retrospective analysis of data was undertaken where appropriate (Hagiwara, 2011b).

No deaths or adverse clinical signs occurred in any treatment group, and all animals were killed according to schedule after 13 weeks of treatment. There were no notable intergroup differences in body weight, feed consumption, water consumption, urine analysis, ophthalmic, hearing, teeth or macroscopic examinations. At 3500 ppm, haemoglobin, erythrocytes and haematocrit values were significantly reduced from week 4 onwards. Also, a significant decrease in total protein levels was recorded from week 4 onwards, and a reduction in albumin level was observed after 8 weeks. During 4 weeks, β-globulin level was significantly reduced, whereas the albumin to globulin ratio remained normal. Relative spleen weights were significantly increased. Microscopic examinations showed siderosis (sediments of iron) in the Kupffer cells of the liver, spleen and bone marrow at 600 and 3500 ppm (Table 12). However, corresponding findings were not seen in the kidney.

The NOAEL was identified as 100 ppm (equal to 3.4 mg/kg bw per day), on the basis of haemosiderin deposits in the liver, spleen and bone marrow at 600 ppm (equal to 21.3 mg/kg bw per day) and above.

The study was not GLP compliant (Leuschner et al., 1971).

A 104-week dietary study was performed at C.H. Boehringer Sohn, not under GLP conditions, and reported in 1974 (von Sandersleben et al., 1974). In 1990, copies of the raw data were forwarded to Inveresk Research International Limited for statistical analysis and report writing under GLP (Goburdhun & Greenough, 1990). The summary below is mainly based on that 1990 report.

Groups of four male and four female Beagle dogs (aged about 10 months,weighing around 14 kg [males] or about 11 kg [females]) were given triforine (batch no. T3/70; purity 96.6%) in the diet at a concentration of 0, 10, 40, 100 or 1000 ppm (equal to 0, 0.23, 0.93, 2.39 and 22.50 mg/kg bw per day for males and 0, 0.25, 0.99, 2.56 and 23.60 mg/kg bw per day for females, respectively) for 2 years. The animals were observed at least once a day for viability and clinical signs. Body weights were determined once a week, and feed consumption was measured every day. Samples were taken from all animals for haematology, blood chemistry and urine analysis during weeks 6, 13, 26, 52, 78 and 104. Ophthalmic examination of all animals was conducted pretreatment and during weeks 13, 26, 52, 78 and 104 of treatment. All surviving animals were killed at the end of treatment and subjected to postmortem examination, organ weight recording and tissue preservation. The major organs and tissues were preserved, embedded and stained with haematoxylin and eosin. The heart was

Table 12. Selected histopathological findings

Dietary concentration (ppm)	Iron staining[a]													
	Liver					Spleen						Bone marrow		
	(+)	+	+/++	++	*P*	(+)	+	+/++	++	+++	*P*	(+)	+	*P*
Males														
0	0	0	0	0	–	4	0	0	0	0	–	0	0	–
100	2	0	0	0	–	2	2	0	0	0	–	0	0	–
600	4	0	0	0	*	0	2	1	0	1	*	4	0	*
3 500	0	0	2	2	*	0	2	2	0	0	*	0	4	*
Females														
0	0	0	0	0	–	4	0	0	0	0	–	0	0	–
100	0	0	0	0	–	4	0	0	0	0	–	0	0	–
600	3	1	0	0	*	0	4	0	0	0	*	4	0	*
3 500	0	0	3	1	*	0	0	3	1	0	*	0	4	*

F: female; M: male; ppm: parts per million; *: $P < 0.05$ (Wilcoxon test)

[a] Grade: (+): insignificant iron sediments; +: slight iron sediments; ++: moderate iron sediments; +++: marked iron sediments.

Source: Leuschner et al. (1971)

additionally stained with Masson trichrome. The Berlin blue method was used for iron determination in heart, lungs, liver, kidney and spleen, and Fat Red 7B was used to identify fat in fixed frozen sections of heart, liver, kidney and adrenal. PAS staining in liver and kidney according to McManus was also performed. Data were analysed statistically where appropriate.

One male dog in the 100 ppm group died of acute pneumonia. There were no other deaths, and there were no treatment-related signs of toxicity, changes in feed consumption, changes in body weight gain or ophthalmoscopic findings. Haematological changes in the dogs at 1000 ppm group included increased MCV in males in week 13 (12%; $P < 0.01$) and in females in week 26 (3.5%; $P < 0.05$) and decreased MCHC in males at week 13 (3.5%; $P < 0.01$) (Table 13). The other changes either were not statistically significant or were inconsistent with respect to time interval, sex or dose.

Examination of femoral bone marrow smears at termination (Table 14) showed a shift in the granulopoietic:erythropoietic ratio towards erythropoiesis in two males and three females at 1000 ppm. The erythropoietic mitotic index was also increased in one female in this group.

No treatment-related changes in blood chemistry or urine analysis parameters were observed. The absolute and relative organ weights were comparable in all groups, and there were no treatment-related gross pathological findings. Microscopically, there were increases in the iron content of the Kupffer cells and bone marrow in the 1000 ppm group (Table 15).

The NOAEL was identified as 100 ppm (equal 2.39 mg/kg bw per day), based on haematological changes, increased erythropoiesis and haemosiderin deposition in the liver and bone marrow in animals of both sexes at 1000 ppm (equal to 22.50 mg/kg bw per day).

The original study was not GLP compliant. However, subsequent analysis of the data was GLP compliant (von Sandersleben et al., 1974; Goburdhun & Greenough, 1990).

Table 13. Group mean values for selected haematological data at weeks 13 and 26

Dietary concentration (ppm)	RBC (10^3/mm^3)	Hct (%)	Hb (g/100 mL)	MCV (µm^3)	MCHC (%)	Retic (pg)
Week 13						
Males						
0	6.95	46	17.2	66.66	37.15	4
10	7.21	50	17.7	68.61	35.71	7**
40	7.04	48	17.5	68.74	36.19	6*
100	7.01	47	17.2	66.43	36.89	5
1 000	6.60	49	16.6	74.46**	33.89**	8***
Females						
0	6.42	44	16.8	67.90	38.65	4
10	6.28	45	16.1	72.17	35.58*	5
40	6.50	47	16.9	72.53	36.01*	6
100	6.56	47	16.6	72.00	35.09**	6
1 000	6.31	46	15.9	73.02	34.45**	6
Week 26						
Males						
0	6.70	49	17.9	72.78	36.58	8
10	7.05	51	18.0	71.76	35.58	7
40	7.18	49	18.3	68.75	37.07	7
100	6.65	47	17.2	71.03	36.36	6
1 000	6.63	48	17.1	71.97	35.94	10
Females						
0	6.51	48	17.3	73.06	36.44	7
10	6.70	48	17.7	71.61	36.82	8
40	7.01	50	18.8	71.54	37.57	8
100	6.47	47	17.2	73.02	36.26	11
1 000	6.32	48	16.9	75.59*	35.35	9

Hb: haemoglobin; Hct: haematocrit; MCHC: mean corpuscular haemoglobin concentration; MCV: mean corpuscular volume; ppm: parts per million; RBC: red blood cells; Retic: reticulocytes; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$

Source: von Sandersleben et al. (1974); Goburdhun & Greenough (1990)

(b) Dermal application

Groups of seven Fischer 344 rats (aged about 5 weeks, weighing around 245.6–290.4 g [males] or 159.7–189.1 g [females]) of each sex received technical-grade triforine (batch no. Ht8/91/1; purity 99.6%) in corn oil (3 mL/kg bw) on a shaved area of each animal's back at a dose of 0, 110, 350 or 1100 mg/kg bw per day, 5 days/week, for 3 weeks. After each application, the treated area was covered with gauze and bandage for 6 hours, then washed and dried.

Table 14. Group mean and individual values for selected bone marrow smears in control and high-dose groups

Dietary concentration (ppm)	Animal no.	M:E		Mit	
		Individual	Mean ± SD	Individual	Mean ± SD
Males					
0	1	1.9	1.4 ± 0.3	0.0	0.8 ± 0.7
	2	1.3		0.7	
	3	1.3		1.7	
	4	1.2		0.6	
1 000	33	1.4	1.2 ± 0.2	0.7	1.1 ± 0.3
	34	1.0		1.2	
	35	1.0		1.2	
	36	1.3		1.3	
Females					
0	5	1.5	1.5 ± 0.0	0.7	0.9 ± 0.3
	6	1.5		1.4	
	7	1.5		0.7	
	8	1.4		0.7	
1 000	37	1.1	0.8 ± 0.2***	0.6	1.7 ± 1.4
	38	0.9		1.0	
	39	0.7		3.8	
	40	0.6		1.3	

M:E: myeloid:erythroid ratio; Mit: erythrocytic mitotic cells; ppm: parts per million; SD: standard deviation; ***: $P \leq 0.001$

Source: von Sandersleben et al. (1974); Goburdhun & Greenough (1990)

No deaths, clinical signs or treatment-related dermal changes were observed, and there were no effects on feed consumption or body weight gain attributable to the treatment. All animals, including the controls, lost some weight, particularly during the first week; this response was attributed to the bandaging procedure. No significant treatment-related variations in organ weights or haematological end-points were seen, and there were no gross or microscopic pathological findings. Female rats at 350 and 1100 mg/kg bw per day showed statistically significant increases in serum cholesterol (13% and 22%, respectively), triglyceride (32% and 40%, respectively) and bilirubin levels (27% and 13%, respectively). A 25% decrease in serum alkaline phosphatase activity was seen in females at 1100 mg/kg bw per day. In males, serum alkaline phosphatase activity was reduced by 15% at 350 mg/kg bw per day and by 21% at 1100 mg/kg bw per day. In rats at 1100 mg/kg bw per day, total serum protein level was increased by 5.7% and serum albumin level by 3.8%. As the changes in bilirubin, cholesterol and triglyceride levels were not accompanied by histopathological changes in the liver and were confined to a single sex, their biological significance is unclear. The increases in alkaline phosphatase activity may reflect a toxicological effect, but the decreases observed in this study are not usually considered to be toxicologically significant.

The NOAEL was 1100 mg/kg bw per day, the highest dose tested, as no toxicity was observed. The study was GLP compliant, and a QA statement was attached (Fokkema, 1992).

Table 15. The incidences of Kupffer cell and bone marrow siderosis in histopathological examination[a]

Dietary concentration (ppm)	Kupffer cell siderosis					Bone marrow siderosis				
	No. of animals	Incidence at grade				No. of animals	Incidence at grade			
		0	+	++	+++		0	+	++	+++
Males										
0	4	3	1	0	0	4	0	1	2	1
10	4	0	2	2	0	4	0	0	3	1
40	4	0	2	0	2	3	1	1	0	1
100	2	1	1	0	0	3	0	1	1	1
1 000	4	0	1	2	1	4	1	0	2	1
Females										
0	4	0	0	3	1	4	2	1	0	1
10	4	0	3	1	0	4	1	1	1	1
40	4	0	2	2	0	4	2	1	0	1
100	4	2	1	1	0	3	0	1	1	1
1 000	4	0	0	1	3	4	0	1	2	1

ppm: parts per million

[a] Grade: 0: none; +: slight; ++: moderate; +++: severe. The numerical values mean the number of animals with applicable histopathological finding and grade.

Source: von Sandersleben et al. (1974); Goburdhun & Greenough (1990)

In another study, groups of 10 male and 10 female Sprague-Dawley rats received topical applications of a 0%, 0.5% or 1.5% aqueous dilution of a 20% triforine emulsion (mean achieved triforine doses of 0, 10 or 30 mg/kg bw per day) on shaved, intact or abraded skin for 4 hours/day daily under occluded conditions for 21 consecutive days. Five males and five females per group were observed for an additional 21 days after the end of the treatment period (recovery).

Very slight reddening and swelling were seen at the site of application in test and control animals. These symptoms reached a maximum degree of 1 and subsided within 30–60 minutes and were most probably associated with the hyperthermia occurring as a result of the covering. There were no deaths and no treatment-related general clinical signs. There was no effect of treatment on body weight, feed and water consumption, haematology, clinical chemistry, urine analysis, eyes, teeth or organ weights in either sex of any group. There were no macroscopic or microscopic findings.

A NOAEL of 30 mg/kg bw per day was identified, based on the absence of adverse findings at the highest dose tested (Leuschner et al., 1972).

2.3 Long-term studies of toxicity and carcinogenicity

Mice

Triforine (lot no. T 3/70; purity 96.6%) was administered to NMRI-EMD-SPF mice (4–5 weeks old, weighing about 24 g [males, mean] or 21 g [females, mean] at the initiation of the study) at a dietary concentration of 0, 30, 150 or 750 ppm (equal to 0, 4.7, 24 and 124 mg/kg bw per day for males and 0, 5.6, 28 and 142 mg/kg bw per day for females, respectively) for 81 weeks. The group mean dose values were not available in the reports but were calculated by Sumitomo Corporation for submission in Japan (to JMAFF).

Morbidity/mortality, clinical signs and behaviour were examined once or twice a day. Body weights were measured once a week during weeks 1–12, once fortnightly during weeks 13–52 and once a month thereafter. Feed consumption was determined each week throughout the study period. After 81 weeks of treatment, all surviving animals were killed and necropsied. Premature decedents were also necropsied. The following organs from all animals were preserved, processed and stained with haematoxylin and eosin, and histological evaluations were performed: heart, lungs, liver with gallbladder, spleen, kidneys with adrenals, gonads, urinary bladder, brain, all macroscopically visible tumours and organs suspected of having a tumour. The heart, lungs, liver, kidney and spleen were additionally stained using Masson's method and Fat Red 7B (except for lungs). Statistical analyses were performed with a Fisher's exact probability test for mortality and Student's *t*-test for body weight and feed consumption.

Cumulative mortality noted at week 81 for the 0 (control), 30, 150 and 750 ppm groups was 32.5%, 20.0%, 35.0% and 50.0% for males and 30.0%, 40.0%, 26.3% and 32.5% for females, respectively. Longevity, clinical symptoms, body weights and feed consumption were not adversely affected by treatment at any dose.

The total number of tumour-bearing animals and the number of benign and malignant neoplasms were not adversely affected by treatment at any dose. The nature and incidence of all individual tumour types were comparable in treated and control groups, with the exception of the incidence of leukaemias in the male group treated at 750 ppm, which was substantially lower than the control incidence.

In view of the above, the NOAEL for general toxicity and carcinogenicity, under the study conditions, was identified as 750 ppm (equal to 124 mg/kg bw per day), the highest dose tested.

The study was not adequate to evaluate carcinogenicity. The study was not GLP compliant (Hofmann et al., 1975).

Another mouse study was initially designed for a period of 78 weeks. However, the dosing period was extended to 105 weeks due to high survival after 78 weeks. Four groups of 50 mice (Crl: CD-1 (ICR) BR) of each sex were given triforine (lot no. 2764; purity 99.1%) in the diet at a concentration of 0, 70, 700 or 7000 ppm (equal to mean achieved doses of 0, 11.4, 117 and 1204 mg/kg bw per day for males and 0, 15.9, 161 and 1570 mg/kg bw per day for females, respectively) for 105 weeks. The animals were approximately 6 weeks old and weighed about 21 ± 2 g (males) or 18 ± 3 g (females) on arrival.

Morbidity/mortality checks were performed twice daily, and clinical signs were examined every day. All animals were observed once each week for a detailed clinical examination and palpation. Body weights and feed consumption were recorded once every week until week 13 and once every 4 weeks thereafter. Water consumption was monitored by visual inspection throughout the treatment period. Blood smears were prepared for determination of a differential white blood cell count at weeks 52/53, 77/78 and 104. After 105 weeks of treatment, all surviving animals were killed and necropsied. Premature decedents were also necropsied. Organ weights of brain, kidneys, liver with gallbladder, spleen, testes and ovaries from 10 males and 10 females in each group were measured, and covariance analysis was performed. A full range of tissues was preserved and stained with haematoxylin and eosin, and histological evaluations were performed in all animals from control and high-dose groups and all premature decedents. The kidneys, liver (with gallbladder), lungs, spleen, colon and rectum were also examined from all other animals. Bone marrow smears were prepared for all animals at the terminal necropsies. Data were analysed statistically where appropriate.

There was no treatment-related effect on survival to 105 weeks at any dose level. There were no treatment-related clinical signs of toxicity or changes in feed and water consumption. Reduced body weight gain was noted in male animals at the end of the study by 11% and 16% at 700 and 7000 ppm, respectively. No significant changes in body weight gain were seen in females. No haematological changes were observed. Only the absolute and relative weights of the liver were increased by 21% ($P < 0.01$) in females at 7000 ppm. At necropsy, thickening or enlargement of the

large intestine (mainly colon and rectum) was observed in males that died before the end of the study in the groups receiving 700 or 7000 ppm, whereas none was seen in the control and low-dose groups. An increased incidence of lung masses at 7000 ppm was observed only in females. This increase was apparent in both terminal kill animals and premature decedents. Histologically, the incidences of enlargement in the colon and rectum and thickening in the rectum were significantly increased in 700 and 7000 ppm males, and the incidence of thickening of the colon was significantly increased in 7000 ppm males. These changes occurred mainly in premature decedents and were considered to be the likely cause of death. Also, ulceration and inflammation in the colon and rectum were observed microscopically, predominantly in male mice that died before the end of the study after receiving 700 or 7000 ppm. The overall occurrence of these findings was none in male controls, 6% at 70 ppm, 16% at 700 ppm and 12% at 7000 ppm, whereas in females they were not significantly increased.

At 7000 ppm, higher incidences of liver and lung tumours were found (Table 16). In males, the incidences of hepatocellular adenoma and carcinoma were higher than control incidences, but not statistically significantly (Table 17). However, the incidence was within the historical control ranges of the performing laboratory and/or the animal supplier (Tables 18 and 19) and was therefore considered not to be related to treatment. There were no notable differences in the incidence of liver tumours in females.

In females at 7000 ppm, there was a marked and treatment-related increase in the incidence of benign alveolar/bronchiolar adenoma, and the incidence of carcinoma was slightly, but significantly, higher than in the controls. However, the etiology of this higher incidence of lung carcinoma was equivocal. The incidences of alveolar/bronchiolar adenoma and carcinoma in males at 7000 ppm were not significantly higher than control values, but the combined incidence of adenoma plus carcinoma was statistically significantly higher on a time-weighted analysis (Table 17). Comparison of the incidences with historical control data (Tables 18 and 19) suggested that lung tumours in males at 7000 ppm were unlikely to be related to treatment with triforine.

Table 16. Incidence of all types of neoplastic findings in liver and lung

	Incidence of finding							
	Males				**Females**			
Organ/lesion	**0 ppm**	**70 ppm**	**700 ppm**	**7 000 ppm**	**0 ppm**	**70 ppm**	**700 ppm**	**7 000 ppm**
All animals								
Liver: no. examined	50	50	50	50	49	50	50	50
Hepatocellular adenoma	6	9	5	11	2	1	1	2
Hepatocellular adenoma multiple	3	3	3	4	1	1	0	0
(Associated) hepatocellular adenoma multiple	0	1	1	4	0	0	0	0
(Associated) hepatocellular adenoma	0	1	0	0	0	0	0	0
Hepatocellular carcinoma	4	7	8	9	1	0	0	0
Metastasizing hepatocellular carcinoma	1	0	0	1	0	0	0	0
Lung: no. examined	50	50	50	50	50	50	50	49
Alveolar/bronchiolar adenoma multiple	4	1	5	3	1	2	1	1
Alveolar/bronchiolar adenoma	10	12	3	14	4	4	6	20***
(Associated) alveolar/bronchiolar adenoma multiple	0	0	0	0	0	1	0	0
(Associated) alveolar/bronchiolar adenoma	3	0	0	1	0	0	0	1

Table 16 (continued)

Organ/lesion	Incidence of finding							
	Males				Females			
	0 ppm	70 ppm	700 ppm	7 000 ppm	0 ppm	70 ppm	700 ppm	7 000 ppm
Alveolar/bronchiolar carcinoma	5	1	1	4	1	2	1	6
Metastasizing alveolar/bronchiolar carcinoma	0	1	3	3	0	0	0	0
Terminal kill								
Liver: no. examined	36	31	15	22	23	23	18	20
Hepatocellular adenoma	5	7	1	5	2	1	0	2
Hepatocellular adenoma multiple	3	3	2	4	0	1	0	0
(Associated) hepatocellular adenoma multiple	0	1	0	1	0	0	0	0
Hepatocellular carcinoma	2	4	2	5	1	0	0	0
Lung: no. examined	36	31	15	22	23	23	18	20
Alveolar/bronchiolar adenoma multiple	4	1	4	2	1	1	1	0
Alveolar/bronchiolar adenoma	9	8	0	7	1	1	2	11***
(Associated) alveolar/bronchiolar adenoma multiple	0	0	0	0	0	1	0	0
(Associated) alveolar/bronchiolar adenoma	1	0	0	1	0	0	0	0
Alveolar/bronchiolar carcinoma	2	1	0	3	1	2	0	1
Premature decedents								
Liver: no. examined	14	19	35	28	26	27	32	30
Hepatocellular adenoma	1	2	4	6	0	0	1	0
Hepatocellular adenoma multiple	0	0	1	0	1	0	0	0
(Associated) hepatocellular adenoma multiple	0	0	1	3	0	0	0	0
(Associated) hepatocellular adenoma	0	1	0	0	0	0	0	0
Hepatocellular carcinoma	2	3	6	4	0	0	0	0
Metastasizing hepatocellular carcinoma	1	0	0	1	0	0	0	0
Lung: no. examined	14	19	35	28	27	27	32	29
Alveolar/bronchiolar adenoma multiple	0	0	1	1	0	1	0	1
Alveolar/bronchiolar adenoma	1	4	3	7	3	3	4	9
(Associated) alveolar/bronchiolar adenoma	2	0	0	0	0	0	0	1
Alveolar/bronchiolar carcinoma	3	0	1	1	0	0	1	5
Metastasizing alveolar/bronchiolar carcinoma	0	1	3	3	0	0	0	0

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ (Fisher's exact probability test)

Source: Heath et al. (1992)

Table 17. Summary of primary adenoma and carcinoma in liver and lungs from all animals

Sex	Lesion	Incidence[a]			
		0 ppm	70 ppm	700 ppm	7 000 ppm
Liver					
Males	*No. examined*	*50*	*50*	*50*	*50*
	Hepatocellular adenoma	6 (12)	9 (18)	5 (10)	11 (22)
	Hepatocellular carcinoma	4 (8)	7 (14)	8 (16)	9 (18)
Lung					
Males	*No. examined*	*50*	*50*	*50*	*50*
	Alveolar/bronchiolar adenoma multiple	4 (8)	1 (2)	5 (10)	3 (6)
	Alveolar/bronchiolar adenoma	10 (2)	12 (24)	3 (6)	14 (28)
	(Associated) alveolar/bronchiolar adenoma multiple	0	0	0	0
	(Associated) alveolar/bronchiolar adenoma	3 (6)	0	0	1 (2)
	Alveolar/bronchiolar carcinoma	5 (10)	1 (2)	1 (2)	4 (8)
	Metastasizing alveolar/bronchiolar carcinoma	0	1 (2)	3 (6)	3 (6)
	Alveolar/bronchiolar adenoma + carcinoma	19 (38)	15 (30)	12 (24)	24^{+1} (48)
Females	*No. examined*	*50*	*50*	*50*	*49*
	Alveolar/bronchiolar adenoma multiple	1 (2)	2 (4)	1 (2)	1 (2)
	Alveolar/bronchiolar adenoma	4 (8)	4 (8)	6 (12)	20*** (41)
	(Associated) alveolar/bronchiolar adenoma multiple	0	1 (2)	0	0
	(Associated) alveolar/bronchiolar adenoma	0	0	0	1 (2)
	Alveolar/bronchiolar carcinoma	1 (2)	2 (4)	1 (2)	6^{+2} (12)
	Metastasizing alveolar/bronchiolar carcinoma	0	0	0	0
	Alveolar/bronchiolar adenoma + carcinoma	6 (12)	8 (16)	8 (16)	27^{+3} (55)

ppm: parts per million; ***: $P < 0.001$ (Fisher's exact probability test); $^{+1}$: $P = 0.035$; $^{+2}$: $P = 0.020$; $^{+3}$: $P = 0.001$ (time-adjusted analysis)

[a] The values in parentheses are percentages.

Source: Heath et al. (1992)

Table 18. Historical control data in IRI: CD-1 strain mice in 74- and 104-week carcinogenicity studies from 1986 to 1994

	Incidence[a]										
	104-week studies								78-week studies		
	1991–1993	**1989–1991**	**1989–1991**	**1991–1993**	**1991–1993**	**1991–1993**	**1989–1991**	**1988–1990**	**1993–1995**	**1993–1995**	**1994–1996**
Male: Hepatocellular neoplastic lesions in liver											
No. examined	*50*	*50*	*14*	*50*	*60*	*100*	*120*	*48*	*50*	*43*	*50*
Hepatocellular adenoma	6 (12)	8 (16)	1 (7.1)	6 (12.0)	15 (25.0)	25 (25.0)	14 (11.7)	10 (20.8)	8 (16.0)	11 (25.6)	7 (14.0)
Hepatocellular carcinoma	11 (22.0)	8 (16.0)	2 (14.3)	5 (10.0)	3 (5.0)	11 (11.0)	7 (5.8)	4 (8.3)	4 (8.0)	2 (4.7)	1 (2.0)
Male: Alveolar/bronchiolar neoplastic lesions in lung											
No. examined	*50*	*50*	*14*	*50*	*60*	*99*	*120*	*49*	*50*	*43*	*50*
Alveolar/bronchiolar adenoma multiple	0	0	0	0	0	0	0	0	0	1 (2.3)	0
Alveolar/bronchiolar adenoma	5 (10)	9 (18)	1 (7.1)	14 (28.0)	17 (28.3)	27 (27.3)	18 (15.0)	9 (18.4)	9 (18.0)	12 (27.9)	6 (12.0)
(Associated) alveolar/bronchiolar adenoma multiple	0	0	0	0	0	0	1 (0.8)	0	0	0	0
(Associated) alveolar/bronchiolar adenoma	0	3 (6.0)	2 (14.3)	0	8 (13.3)	3 (3.0)	2 (1.7)	0	0	0	0
Alveolar/bronchiolar carcinoma	8 (16.0)	10 (20.0)	3 (21.4)	8 (16.0)	12 (20.0)	9 (9.1)	10 (8.3)	6 (12.2)	3 (6.0)	0	0
Alveolar/bronchiolar carcinoma multiple	0	0	0	2 (4.0)	0	0	5 (4.2)	0	0	0	0
Alveolar/bronchiolar adenoma + carcinoma	13 (26.0)	22 (44.0)	6 (42.9)	24 (48.0)	37 (61.7)	39 (39.4)	36 (30.0)	15 (30.6)	12 (24.0)	13 (30.2)	6 (12.0)
Female: Alveolar/bronchiolar neoplastic lesions in lung											
No. examined	*49*	*50*	*19*	*50*	*60*	*100*	*120*	*50*	*50*	*32*	*50*
Alveolar/bronchiolar adenoma multiple	0	0	0	0	0	0	2 (1.7)	0	0	0	0
Alveolar/bronchiolar adenoma	4 (8.2)	7 (14.0)	3 (15.8)	9 (18.0)	9 (15.0)	11 (11.0)	12 (10.0)	9 (18.0)	4 (8.0)	1 (3.1)	6 (12.0)
(Associated) alveolar/bronchiolar adenoma multiple	0	0	0	0	0	0	0	0	1 (2.0)	0	0
(Associated) alveolar/bronchiolar adenoma	0	0	0	0	1 (1.7)	1 (1.7)	0	0	0	0	0
Alveolar/bronchiolar carcinoma	7 (14.3)	3 (6.0)	0	7 (14.0)	3 (5.0)	5 (5.0)	6 (5.0)	2 (4.0)	2 (4.0)	0	0

	Incidence[a]										
	104-week studies							78-week studies			
	1991–1993	1989–1991	1989–1991	1991–1993	1991–1993	1991–1993	1989–1991	1988–1990	1993–1995	1993–1995	1994–1996
Alveolar/bronchiolar carcinoma multiple	0	0	0	0	0	0	2 (1.7)	0	0	0	0
Alveolar/bronchiolar adenoma + carcinoma	11 (22.4)	10 (20.0)	3 (15.8)	16 (32)	13 (21.7)	17 (17.0)	22 (18.3)	11 (22.0)	7 (14.0)	1 (3.1)	6 (12.0)

IRI: Inveresk Research Internationa Limited

[a] The values in parentheses are percentages.

Source: Heath et al. (1992)

Table 19. Historical control data in Charles River: Crl:CD-1®BR strain mice in 24-month studies

		Control incidence[a] in study started in year:						
Sex	**Lesion**	**DZ[b]: 1990**	**CP: 1985**	**BX: 1981**	**DN: 1988**	**CX: 1983**	**DU: 1989**	**EG: 1989**
Hepatocellular neoplastic lesions in liver								
Males	*No. examined*	*49*	*50*	*52*	*48*	*72*	*50*	*50*
	Hepatocellular adenoma	2 (4.08)	9 (18.00)	6 (11.54)	10 (20.83)	27 (37.50)	9 (18.00)	9 (18.00)
	Hepatocellular carcinoma	8 (16.33)	1 (2.00)	9 (17.31)	4 (8.33)	12 (16.67)	7 (14.00)	5 (10.00)
Females	*No. examined*	*49*	*51*	*52*	*49*	*71*	*50*	*49*
	Hepatocellular adenoma	0 (0.0)	0 (0.0)	2 (3.85)	1 (2.04)	8 (11.27)	0 (0.0)	3 (6.12)
	Hepatocellular carcinoma	0 (0.0)	1 (1.96)	1 (1.92)	0 (0.0)	0 (0.0)	2 (4.00)	1 (2.40)
Alveolar/bronchiolar neoplastic lesions in lung								
Males	*No. examined*	*50*	*51*	*52*	*49*	*72*	*50*	*50*
	Alveolar/bronchiolar adenoma	9 (18.00)	7 (13.73)	12 (23.08)	9 (18.37)	7 (9.72)	11 (22.00)	14 (28.00)
	Alveolar/bronchiolar carcinoma	8 (16.00)	1 (1.96)	1 (1.92)	6 (12.24)	10 (13.89)	8 (16.00)	5 (10.00)
Females	*No. examined*	*50*	*51*	*52*	*49*	*71*	*50*	*50*
	Alveolar/bronchiolar adenoma	2 (4.00)	5 (9.80)	5 (9.62)	9 (18.37)	5 (7.04)	4 (8.00)	5 (10.00)
	Alveolar/bronchiolar carcinoma	5 (10.00)	3 (5.88)	7 (13.46)	2 (4.08)	7 (9.86)	5 (10.00)	1 (2.00)

[a] The values in parentheses are percentages.
[b] Study code.

Source: Heath et al. (1992)

There was no other excessive incidence of tumours in any other tissue or organ.

The NOAEL for systemic toxicity was 70 ppm (equal to 11.4 mg/kg bw per day), on the basis of a slight decrease in body weight gain and changes in the large intestine of males fed 700 ppm (equal to 117 mg/kg bw per day) and above. The NOAEL for carcinogenicity was 700 ppm in females (equal to 161 mg/kg bw per day), on the basis of the increased incidence of lung tumours (predominantly adenomas) in females at 7000 ppm (equal to 1570 mg/kg bw per day).

The study was GLP compliant, and a QA statement was attached (Heath et al., 1992).

Rats

In a chronic toxicity study, two groups of 50 Wistar (Chbb: THOM (SPF)) rats of each sex (control and high dose) and three groups of 35 rats of each sex for low, low-intermediate and high-intermediate dose groups were given triforine (lot no. T3/70; purity 96.6%) in the diet at a concentration of 0, 25, 125, 625 or 3125 ppm (equal to mean achieved doses of 0, 1.2, 6.2, 31.2 and 158.7 mg/kg bw per day for males and 0, 1.5, 7.8, 38.6 and 195.4 mg/kg bw per day for females, respectively) for 2 years. The animals were 8 weeks old and weighed about 210 g (males) or about 165 g (females) at the initiation of the study. The animals were fed the diets ad libitum for at least 104 weeks.

The animals were observed once daily for viability, physical condition and behaviour. Body weights were recorded weekly for 26 weeks and every 2 weeks thereafter. Feed consumption was recorded weekly during the treatment period, and water consumption was measured at weeks 52 and 104. Blood samples were taken for haematological examination and clinical chemistry during weeks 0, 6, 13, 26, 52, 78 and 104 from 20 male and 20 female rats per group. Urine was analysed before and at the end of the study in 10 animals of each sex from the control and high-dose groups. All animals were examined postmortem. Complete histopathological examinations were performed at the end of the study on 20 rats of each sex from the control and high-dose groups, on 15 rats of each sex from the other groups, on rats that died before the end of the study and on all tumour-bearing rats.

Mortality, clinical signs, body weight development and clinical chemistry and urine analysis parameters were unaffected by treatment at all dose levels. Temporary reductions in the number of erythrocytes, haemoglobin concentrations and haematocrit values in males and increased numbers of reticulocytes in both sexes (39% in males, 29% in females) were seen in rats at 3125 ppm during week 6, the degree of change being more pronounced among male rats (Table 20). However, there was no evidence of anaemia in either sex at 104 weeks.

There were no significant differences in organ weights at any dose level. Haemosiderosis was observed in the spleens of both control and treated rats, but the incidence and degree of severity did not differ significantly between the groups. Sinuses of the adrenals of females in all groups showed cavernous dilatation, and some were thrombosed. These changes occurred more frequently in treated than in control animals, but they are common findings in Wistar rats. All other non-neoplastic histopathological observations and all neoplastic alterations were considered to be etiologically spontaneous in origin. No significant difference in tumour incidences between the groups was found.

A NOAEL for systemic toxicity was identified as 625 ppm (equal to 31.2 mg/kg bw per day), based on the temporary slight anaemia observed at 3125 ppm (equal to 158.7 mg/kg bw per day). A NOAEL for carcinogenicity was identified as 3125 ppm (equal to 158.7 mg/kg bw per day), the highest dose tested, as no histopathological alterations, including neoplastic alterations, were observed at any dose.

The study was not GLP compliant (Hill, 1974; Stötzer & Notman, 1978).

Triforine (lot no. 2764; purity 99.1 ± 0.9%) was administered to four groups of 70 Sprague-Dawley (Crl:CD(SD) BRf) rats (aged approximately 7 weeks, weighing around 216–225 g [males] or 141–147 g [females]) of each sex in the diet at a concentration of 0, 200, 2000 or 20 000 ppm (equal to 0, 10.3, 101 and 1038 mg/kg bw per day for males and 0, 13.1, 136 and 1436 mg/kg bw per day in females, respectively) for 2 years. Twenty rats of each sex per group were killed after 52 weeks, and

Table 20. Selected haematological investigation data

Dietary concentration (ppm)	RBC (10^3/mm^3)	Hct (%)	Hb (g/100 mL)	MCV (µm^3)	MCH (pg)	MCHC (%/100 mL)	Retic (‰)	Platelets (10^3/mm^3)
Week 6								
Males								
0	8.073	48.90	17.15	60.59	21.26	35.10	21.4	670.5
25	7.823	43.40	16.93	61.93*	21.65	34.98	24.9**	672.7
125	7.901	49.13	16.70	62.21	21.14	34.00	24.4*	662.0
625	7.913	48.53	17.07	61.37	21.59	35.22	28.3**	670.7
3 125	7.327**	47.00	16.04**	64.27**	21.95	34.15	29.9**	663.5
Females								
0	7.483	46.25	15.66	61.83	20.94	33.88	22.8	658.0
25	7.423	46.00	15.34	62.01	20.67	33.35	26.1**	664.0
125	7.589	45.73	15.85	60.28	20.89	34.65*	25.5*	684.7
625	7.395	45.27	15.31	61.26	20.72	33.84	26.1**	667.3
3 125	7.327	45.55	15.53	62.18	21.20	34.10	29.4**	657.5
Week 13								
Males								
0	8.901	49.21	17.41	55.34	19.58	35.38	21.2	678.4
25	8.958	48.80	17.40	54.51	19.43	35.66	21.1	676.0
125	8.627	48.13	17.00	55.85	19.73	35.34	22.4	682.0
625	8.899	49.00	17.09	55.10	19.21	34.88	23.5	678.7
3 125	8.648	48.95	17.11	56.69	19.81	34.97	21.9	679.0
Females								
0	8.003	47.80	16.51	59.78	20.64	34.54	20.0	642.0
25	8.081	47.87	16.76	59.29	20.76	35.01	23.1**	659.3
125	8.045	47.40	16.39	58.98	20.38	34.57	26.0**	666.0
625	7.827	46.67	15.81*	59.63	20.21	33.89	24.3**	664.0
3 125	7.737	47.45	16.51	61.42	21.36*	34.79	23.9**	667.0
Week 26								
Males								
0	8.971	50.37	17.61	56.18	19.64	34.97	18.4	777.9
25	8.799	49.50	17.34	56.25	19.71	35.07	19.2	790.7
125	9.085	51.20	17.91	56.39	19.72	34.98	19.4	792.0
625	8.789	50.80	17.71	51.81	20.16*	34.88	18.9	750.0
3 125	8.701	49.00	17.20	56.35	19.78	35.10	20.3*	804.0
Females								
0	7.685	47.10	17.04	61.29	22.20	36.22	16.9	678.0
25	7.721	48.87	17.07	60.73	22.13	36.44	18.1	672.0

Table 20 (continued)

Dietary concentration (ppm)	RBC (10^3/mm^3)	Hct (%)	Hb (g/100 mL)	MCV (µm^3)	MCH (pg)	MCHC (%/100 mL)	Retic (‰)	Platelets (10^3/mm^3)
125	7.781	48.13	17.37	61.88	22.34	36.10	17.5	728.0
625	7.737	47.21	16.73	61.03	21.64	35.47	19.9**	715.0
3 125	7.739	46.90	16.46**	60.61	21.28**	35.12	22.5**	703.5
Week 52								
Males								
0	8.834	51.17	16.79	57.94	19.00	32.80	17.6	701.7
25	8.563	49.14*	16.23	57.38	18.94	33.02	18.0	727.9
125	8.620	50.20	16.45	58.25	19.09	32.78	19.7*	714.7
625	8.669	50.69	16.61	58.49	19.16	32.76	18.6	708.5
3 125	8.580	50.00	16.03	58.32	18.69	32.05	21.4**	701.5
Females								
0	8.158	50.25	16.00	61.61	19.62	31.85	20.3	684.0
25	8.117	49.40	16.06	60.85	19.79	32.53	20.7	708.0
125	8.053	49.20	16.15	61.08	20.06*	32.84**	20.8	695.3
625	7.889*	48.21**	15.81	61.12	20.05*	32.81**	22.1	696.4
3 125	7.703**	47.75**	15.46**	62.02	20.09*	32.39	20.4	694.5
Week 78								
Males								
0	8.302	49.76	16.32	60.09	19.69	32.78	19.7	816.5
25	8.224	48.38	15.96	58.99	19.46	33.00	22.0	735.4*
125	8.111	48.67	15.85	61.53	19.62	31.90**	23.9*	783.3
625	8.247	49.58	15.71	60.16	19.06	31.68**	18.3	786.7
3 125	7.900	48.70	15.62*	61.76	19.82	32.09*	19.8	808.5
Females								
0	7.003	47.45	15.23	67.98	21.80	32.10	20.5	758.5
25	7.281	46.21	15.37	63.63	21.18	33.27	23.9	709.3
125	7.233	47.07	15.22	65.95	21.28	32.36	21.6	706.4
625	6.789	46.14	14.64	68.95	21.85	31.74	20.6	715.7
3 125	6.757	44.63**	14.25*	67.52	21.52	31.92	23.6	748.9
Week 104								
Males								
0	7.886	49.92	15.66	63.70	19.96	31.36	25.7	740.8
25	7.732	49.25	15.21	63.71	19.67	30.88	22.9	785.8
125	8.122	50.67	15.92	62.45	19.63	31.43	24.4	781.7
625	7.865	50.91	15.45	65.19	19.68	30.33	23.3	832.7
3 125	7.875	47.58*	15.06	60.69	19.19	31.64	24.8	785.8

Dietary concentration (ppm)	RBC (10^3/mm^3)	Hct (%)	Hb (g/100 mL)	MCV (µm^3)	MCH (pg)	MCHC (%/100 mL)	Retic (‰)	Platelets (10^3/mm^3)
Females								
0	7.035	44.28	14.06	63.66	20.10	31.72	24.1	691.7
25	6.823	42.25	13.52	62.25	19.83	31.91	25.6	733.3
125	7.468	45.00	14.97	60.56	20.09	33.35	22.1	762.5
625	6.757	43.13	13.92	63.88	20.60	32.30	22.5	733.8
3 125	6.675	41.53	13.29	62.36	19.88	31.90	23.6	766.7

Hb: haemoglobin; Hct: haematocrit; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; MCV: mean corpuscular volume; ppm: parts per million; RBC: red blood cells; Retic: reticulocytes; *: $P < 0.05$; **: $P < 0.01$

Source: Hill (1974); Stötzer & Notman (1978)

the remaining animals were killed after 104 weeks of treatment. Blood and urine samples were collected for analysis from 10 animals of each sex per group at weeks 26 (females), 28 (males), 52 and 104. The eyes of all control and high-dose animals were examined before treatment started and during week 51, using an indirect ophthalmoscope. At necropsy, organ weights were recorded from all animals killed after 52 weeks and from 10 animals of each sex per group killed at 104 weeks. Histopathological examinations were performed on all tissues from all control and high-dose animals killed after 52 or 104 weeks, on all premature decedents and on gross lesions from all animals. Tissues with histological alterations in the high-dose group were also examined in all animals.

Survival to 104 weeks was unaffected by treatment with triforine at all dose levels. There were no treatment-related clinical signs of toxicity throughout the treatment period, but reduced body weight gain occurred in males treated at 2000 or 20 000 ppm for 104 weeks (−7%) and in females treated at 20 000 ppm for 52 weeks (−21%) or 104 weeks (−3%). There were no significant differences in the mean feed or water consumption at any dose level. There were no ocular changes at 51 weeks in animals treated at 20 000 ppm and no effects at any dose level on the urine analysis profiles throughout the study.

Treatment-related effects on the haematological profile were confined to transient signs of minimal anaemia in both sexes at 20 000 ppm in weeks 28/26 only (Table 21).

There were no toxicologically relevant changes in the clinical chemistry profile in either sex at any dose level. Minor, but statistically significant, changes in blood chemistry occurred in the group treated at 20 000 ppm, including slightly raised concentrations of plasma sodium and calcium in males at week 28, slightly raised calcium and cholesterol levels in females at week 104, raised total protein levels in males at week 104 and a slightly lower concentration of glucose in males at week 52. Females at 2000 ppm also had raised calcium levels at week 104. However, these findings were considered not to be toxicologically significant because they were not correlated with any adverse in-life or histological findings.

There were no treatment-related gross pathological findings at any dose level either in decedents or in animals killed after 52 or 104 weeks of treatment.

Treatment-related effects on organ weights comprised increased absolute and relative spleen weights in females treated at 2000 and 20 000 ppm for 52 weeks and increased absolute and/or relative liver weights in females treated at 2000 and 20 000 ppm after 104 weeks and in both sexes treated at 20 000 ppm after 52 weeks. Increased liver weight in females in the 200 and 2000 ppm groups at week 104 was considered not to represent an adverse effect of treatment because the changes were not detected in both absolute and relative weights, there were no toxicologically significant findings in clinical pathology, necropsy or histological examinations to correlate with this observation, and the terminal body weights in both groups were higher than control values.

Table 21. Group mean values for selected haematological data

Dietary concentration (ppm)	RBC ($\times 10^{12}$/L)	Hct (L/L)	Hb (g/100 mL)	MCV (fL)	MCHC (g/100 mL)
52-week groups: week 28 (male)/26 (female)					
Males					
0	7.51	0.414	15.6	55	38.0
200	7.45	0.406	15.3	55	37.8
2 000	7.19	0.399	15.0*	56	37.7
20 000	7.24	0.399	14.8**	56	37.2**
Females					
0	7.07	0.392	14.8	55	37.9
200	6.79	0.381	14.3	56	37.8
2 000	6.84	0.388	14.4	57	37.3*
20 000	6.57**	0.380	14.0	58**	37.0***
52-week groups: week 51					
Males					
0	7.64	0.417	16.1	55	38.2
200	7.37	0.408	15.5*	55	37.8
2 000	7.36	0.404	15.4*	55	37.9
20 000	7.35	0.402	15.2**	55	37.7
Females					
0	6.77	0.391	14.7	57	38.0
200	6.76	0.388	14.5	57	37.8
2 000	6.66	0.393	14.7	59	37.9
20 000	6.58	0.387	14.3	59	37.4
104-week groups: week 103					
Males					
0	6.23	0.341	13.0	55	38.3
200	6.47	0.374	14.1	59	38.0
2 000	6.36	0.356	13.5	57	38.5
20 000	6.81	0.377	14.2	55	38.1
Females					
0	6.33	0.362	13.7	58	38.3
200	6.36	0.367	13.8	58	38.0
2 000	6.35	0.365	13.8	58	38.2
20 000	5.89	0.342	12.8	59	37.9

Hb: haemoglobin; Hct: haematocrit; MCHC: mean corpuscular haemoglobin concentration; MCV: mean corpuscular volume; ppm: parts per million; RBC: red blood cells; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$

Source: Perry, Mulhern & Finch (1992)

Furthermore, in a previously conducted 2-year chronic toxicity and carcinogenicity study in rats (Hill, 1974; Stötzer & Notman, 1978), organ weight changes were not observed up to 195.4 mg/kg bw per day, which was higher than the actual dose level of 2000 ppm of the current study (101 and 136 mg/kg bw per day for males and females, respectively). Therefore, changes in organ weight were considered not to be treatment related.

Males at 20 000 ppm showed increased relative thymus weight at 104 weeks, but there was no histological correlate. Increased kidney weights occurred in the 20 000 ppm group of females at 52 weeks, but without histological correlate, and not at 104 weeks. Adrenal weights were reduced in all treated male groups, but were increased in females. These findings were considered incidental to treatment with triforine.

Microscopic examination showed an increase in the incidences and/or severity of Kupffer cell pigmentation/pigmented macrophages, pale cell foci and bile duct hyperplasia in liver and haemosiderin deposits in spleen in one or both sexes treated at 20 000 ppm for 52 weeks (Table 22). Although females at 2000 ppm also showed an increase in the incidence of Kupffer cell pigmentation/pigmented macrophages, there was no statistical significance. After 104 weeks of treatment, the hepatic changes seen at 52 weeks were no longer apparent, but an increased incidence of haemosiderin deposits in the spleen occurred in females at 2000 or 20 000 ppm. The incidence of focal alveolitis in lungs was significantly increased in both males and females at 20 000 ppm. The lesion is characterized by accumulations of alveolar macrophages, thickening of the alveolar wall and low-grade chronic inflammatory cell infiltrates. The findings in the liver and spleen may be related to changes in the haemoglobin concentration and associated haematological parameters. No other treatment-related or toxicologically significant non-neoplastic findings were observed.

The total number of tumour-bearing animals and the numbers of benign and malignant neoplasms in the treated groups were comparable with the control incidences in both sexes (Table 23).

A treatment-related increase in incidence, number, nature or type of tumour was not apparent at any dose level in relevant animals.

In view of the above, the NOAEL for carcinogenic effects was identified as 20 000 ppm (equal to 1038 mg/kg bw per day), the highest dose tested, based on no significant increase in tumour incidence observed at any dose level employed in the study.

The NOAEL for systemic effects was identified as 200 ppm (equal to 10.3 mg/kg bw per day), based on transient signs of slight anaemia, increased spleen weights and increased haemosiderin deposition in spleen at 2000 ppm (equal to 101 mg/kg bw per day) and above.

The study was GLP compliant, and a QA statement was attached (Perry, Mulhern & Finch, 1992).

2.4 Genotoxicity

Triforine was tested for genotoxicity in seven in vitro and three in vivo studies. All studies complied with GLP, and QA statements were attached. On the basis of these studies, it is concluded that triforine is unlikely to be genotoxic. A summary of these studies is given in Table 24.

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

In a two-generation reproduction study, groups of 28 male and 28 female Crl:CD (SD) BR F_0 rats (aged about 6 weeks, weighing approximately 68–140 g on arrival) and groups of 24 F_1 generation rats of each sex were given triforine (lot no. 2764; purity 99.1 ± 0.9%) in the diet at a concentration of 0, 500, 3000 or 20 000 ppm for 10/11 weeks before mating and through lactation until weaning of the F_1 or F_2 offspring (24 days of lactation). The study was completed after weaning of the F_2 rats. The dietary concentrations of 0, 500, 3000 and 20 000 ppm were equal to 0, 39.4, 252.5 and 1768.3 mg/kg bw per day for males and 0, 54.6, 323.3 and 2209.2 mg/kg bw per day for females

Table 22. Incidence of treatment-related non-neoplastic alterations

		Incidence of finding							
		Males				Females			
Organ/lesion	Grade[a]	0 ppm	200 ppm	2 000 ppm	20 000 ppm	0 ppm	200 ppm	2 000 ppm	20 000 ppm
52-week groups									
Liver: no. examined		*20*	*20*	*19*	*20*	*20*	*20*	*20*	*20*
Pale cell focus	Total	2	5	2	9*	1	4	0	2
	+/–	1	5	1	5	1	3	0	2
	+	1	0	1	4	0	1	0	0
Kupffer cell pigmentation/pigmented macrophages	Total	6	10	5	11	3	3	9	10*
	+/–	6	7	3	5	3	3	5	8
	+	0	3	1	5*	0	0	4	2
	++	0	0	1	1	0	0	0	0
Bile duct hyperplasia	Total	4	7	8	4	2	4	5	8
	+/–	3	6	6	1	2	4	3	3
	+	1	1	2	2	0	0	2	5*
	++	0	0	0	1	0	0	0	0
Spleen: no. examined		*20*	*19*	*19*	*20*	*20*	*20*	*20*	*20*
Increased brown pigment deposit[b]	Total	4	8	11**	18***	18	16	14	17
	+	3	8*	11**	14***	14	12	11	3**
	++	1	0	0	4	4	4	3	13**
	+++	0	0	0	0	0	0	0	1
104-week groups: All animals									
Lung: no. examined		*50*	*50*	*50*	*50*	*49*	*50*	*50*	*50*
Focal alveolitis	Total	12	6	8	24*	6	7	13	17*
	+/–	1	0	1	0	0	1	2	1
	+	6	5	6	19**	3	4	8	14**
	++	4	1	1	5	2	2	3	1
	+++	1	0	0	0	1	0	0	1
Spleen: no. examined		*50*	*50*	*50*	*50*	*49*	*50*	*50*	*50*
Increased brown pigment deposit(s)	Total	15	21	27*	22	28	30	41**	43**
	+/–	0	5	4	0	0	3	1	0
	+	8	9	10	12	17	8*	24	18
	++	7	7	11	10	10	18	14	23*
	+++	0	0	2	0	1	1	2	2
104-week groups: Terminal kill at 104 weeks									
Lung: no. examined		*25*	*26*	*31*	*31*	*28*	*30*	*29*	*27*
Focal alveolitis	Total	8	5	7	18	5	5	8	10
	+/–	1	0	1	0	0	1	2	0

		Incidence of finding							
		Males				Females			
Organ/lesion	Grade[a]	0 ppm	200 ppm	2 000 ppm	20 000 ppm	0 ppm	200 ppm	2 000 ppm	20 000 ppm
	+	4	4	6	14*	3	2	5	8
	++	3	1	0	4	2	2	1	1
	+++	0	0	0	0	0	0	0	1
Spleen: no. examined		*25*	*26*	*31*	*31*	*28*	*30*	*29*	*27*
Increased brown pigment deposit(s)	Total	5	9	10	11	11	17	24**	27***
	+/–	0	5	4	0	0	3	1	0
	+	3	3	4	9	10	6	16	14
	++	2	1	2	2	1	8*	6	13***
	+++	0	0	0	0	0	0	1	0
104-week groups: Premature decedents									
Lung: no. examined		*25*	*24*	*19*	*19*	*21*	*20*	*21*	*23*
Focal alveolitis	Total	4	1	1	6	1	2	5	7*
	+/–	0	0	0	0	0	0	0	1
	+	2	1	0	5	0	2	3	6*
	++	1	0	1	1	0	0	2	0
	+++	1	0	0	0	1	0	0	0
Spleen: no. examined		*25*	*24*	*19*	*19*	*21*	*2*	*21*	*23*
Increased brown pigment deposit(s)	Total	10	12	17**	11	17	13	17	16
	+	5	6	6	3	7	2	8	4
	++	5	6	9	8	9	10	8	10
	+++	0	0	2	0	1	1	1	2

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$

[a] Grade: (−): none; (+): slight; (++): moderate; (+++): severe.

[b] This finding in 52-week groups was described as "increased haemosiderin" in the report.

Source: Perry, Mulhern & Finch (1992)

Table 23. Incidence of selected neoplastic findings

		Incidence of tumour							
		Males				Females			
Organ/lesion		0 ppm	200 ppm	2 000 ppm	20 000 ppm	0 ppm	200 ppm	2 000 ppm	20 000 ppm
All animals									
Pituitary: no. examined		*49*	*24*	*19*	*50*	*49*	*20*	*21*	*50*
Adenoma (B)		33	17	16	31	36	19	16	37
Thyroid: no. examined		*49*	*23*	*18*	*49*	*49*	*20*	*21*	*50*
C-cell adenoma (B)		5	1	2	6	2	3	8	4

Table 23 (continued)

Organ/lesion	Incidence of tumour							
	Males				Females			
	0 ppm	200 ppm	2 000 ppm	20 000 ppm	0 ppm	200 ppm	2 000 ppm	20 000 ppm
Terminal kill at 104 weeks								
Pituitary: no. examined	*25*	–	–	*31*	*28*	–	–	*27*
Adenoma (B)	16	–	–	19	22	–	–	21
Thyroid: no. examined	*25*	–	–	*31*	*28*	–	–	*27*
C-cell adenoma (B)	5	–	–	5	2	–	–	4
Premature decedents								
Pituitary: no. examined	*24*	*24*	*19*	*19*	*21*	*20*	*21*	*23*
Adenoma (B)	17	17	16	12	14	19*	16	16
Thyroid: no. examined	*24*	*23*	*18*	*18*	*21*	*20*	*21*	*23*
C-cell adenoma (B)	0	1	2	1	0	3	8**	0

–: not examined; (B): benign neoplasms; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$

Source: Perry, Mulhern & Finch (1992)

Table 24. Summary of genotoxicity studies on triforine

Study	Strain/species	Concentration/dose	Purity (%)	Result	Reference
In vitro					
Bacterial reverse mutation assay (Ames test)	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537, TA1538	10, 50, 250, 1 250, 2 500 and 5 000 µg/plate	99.9	Negative (±S9)	Kramer (1985)
Bacterial reverse mutation assay (Ames test)	*S. typhimurium* TA98, TA100	0.002, 0.02, 0.2, 2.0, 10.0, 20.0, 100.0 and 200.0 µmol/L	99.2	Negative (±S9)	Obermeier (1977)
Reverse mutation	*S. typhimurium* TA100, TA1535	100 µg/plate	NR	Negative (±S9)	Röhrborn (1977)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538; *Escherichia coli* WP2hcr	5 000 µg/plate	NR	Negative (±S9)	Moriya et al. (1983)
Chromosomal aberration assay in mammalian cells	Chinese hamster cell line V79	7 h: 50 µg/mL (–S9) 7 h: 40 µg/mL (+S9) 18 h: 5–50 µg/mL (–S9) 18 h: 4–40 µg/mL (+S9) 28 h: 50 µg/mL (–S9) 28 h: 40 µg/mL (+S9)	99.9	Negative (±S9)	Miltenburger (1985a)

Study	Strain/species	Concentration/dose	Purity (%)	Result	Reference
Chromosome studies using cultured CHO cells; cytogenetics	CHO cells	24 h: 23.44–300 µg/mL (−S9)	99.8	Negative	Brooks & Wiggins (1994)
		48 h: 75–150 µg/mL (−S9)		Positive	
		3 h (21 h recovery): 18.75–200 µg/mL (+S9)		Positive	
		3 h (45 h recovery): 200 µg/mL (+S9)		Positive	
Chromosomal aberration test	Cultured CHL cells	24 h: 65.0–520 µg/mL (−S9)	99.6	Positive[a]	Nakajima (1992)
		24 h: 51.8–400 µg/mL (−S9)		Positive[a]	
		48 h: 65.0–520 µg/mL (−S9)		Positive	
		6 h (18 h recovery): 65.0–520 µg/mL (+S9)		Negative	
HGPRT assay	Chinese hamster cell line V79	5–50 µg/mL (−S9) 5–50 µg/mL (+S9)	99.8	Negative	Miltenburger (1984)
HGPRT assay	CHO cells	25, 50, 75, 100, 150 and 200 µg/mL	99.8	Negative	Adams et al. (1993)
Unscheduled DNA synthesis	Male rat hepatocytes	0.5, 1, 10, 25 and 50 µg/mL	99.8	Negative	Miltenburger (1985b)
DNA repair test	Rat hepatocytes	8 concentrations ranging from 0.063 to 200 µg/mL	99.8	Negative	Proudlock et al. (1993)
In vivo					
Micronucleus test	NMRI KFM mouse	5 000 mg/kg bw (three administrations: 24 h, 48 h, 72 h)	98.8	24, 72 h: Negative 48 h: Positive[b] (female)	Guernard (1984a)
Micronucleus test	NMRI KFM mouse	0 (solvent), 200, 1 000 and 5 000 mg/kg bw (single administration)	98.8	Negative	Guernard (1984b)
Micronucleus test	BDF1 mouse	0 (solvent), 4 100, 8 200 and 16 400 mg/kg bw	99.4	Negative	Sasaki (1984)

bw: body weight; CHL: Chinese hamster lung; CHO: Chinese hamster ovary; DNA: deoxyribonucleic acid; HGPRT: hypoxanthine-guanine phosphoribosyltransferase; NR: not reported; S9: 9000 × *g* supernatant fraction from rat liver homogenate

[a] There was no clear increase in structural chromosomal aberrations compared with the control cultures in the cells exposed for 24 hours. The incidences of chromosomal aberrations in the cells exposed to triforine at levels of 65.0, 130 and 260 µg/mL were 3.5%, 2.0% and 3.0% (including gaps) and 2.5%, 1.0% and 2.5% (excluding gaps), respectively, compared with dimethyl sulfoxide control values of 1.0% and 0.5%. Severe cytotoxic effects were also seen at 520 µg/mL for 24- and 48-hour exposures in the main study. The incidence of numerical abnormalities in cells treated with 65.0, 130 and 260 µg/mL were 8.0%, 17.5% and 1.5%, respectively, showing positive reaction to the exposure to triforine, although the incidence declined at the highest dose level. In the additional 24-hour assay, the incidences of numerical abnormalities in cells exposed to 51.8, 86.4, 144 and 240 µg/mL were 1.0%, 20.5%, 22.5% and 2.0%, respectively. Severe toxic effects were seen at 400 µg/mL. The results of the additional assay confirmed the results of the main study. After 48 hours of exposure, the incidences of chromosomal aberrations in the cells exposed to 65.0, 130 and 260 µg/mL were 3.0%, 3.5% and 6.0% (including gaps) and 2.5%, 3.0% and 5.0% (excluding gaps), respectively. The results suggested a slight increase in clastogenicity, but were inconclusive according to the above criteria. The incidences of numerical

Table 24 (continued)

abnormalities in cells treated with 65.0, 130 and 260 µg/mL were 3.5%, 33.0% and 56.5%, showing a clear positive reaction to the exposure to the test substance.

[b] At 48 hours post-administration, a statistically significant increase in mean micronucleated polychromatic erythrocytes was observed in the female treated group when compared with the corresponding negative control group ($P < 0.01$). As only 2/5 females treated with triforine and killed after 48 hours showed a higher number of micronucleated polychromatic erythrocytes than the control range, it is unclear if this finding represents a real effect of treatment or an incidental effect. No significant increase in incidences was found in male groups at this examination time or when male and female animal data were pooled. The polychromatic erythrocyte/normochromatic erythrocyte ratios for the 24-, 48- and 72-hour groups were not affected by triforine treatment.

(mean values for F_0 and F_1 generations). These intake values were not available in the reports but were calculated by Sumitomo Corporation for submission in Japan (to JMAFF).

In F_0 and F_1 parental animals, morbidity/mortality and clinical signs were checked at least once daily; detailed clinical examination, body weight and feed consumption were assessed approximately weekly. Duration of gestation and fertility and gestation indices were calculated. In addition, any deficiencies in maternal care during lactation were recorded. The F_1 and F_2 offspring were examined daily during the lactation period for clinical signs, and the number of pups born and the number found dead in each litter were recorded at birth. The live pups were sexed, counted, examined for the presence of milk in the stomach and external abnormalities and weighed during lactation. The birth index, live birth index, viability index, lactation index and overall survival index were calculated. Where practical, any pups found dead or killed as a result of morbidity were also sexed and examined as above.

All F_0 and F_1 parental animals were subjected to a necropsy and gross pathological examination. The liver, kidneys, thyroid, spleen and reproductive organs were weighed. The number of implantation sites in the uterus was recorded, and post-implantation loss was evaluated. Histopathological examinations were performed on the reproductive organs (high-dose and control groups only) and the liver, kidney, spleen, thyroid and all abnormal tissues from both generations. In addition, the reproductive tract of non-parturient females was examined for signs of pregnancy and examined histopathologically. Non-siring males were also examined histopathologically. F_1/F_2 offspring found dead or killed on or after day 14 of lactation were necropsied and examined macroscopically for external abnormalities, and organs and tissues of the thoracic and abdominal cavities were examined in situ. Those found dead before day 14 of lactation were examined for externally visible abnormalities and for the presence of milk in the stomach. F_1 weanlings not selected as parents and F_2 pups were killed and discarded after external examination.

F_0/F_1 parental animals: One F_0 male and one F_0 female at 500 ppm were found dead during the study, and one F_1 female at 20 000 ppm was killed prematurely because of dystocia. None of these deaths was related to treatment. No treatment-related clinical signs were noted in either the F_0 or F_1 generation. Slight reductions in body weight gain and feed consumption were found in both sexes at 3000 and 20 000 ppm in both generations, and body weight gains during gestation were reduced at 20 000 ppm in the F_0 generation. There were no effects on the body weight gain of F_1 females at any dose level during gestation or lactation. There were no gross pathological findings attributable to treatment in parental animals. The relative kidney weights were slightly increased in F_0 males and females at 3000 and 20 000 ppm and in F_1 males at 20 000 ppm. Treatment-related increases in relative liver weights were observed in males and females in both generations at 3000 and 20 000 ppm, and treatment-related increases in relative spleen weights were seen in F_1 males at 20 000 ppm and in females in both generations at 3000 and 20 000 ppm. Triforine treatment did not affect mating performance, fertility, duration of gestation, gestation index, post-implantation loss, litter size or pup survival in either the F_0 or F_1 generation.

There were treatment-related increases in the incidences of microscopic changes in the spleen, kidneys, thyroid and liver (Table 25). A dose-related increase in haemosiderin deposition in spleen occurred, partly accompanied by dose-related increases in extramedullary haematopoiesis and in spleen weight for both sexes treated with 3000 and 20 000 ppm in both generations. The severity of

Table 25.1 Incidence of treatment-related histopathological alterations in a reproductive toxicity study in rats

	Incidence of finding							
	Males				Females			
Organ/lesion[a]	**0 ppm**	**500 ppm**	**3 000 ppm**	**20 000 ppm**	**0 ppm**	**500 ppm**	**3 000 ppm**	**20 000 ppm**
F_0 generation								
Kidney: no. examined	*28*	*28*	*28*	*28*	*26*	*28*	*28*	*28*
Nephropathy								
Total	8	6	16	14	0	0	0	2
Grade +/–	7	3	7	8	0	0	0	2
Grade +	1	2	7	2	0	0	0	0
Grade ++	0	1	2	3	0	0	0	0
Grade +++	0	0	0	1	0	0	0	0
Pelvic dilatation	3	8	3	11*	11	10	4*	16
Mineral deposit (overall)	–	–	–	–	0	10***	8**	8**
Cortical	0	1	0	1	0	3	5	0
Corticomedullary	0	0	0	0	0	1	0	0
Papillary	1	2	1	1	0	2	1	1
Pelvic mineral deposit	0	1	0	0	0	5	1	7*
Cortical subcapsular mineral deposit	0	1	0	0	0	1	1	2
Liver: no. examined	*28*	*28*	*27*	*28*	*26*	*26*	*25*	*28*
Centrilobular hepatocellular hypertrophy	0	0	2	0	0	0	0	1
Spleen: no. examined	*28*	*27*	*27*	*28*	*26*	*27*	*27*	*28*
Increased haemosiderin	5	8	21***	26***	1	4	25***	28***
Increased extramedullary haematopoiesis	1	7*	2	6	0	1	4	7*
Thyroid: no. examined	*28*	*27*	*27*	*28*	*26*	*27*	*27*	*28*
Very active appearance	10	4	6	13	3	1	4	15**
F_1 generation								
Kidney: no. examined	*24*	*24*	*24*	*24*	*24*	*24*	*23*	*24*
Nephropathy								
Total	1	8*	7*	16***	0	0	1	0
Grade +/–	1	5	2	8*	0	0	1	0
Grade +	0	2	3	5*	0	0	0	0
Grade ++	0	1	2	3	0	0	0	0
Grade +++	0	0	0	0	0	0	0	0
Pelvic dilatation	3	3	2	2	3	1	1	2
Mineral deposit (overall)	–	–	–	–	0	3	4*	5*

Table 25 (continued)

Organ/lesion[a]	Incidence of finding							
	Males				Females			
	0 ppm	500 ppm	3 000 ppm	20 000 ppm	0 ppm	500 ppm	3 000 ppm	20 000 ppm
Cortical	0	0	0	0	0	0	0	0
Corticomedullary	0	0	0	0	0	0	1	1
Papillary	1	0	1	0	0	1	3	1
Pelvic mineral deposit	1	0	0	2	0	1	1	3
Cortical subcapsular mineral deposit	0	0	0	0	0	1	0	0
Liver: no. examined	*24*	*24*	*24*	*24*	*24*	*24*	*23*	*24*
Centrilobular hepatocellular hypertrophy	0	5*	0	0	0	0	3	0
Spleen: no. examined	*24*	*24*	*24*	*24*	*24*	*24*	*23*	*24*
Increased haemosiderin	4	13*	21***	24***	3	8	21***	24***
Increased extramedullary haematopoiesis	0	4	5*	12***	1	4	7*	10**
Thyroid: no. examined	*24*	*24*	*24*	*24*	*24*	*24*	*24*	*24*
Very active appearance	6	3	6	12	6	2	2	18**

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$

[a] Grade: +/–: mildest to +++: most severe.

Source: McCay & Hazelden (1991); Hazelden & Aitken (1993)

spontaneously occurring nephropathy in males and nephrocalcinosis in females was dose-dependently increased, and a small increase in absolute kidney weight was found. These changes were associated with common age-related background lesions in male and female rats. The incidences at 500 ppm were similar to the incidences in control animals. In addition, females in both generations receiving 20 000 ppm showed very active secreting glands in the thyroid, consisting of numerous very small acini with little or no lumina and scant stored secretion and lined by cuboidal or columnar epithelium with pale, foamy cytoplasm. The severity of these effects did not appear to be increased in the F_1 generation. An adaptive increase in absolute liver weight was detected together with a sporadic incidence of centrilobular hepatocyte hypertrophy.

F_1/F_2 offspring: There was a statistically significant reduction in both F_1 and F_2 preweaning pup weight and weight gain in the 20 000 ppm group and in F_1 males at 3000 ppm on day 14 of lactation. No differences in litter size, survival or clinical signs in both F_1 and F_2 pups were observed.

A NOAEL for reproductive toxicity was determined to be 20 000 ppm (equal to 1768.3 mg/kg bw per day), the highest dose tested, based on the absence of reproductive effects at all doses. The NOAEL for parental toxicity was identified as 500 ppm (equal to 39.4 mg/kg bw per day), based on the occurrence of reduced weight gain, increased spleen weight and haemosiderin deposition in the spleen at 3000 ppm (equal to 252.5 mg/kg bw per day) and above. The NOAEL for offspring toxicity was also 500 ppm (equal to 39.4 mg/kg bw per day), based on the occurrence of reduced preweaning body weight gain at 3000 ppm (equal to 252.5 mg/kg bw per day) and above.

The study was GLP compliant, and a QA statement was attached (McCay & Hazelden, 1991; Hazelden & Aitken, 1993).

In a non-GLP three-generation reproductive toxicity study, groups of 10 male and 20 female Chbb: Thom rats were given triforine in the diet at a concentration of 0, 100, 500 or 2500 ppm for 9–10 weeks (F_0) or 9 weeks (F_1 and F_2) prior to mating and throughout lactation until weaning of the offspring from the second mating, which were described as F_{1B}, F_{2B} and F_{3B} offspring. The offspring from the first mating in each generation were described as F_{1A}, F_{2A} and F_{3A} offspring. The dietary concentrations of 0, 100, 500 and 2500 ppm were equal to mean achieved doses of 0, 9.2, 46.8 and 233.0 mg/kg bw per day for males and 0, 9.8, 49.2 and 246.4 mg/kg bw per day for females, respectively (mean values for F_0, F_1 and F_2 generations). These intake values were not available in the report, but were calculated by Sumitomo Corporation for submission in Japan (to JMAFF). A 2-week period, during which the animals continued on the experimental diets, was allowed between weaning of the first offspring and second mating. After weaning of the F_{1B} and F_{2B} offspring, 10 males and 20 females were selected to constitute groups for each treatment level. The study was completed after weaning of the F_{3B} offspring. Pups were not culled during the study.

In $F_0/F_1/F_2$ parental animals, morbidity/mortality and behaviour were checked. Body weights were recorded weekly before mating and throughout the gestation period. Feed consumption of the F_0, F_{1B} and F_{2B} generations was recorded each week during the premating period. Fertility and the period of gestation were determined.

In $F_1/F_2/F_3$ offspring, body weights were measured at birth and on days 4, 14 and 21 of lactation. Sex ratio and the percentage of pups that died were recorded during the lactation period. All surviving parental animals, F_{1A}, F_{2A} and F_{3A} offspring and non-selected F_{1B} and F_{2B} pups were killed at weaning and examined macroscopically. Those pups found dead or killed were also examined macroscopically. The pups with suspected malformations were prepared for further examination. For F_{3B} offspring, 10 males and 10 females per group were killed at weaning and necropsied, and the principal organs were weighed. Heart, lungs, liver, kidney, thymus, spleen, male gonads, salivary gland and brain were evaluated histopathologically for the control and high-dose animals. A further 10 males and 10 females per group were fixed, stained with alizarin red and examined for skeletal abnormalities. The remaining pups were necropsied and examined macroscopically for abnormalities of organs. Data were analysed statistically where appropriate.

$F_0/F_1/F_2$ parental animals: There were no treatment-related deaths or clinical signs and no changes in feed consumption or reproductive parameters at any dose level in all generations. Although reduced body weight gains were observed in all treated groups in F_0 males and at 2500 ppm in F_{1B} males, these changes were not considered to be treatment related, because the reduced body weight gains were not observed in the F_2 generation. There were no effects on body weight gain during gestation or the lactation period at any dose level in all generations.

$F_{1A,B}/F_{2A,B}/F_{3A}$ offspring: Statistically significantly reduced mean weights of offspring at birth were recorded only in F_{3A} offspring in all treated groups. However, this was considered to be a consequence of increased litter size. There was no treatment-related increase in malformations at any dose level in all generations.

F_{3B} offspring: There were no treatment-related changes in macropathological or micropathological examinations or skeletal investigations at any dose level. Increased absolute spleen weight in males at 2500 ppm and decreased absolute pituitary weight in females were observed at all dose levels (Table 26). However, these differences might be attributable to imprecise processing of the organs rather than treatment-related changes, because there were no histopathological correlates and because individual pituitary weights in treated females were largely within the concurrent control range.

The NOAEL for reproductive toxicity, parental toxicity and offspring (F_1, F_2 and F_3 weaned offspring) toxicity was identified as 2500 ppm (equal to 233.0 mg/kg bw per day), the highest dose tested, based on the absence of treatment-related effects at all dose levels (Niggeschulze, Hill & Stötzer, 1974).

Table 26. Group mean organ weights in F_{3B} offspring

		Absolute organ weight (mg)			
Sex	**Organ**	**0 ppm**	**100 ppm**	**500 ppm**	**2 500 ppm**
Males	*Body weight (g)*	*33.50*	*35.90*	*35.10*	*35.40*
	Spleen	90.80	95.65	95.35	112.60**
Females	*Body weight (g)*	*30.40*	*31.70*	*31.30*	*31.70*
	Pituitary	2.00	1.06*	1.30*	1.25*

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$

Source: Niggeschulze, Hill & Stötzer (1974)

(b) Developmental toxicity

Rat

In a preliminary embryotoxicity study, four groups of eight mated Crl:CD (SD) BR rats (approximately 8–12 weeks old and with a body weight of 174–204 g) were treated orally by gavage with triforine at a dose of 0, 250, 500 or 1000 mg/kg bw per day from day 6 to day 15 of gestation at a dose volume of 10 mL/kg bw in distilled water. The animals were killed on day 20 of gestation for assessment of maternal toxicity and effects on fetal development. Mortality, clinical signs, body weight and feed consumption were recorded. Dams were examined macroscopically at necropsy on day 20 of gestation. All fetuses were removed and also examined macroscopically at maternal necropsy and subsequently by detailed internal visceral or skeletal examination.

There were no treatment-related deaths, clinical signs of toxicity or gross pathological changes. There was a slight reduction in body weight gain from day 9 to day 12 post-coitum in the rats at 500 and 1000 mg/kg bw per day. This finding was considered to be treatment related. Necropsy did not reveal any treatment-related effect. There was no effect on preimplantation or post-implantation loss. Treatment-related malformations or variations were not detected during external fetal examination.

In view of the above, this study showed that no maternal toxicity, embryotoxicity or externally detected signs of teratogenicity were observed at 250 mg/kg bw per day, and the doses of this study appeared suitable for the subsequent main study.

The study was GLP compliant, and a QA statement was attached (Fuchs, 1992).

In the main study, four groups of 30 mated Crl:CD (SD) BR rats (approximately 8–12 weeks old and with a body weight of 181–247 g) were treated with triforine (lot no. Ht 08/91/1; purity 99.8%) orally by gavage at a dose of 0, 200, 500 or 1000 mg/kg bw per day from day 6 to day 15 of gestation at a dose volume of 10 mL/kg bw in distilled water. The animals were killed on day 20 of gestation for assessment of maternal toxicity and effects on fetal development. Mortality, clinical signs, body weight and feed consumption were recorded. Dams were examined macroscopically at necropsy on day 20 of gestation. All fetuses were removed and also examined macroscopically at maternal necropsy and subsequently by detailed internal visceral or skeletal examination.

There were no treatment-related deaths, clinical signs of toxicity or gross pathological changes. Feed consumption was minimally reduced at 1000 mg/kg bw per day, but this was not accompanied by a significant decrease in body weight gain. Therefore, this change was not considered to be of biological significance. No gross pathological changes were seen in fetuses removed on day 20 of gestation. No reproductive parameters were affected by treatment, and there were no indications of treatment-related embryotoxicity or teratogenicity.

The NOAEL for maternal and embryo/fetal toxicity was identified as 1000 mg/kg bw per day, the highest dose tested, based on a lack of treatment-related effects at all doses.

The study was GLP compliant, and a QA statement was attached (Fuchs, 1993a).

In an old study, four groups of 20 gravid Sprague-Dawley rats (approximately 95 ± 5 days old and with a body weight of 201–257 g) were treated with triforine (lot no. 1; purity 99.4%) orally by gavage at a dose of 0, 100, 400, 800 or 1600 mg/kg bw per day from day 6 to day 15 of gestation at a dose volume of 5 mL/kg bw in 1% carboxyethyl cellulose gel. The animals were killed on day 19 of gestation for reproductive assessment and fetal examination. Clinical signs, body weight and feed consumption were recorded. Dams were examined macroscopically at necropsy on day 19 of gestation, and all fetuses were removed and also examined macroscopically and subsequently by detailed internal visceral or skeletal examination. Data were reanalysed statistically according to JMAFF requirements (Hagiwara, 2011a).

There were no deaths, clinical signs, effects on feed and water consumption, or macroscopic findings at any dose level. A statistically significantly lower body weight was observed at 1600 mg/kg bw per day on day 19 of gestation, which was attributed to the small number of fetuses. There was a significant reduction in the number of fetuses and a corresponding increase in the number of early resorptions at 1600 mg/kg bw per day. The post-implantation loss in the 1600 mg/kg bw per day group was 39.4%, which was higher than in controls (8.1%) (Table 27). There were no treatment-related changes in the sex or weight of fetuses (Table 28) and no increase in the incidence of malformations (Table 29). An increase in the skeletal variation rate, mainly an increase in retarded ossification in sternebrae, was found at 800 and 1600 mg/kg bw per day.

Table 27. Implantation data from a developmental toxicity study in rats

Dose (mg/kg bw per day)	No. mated	No. gravid	Mean no. of CL	Mean no. of IP	Implantation loss (%)		Resorptions			
					Pre-	Post-	Total no. early	Total no. late	Mean total/dam	Rate (%)
0	22	20	14.0	13.6	2.9	8.1	19	3	1.1	8.1
100	24	20	13.2	13.1	1.1	5.0	11	2	0.7	5.0
400	21	20	14.5	14.1	2.8	8.5	17	7	1.2	8.5
800	23	20	13.1	12.8	1.9	7.4	17	4	1.1	8.2
1 600	23	20	13.9	13.5	2.9	39.4**	67*	36*	5.2**	38.3*

bw: body weight; CL: corpora lutea; IP: implantations; *: $P \leq 0.05$; **: $P \leq 0.01$

Source: Leuschner (1972)

Table 28. Fetal data from a developmental toxicity study in rats

Dose (mg/kg bw per day)	Number of fetuses			Overall mean fetal weight (g)	Sex ratio (% male)
	Total	Total/dam	Dead/dam		
0	249	12.5	0	3.5	51
100	248	12.4	0	3.5	49
400	258	12.9	0	3.5	50
800	237	11.9	0	3.5	50
1 600	166	8.3*	0.2	3.5	57

bw: body weight; *: $P \leq 0.01$

Source: Leuschner (1972)

Table 29. Incidence of abnormalities in a developmental toxicity study in rats

	Incidence of abnormality				
Abnormality	**0 mg/kg bw per day**	**100 mg/kg bw per day**	**400 mg/kg bw per day**	**800 mg/kg bw per day**	**1 600 mg/kg bw per day**
Number of fetuses with runts[a]	0	0	1	0	0
Number of fetuses examined viscerally	85	80	80	80	54
Number of fetuses examined skeletally	164	161	166	157	112
Number of fetuses with visceral malformations	0	0	0	0	0
Number of fetuses with skeletal malformations	0	0	0	0	0
Number of fetuses with visceral variations	0	0	0	0	0
Number of fetuses with skeletal variations	16	16	18	27	28
Rate (%)	9.8	9.9	10.8	17.2	25.0
Incidence of retarded ossification					
Phalanges	1	0	1	2	1
Sternebrae	14	14	17	26	27
Cranium	0	0	1	2	3
Incidence of hypoplasia or aplasia of the 13th costal pair of ribs	2	4	6	6	4

bw: body weight

[a] Fetuses weighing less than 70% of the mean litter weight.

Source: Leuschner (1972)

The NOAEL for maternal toxicity was identified as 800 mg/kg bw per day, based on the reduction in maternal body weight gain. The NOAEL for embryo and fetal toxicity was identified as 400 mg/kg bw per day, based on the increased incidence of skeletal variations at 800 mg/kg bw per day and higher. No teratogenicity was observed in this study.

The study was not GLP compliant (Leuschner, 1972).

Rabbits

Four groups of 15 mated rabbits (Chbb:HM (Himalayan; SPF-quality)) (approximately 140–210 days old at dosing and with a body weight of 1.94–2.54 kg) were treated with triforine (batch no. 2151; purity not given) orally by gavage at a dose of 0, 5, 25 or 125 mg/kg bw per day from day 6 to day 18 of gestation at a dose volume of 5 mL/kg bw in 0.5% carboxymethyl cellulose. The animals were killed on day 29 of gestation for reproductive assessment and fetal examination. Clinical signs, body weight and feed consumption were recorded. Dams were examined macroscopically at necropsy on day 29 of gestation, and all fetuses were removed and also examined macroscopically and subsequently by detailed internal visceral or skeletal examination (by X-ray).

There were no deaths, no treatment-related clinical signs and no macroscopic findings at any dose. For most of the treatment period, body weight was reduced in rabbits at 25 and 125 mg/kg bw per day (although not statistically significantly) as a result of reduced feed consumption during gestation days 6–9. Towards the end of treatment, these groups regained weight (Table 30). Dose-related statistically significant decreases in feed consumption were observed in all treated groups on days 7–10 (Table 31). However, the reduction at 5 mg/kg bw per day was not considered to be toxicologically significant, because the body weight was not affected by treatment.

Table 30. Selected group mean body weights in a developmental toxicity study in rabbits

Dose (mg/kg bw per day)	Mean body weight (kg)					
	Day 3	Day 6	Day 10	Day 11	Day 18	Day 29
0	2.30	2.32	2.30	2.29	2.31	2.46
5	2.27	2.24	2.23	2.23	2.22	2.38
25	2.23	2.26	2.19	2.18	2.16	2.27
125	2.25	2.26	2.12*	2.12*	2.21	2.29

bw: body weight; *: $P < 0.05$

Source: Gleich et al. (1981)

Table 31. Selected group mean feed consumption in a developmental toxicity study in rabbits

Dose (mg/kg bw per day)	Feed consumption (g)				
	Days 0–3	Days 4–6	Days 7–10	Days 11–14	Total
0	285	284	341	249	2 513
5	272	244	224*	236	2 090
25	295	265	203**	164	2 134
125	298	277	87**	136	1 760

bw: body weight; *: $P < 0.05$; **: $P < 0.01$

Source: Gleich et al. (1981)

No reproductive parameters were affected by treatment, and there were no indications of treatment-related embryotoxicity or fetotoxicity at any dose.

The NOAEL for maternal toxicity was identified as 25 mg/kg bw per day, based on the reduction in body weight and feed consumption at 125 mg/kg bw per day during the first days of treatment. The NOAEL for embryo/fetal toxicity was 125 mg/kg bw per day, the highest dose tested, as no treatment-related effects were observed at any dose. No evidence of teratogenicity was observed in this study.

The study was GLP compliant, and a QA statement was attached (Gleich et al., 1981).

In another study, four groups of 18 mated New Zealand White rabbits (approximately 14–17 weeks old at dosing and with a body weight of 3.1–4.0 kg) were treated with triforine (lot no. 2764; purity 99.1 ± 0.9%) orally by gavage at a dose of 0, 6, 30 or 150 mg/kg bw per day from day 6 to day 18 of gestation at a dose volume of 10 mL/kg bw in distilled water. The animals were killed on day 28 of gestation for reproductive assessment and fetal examination. Mortality, clinical signs, body weight and feed consumption were recorded. Dams were examined macroscopically for reproductive parameters at necropsy on day 28 of gestation, and all fetuses were removed and also examined externally and subsequently by detailed internal visceral or skeletal examination.

There were no treatment-related deaths or clinical signs of toxicity. Decreased feed consumption was observed at 150 mg/kg bw per day on days 12–18, together with a slight, but not statistically significant, reduction in body weight gain. No gross pathological changes were seen in fetuses removed on day 28 of gestation. No reproductive parameters were affected by treatment, and there were no indications of treatment-related embryotoxicity or teratogenicity.

The NOAEL for maternal toxicity was identified as 30 mg/kg bw per day, based on the reduction of feed consumption and body weight gain at 150 mg/kg bw per day. The NOAEL for

embryo/fetal toxicity was identified as 150 mg/kg bw per day, the highest dose tested, based on the lack of treatment-related effects at all doses.

The study was GLP compliant, and a QA statement was attached (Müller, 1989, 1995).

A preliminary oral embryotoxicity study was undertaken to provide information on the maternal toxicity and embryotoxicity of triforine when administered to rabbits orally during organogenesis. Groups of eight sexually mature and mated New Zealand White rabbits (approximately 14–17 weeks old at dosing and with a body weight of 2.9–4.1 kg) were treated with triforine (lot no. and purity not given) orally by gavage at a dose of 0, 250, 500 or 1000 mg/kg bw per day from day 6 to day 18 of gestation. The animals were killed on day 28 of gestation for reproductive assessment and fetal examination.

No mortalities were observed in this study. One abortion, which occurred at 500 mg/kg bw per day, was considered to be incidental. No treatment-related clinical observations were noted in any dose group. However, there was a slight reduction and a moderate reduction in mean fetal weight in the 500 and 1000 mg/kg bw dose groups, respectively. No gross pathological changes were seen in fetuses removed on day 28 of gestation. No reproductive parameters were affected by treatment, and there were no indications of treatment-related embryotoxicity or teratogenicity.

In view of the above, it was concluded that the 1000 mg/kg bw per day dose elicited slight maternal toxicity and embryotoxicity. Hence, the high dose was suitable for future studies.

The study was GLP compliant, and a QA statement was attached (Müller, 1991).

In another study, two groups of 18 mated New Zealand White rabbits (approximately 14–17 weeks old at dosing and with a body weight of 2.9–4.3 kg) were treated with triforine orally by gavage at a dose level of 0 or 1000 mg/kg bw per day from day 6 to day 18 of gestation at a dose volume of 10 mL/kg bw in distilled water. The animals were killed on day 28 of gestation for reproductive assessment and fetal examination. Mortality, clinical signs, body weight and feed consumption were recorded. Dams were examined macroscopically for reproductive parameters at necropsy on day 28 of gestation; all fetuses were removed and examined macroscopically and subsequently by detailed internal visceral or skeletal examination.

There were no treatment-related deaths or clinical signs of toxicity. Reductions in feed consumption and body weight gain were observed at 1000 mg/kg bw per day, mainly during the early period of treatment, and continued until the day of necropsy. No gross pathological changes were observed, and reproductive parameters were not affected by treatment. The mean fetal weights were slightly reduced, and this indication of fetotoxicity was accompanied by reduced ossification of the bones of the extremities and pelvis. However, there were no indications of teratogenicity at 1000 mg/kg bw per day.

A NOAEL for maternal toxicity and embryotoxicity was not established, because reduction of body weight gain and feed consumption for dams, a slight reduction in mean fetal weight and a delay in bone ossification in fetuses were observed at 1000 mg/kg bw per day, the only dose tested. There was no evidence of teratogenicity.

The study was GLP compliant, and a QA statement was attached (Fuchs, 1993b).

2.6 Special studies

(a) Immunotoxicity

Mice

In a T cell–dependent antibody response assay using sheep red blood cells (sRBCs) in mice, four groups of 10 female CD-1 (Crl:CD-1 (ICR), SPF-quality) mice (approximately 6–8 weeks of age and weighing around 24–29 g) were treated orally, by diet admixture, with triforine (batch no.

TRF0912007; purity 100.2%) at a dose of 0, 280, 1400 or 7000 ppm (equal to 0, 44, 211 and 1115 mg/kg bw per day, respectively) for at least 28 days. A similar group of mice, given an intraperitoneal injection of cyclophosphamide at 20 mg/kg bw per day from day 25 to day 28, acted as a positive control group. All animals received a sensitizing intravenous dose of sRBCs in sterile phosphate-buffered saline 4 days prior to termination.

All animals were examined at least twice daily for mortality, and clinical signs were observed at least once daily. Body weights and feed consumption were measured weekly. In addition, body weights were measured on day 25. Water consumption was not measured, but subjective appraisal was maintained during the study. Blood samples for antibody response were collected from retro-orbital sinus under anaesthesia on day 1 (pretreatment) and day 29 (end of treatment). The antibody response to the immunization with sRBCs was determined by measuring the anti-sRBC immunoglobulin M (IgM) levels in serum using an enzyme-linked immunosorbent assay (ELISA). All animals were killed after 29 days of treatment and subjected to full postmortem examination. Spleen and thymus weights as well as final body weight were recorded. Histopathological examination was not performed. Animals were not fasted prior to blood sampling and necropsy. Data were analysed statistically where appropriate.

There were no deaths, clinical signs, macroscopic findings or effects on body weights, feed consumption or organ weights at any dose of triforine.

At the end of the treatment period (4 days after immunization), there was an approximately 10- to 30-fold increase in average anti-sRBC IgM levels in the control group and in animals treated with triforine when compared with the average anti-sRBC IgM levels at pretreatment. There was no clear difference in response between these groups.

In the positive control group, statistically significantly decreased absolute spleen and thymus weights and relative thymus weight were noted. Almost no anti-sRBC IgM response was noted 4 days after immunization: anti-sRBC IgM levels were almost in the same range as pretreatment values.

In view of the above, it is concluded that triforine has no effect on the T cell–dependent antibody response in mice up to 7000 ppm. There were no treatment-related changes at any dose level.

Therefore, the NOAEL for immunotoxic effects was 7000 ppm (equal to 1115 mg/kg bw per day), the highest dose tested.

The study was GLP compliant, and a QA statement was attached (van Tuyl, 2011a).

Rats

In a T cell–dependent antibody response assay using sRBCs, four groups of 10 female Wistar Han rats (approximately 6–8 weeks of age and weighing about 130–166 g) were treated orally, by diet admixture, with triforine (batch no. TRF0912007; purity 100.2%) at a dose of 0, 560, 2800 or 14 000 ppm (equal to 0, 46, 231 and 1151 mg/kg bw per day, respectively) for at least 28 days. A similar group of rats, given an intraperitoneal injection of cyclophosphamide at 10 mg/kg bw per day from day 24 to day 28, acted as a positive control group. All animals received a sensitizing intravenous dose of sRBCs in sterile phosphate-buffered saline 5 days prior to termination.

All animals were examined at least twice daily for mortality, and clinical signs were observed at least once daily. Body weights were measured twice weekly, and feed consumption was measured weekly. Water consumption was not measured, but subjective appraisal was maintained during the study. Blood samples for antibody response were collected from retro-orbital sinus under anaesthesia on day 1 (pretreatment) and day 29 (end of treatment). The antibody response to the immunization with sRBCs was determined by measuring the anti-sRBC IgM levels in serum using an ELISA.

All animals were killed after 29 days of treatment and subjected to full postmortem examination. Spleen and thymus weights as well as final body weight were recorded. Histopathological examination was not performed. Animals were not fasted prior to blood sampling and necropsy. Data were analysed statistically where appropriate.

There were no deaths, no treatment-related clinical signs and no effects on feed or water consumption at any dose of triforine or in the positive control group. Slightly lower body weight and body weight gain were noted in the 14 000 ppm and positive control groups. There was also a trend towards slightly lower body weight and body weight gain in the 2800 ppm group.

At the end of the treatment period (5 days after immunization), there was an approximately 50- to 100-fold increase in average anti-sRBC IgM levels in the control group and in animals treated with triforine when compared with average anti-sRBC IgM levels at pretreatment. There was no clear difference in response between these groups. In the positive groups, almost no anti-sRBC IgM response was noted 5 days after immunization; anti-sRBC IgM levels were almost in the same range at pretreatment.

At necropsy, reduced size of thymus was noted in all animals in the positive control group. No treatment-related effects were noted in the triforine-treated groups.

Slightly increased absolute and relative spleen weights were noted in the 2800 and 14 000 ppm groups. In the positive control group, reduced absolute and relative spleen and thymus weights were noted.

In view of the above, it is concluded that triforine has no effect on the T cell–dependent antibody response in rats up to 14 000 ppm, although slight reduction of body weight gain at 14 000 ppm and slight increase of spleen weight at 2800 and 14 000 ppm were noted. Therefore, the NOAEL for immunotoxic effects was 14 000 ppm (equal to 1151 mg/kg bw per day), the highest dose tested.

The study was GLP compliant, and a QA statement was attached (van Tuyl, 2011b).

(b) Hepatic enzyme assay

To examine the effect of triforine on hepatic xenobiotic metabolizing enzymes, two groups of 35-day-old Sprague-Dawley rats (six of each sex per group) were given triforine in the diet at a concentration of 0 or 20 000 ppm (mean achieved doses of 1956.9 mg/kg bw per day for males and 2093.8 mg/kg bw per day for females) for 28 days. Two groups of 42-day-old CD-1 mice (eight of each sex per group) were given triforine in the diet at a concentration of 0 or 7000 ppm (mean achieved doses of 1555.3 mg/kg bw per day for males and 1998.2 mg/kg bw per day for females) for 28 days. Positive control groups of equal size were included in the study; rats and mice received diets containing 500 ppm sodium phenobarbitone. All animals were examined daily for mortality and variations in behaviour and condition, and detailed examinations were conducted weekly. Body weights were measured 3 days before treatment, on the first day of treatment (day 0) and subsequently twice weekly. Feed consumption was measured over the intervals between body weight measurements.

All animals were weighed and killed after 28 days of treatment and subjected to necropsy. The livers were removed and weighed. Small samples from each of the four lobes of the liver were taken from all rats and six mice of each sex for microscopic examination. A cube (approximately 1 cm^3) of liver from two rats or two mice of each sex in each group was taken from a consistent anatomical position, stained with toluidine blue and examined by light microscopy. Two centrilobular and two periportal areas were selected from the livers of each animal in the triforine-treated groups, and each one was selected from the negative and positive control groups in order to be sectioned for examination by transmission electron microscopy. The remainder of the liver was used for the biochemical analysis. Whole homogenates of liver samples from each animal (approximately 0.25 g fresh tissue/mL) were analysed for protein concentration, deoxyribonucleic acid (DNA) concentration and cyanide-insensitive palmitoyl coenzyme A oxidation activity. A portion of each whole homogenate was centrifuged 3 times – at 10 000 *g* for 20 minutes, at 158 000 *g* for 40 minutes and at 158 000 *g* for 40 minutes – to assay for cytochrome P450 (CYP) content and activities of erythromycin *N*-demethylase, 7-pentoxyresorufin *O*-dependentylase, 7-ethoxyresorufin *O*-deethylase and microsomal lauric acid 11- and 12-hydroxylase; the content of microsomal protein was also determined. The activities of 7-ethoxyresorufin *O*-deethylase, 7-pentoxyresorufin *O*-dependentylase, erythromycin *N*-demethylase and lauric acid 11-/12-hydroxylase were measured as markers of

induction of cytochrome P450 isoenzymes in the CYP1A, CYP2B, CYP3A and CYP4A subfamilies, respectively.

There were no adverse effects on the condition or behaviour of any animal. Body weights of male rats fed triforine were significantly lower than those of controls over the majority of the study, and those of female rats in the same group were also lower, but less markedly. No body weight changes in mice fed triforine were noted. Feed consumption in both species was unaffected by treatment with triforine. Relative liver weights were increased in male (23%) and female (26%) rats and male (12%) and female (16%) mice. There were no major differences between the relevant control and treated groups with regard to hepatic homogenate protein or DNA content or in cyanide-insensitive palmitoyl coenzyme A oxidation activity (as a measure of peroxisomal fatty acid oxidizing enzyme activity and peroxisomal proliferation). The microsomal cytochrome P450 content was slightly reduced in male (14%) and female (13%) rats and slightly increased only in male (28%) mice fed triforine. 7-Ethoxyresorufin *O*-deethylase activity was not affected in mice, but was reduced in both male (54%) and female (60%) rats. 7-Pentoxyresorufin *O*-dependentylase activity was not affected in any group, whereas erythromycin *N*-demethylase activity was increased only in male rats (52%) and mice (51%). Lauric acid 12-hydroxylase activity was not affected in male rats or female mice, whereas there was a 36% reduction in female rats and a 30% increase in male mice. At microscopic examination, centrilobular hypertrophy was not detected in either rat or mouse. The results from electron microscopic examination showed an increased amount of smooth endoplasmic reticulum in male and female rats but not mice following treatment with triforine. However, these findings were not consistent with the biochemical changes reported for the rat; although there was an increase in liver weight, the apparent increase in smooth endoplasmic reticulum was not accompanied by an increase in P450 or a clear increase in the activity of the mixed-function oxidases.

In positive control groups, there were no changes in clinical signs, body weight or feed consumption in either species. Liver weights were increased markedly in males and females in both species. Microsomal protein and cytochrome P450 contents were statistically significantly increased in males and females in both species. Whereas lauric acid 11-hydroxylase activity was induced in males and females in both species, the activity of lauric acid 12-hydroxylase was decreased in male rats and unaffected in female rats and mice of both sexes. The activities of 7-ethoxyresorufin *O*-deethylase, 7-pentoxyresorufin *O*-dependentylase and erythromycin *N*-demethylase were statistically significantly higher than in controls in males and females of both species, except that erythromycin *N*-demethylase activity was unaffected in female mice. The incidences of centrilobular hypertrophy and smooth endoplasmic reticulum were increased in almost all rats and mice.

In conclusion, triforine produced an increase in liver weight in male rats and mice and also minor changes in some of the xenobiotic metabolizing enzyme activities. Ultrastructural morphological changes were confined to the rat, but triforine did not produce centrilobular hepatic hypertrophy in either rats or mice. In contrast, sodium phenobarbitone produced centrilobular hypertrophy, which was associated with a marked induction of hepatic xenobiotic metabolism in both rats and mice. It was concluded that triforine does not appear to have any marked stimulatory or inhibitory effect on hepatic xenobiotic metabolism and does not appear to produce hepatic peroxisome proliferation in either rats or mice.

The study was GLP compliant, and a QA statement was attached (Robbins, 1994).

(c) Studies on metabolites

Triforine is the major component of metabolites found in plants and in domestic animals. Therefore, no toxicological studies were considered necessary for metabolites of triforine.

3. Observations in humans

3.1 Medical surveillance on manufacturing plant personnel

Medical reports from three companies that synthesize triforine were available. The German company Boehringer Ingelheim examined 12 workers repeatedly between 1970 and 1983 for their

general health conditions and reported no haematological or blood chemistry results considered to be related to exposure to triforine (Celamerck, 1983a,b).

The industrial medical service of E. Merck Co., Darmstadt, found no adverse systemic, dermal or mucosal effects of exposure to triforine among production workers during routine examinations performed in conformity with the requirements of the German Chemical Trade Union (Merck, 1984).

No adverse effects were observed during triforine production at Shell Agrar GmbH in Spain between 1987 and 1993 (Toubes, 1993).

In addition, medical surveillance reports that were prepared by manufacturing companies in India and Japan were available. In the Indian company that has been manufacturing triforine technical since 2005, production workers involved in the manufacture of triforine work an average 8 hours/day, and the production run continues up to 18 weeks, depending on the quantity. In the formulation manufacturing company in Japan, 8–13 workers per year were involved in a campaign lasting 1–3 days by shift work from 2006 to 2011. There were no reports of health impairment associated with triforine in the employees involved in the production of triforine technical (Patel, 2013) or triforine formulation (Shiota, 2012).

3.2 Direct observation

There have not been any epidemiological studies conducted with this compound, nor have there been any known clinical cases or poisoning incidents.

Comments

Biochemical aspects

In single-dose or repeated-dose studies using a dose of 10 mg/kg bw, more than 80% of administered radiolabelled triforine was rapidly absorbed by both male and female rats. In a study using a single dose of 1000 mg/kg bw, only about 10–20% was absorbed. The AUC was about 2 times greater in male rats than in female rats. Triforine was widely distributed in the body. After a single low dose (10 mg/kg bw), more than 78% was excreted in urine, 5–6% in expired air and 12–14% in faeces (9–13% in bile). After a single high dose (1000 mg/kg bw), more than 77% was excreted in faeces and 11–19% in urine. The terminal elimination half-lives at 10 mg/kg bw were 125 hours in males and 95.7 hours in females. After 168 hours, the highest residues were seen in liver, red blood cells and kidney.

Triforine was extensively metabolized by cleavage of one of the two side-chains, followed by oxidation and conjugation of the side-chain metabolites with glucuronic acid or glutathione.

Toxicological data

Triforine is of low acute toxicity, with oral LD_{50}s greater than 5000 mg/kg bw in rats and mice. The dermal LD_{50} in rats was greater than 2000 mg/kg bw. The inhalation LC_{50} in rats was greater than 5.12 mg/L. Triforine was not irritating to the eye or skin of rabbits. It was not a dermal sensitizer in guinea-pigs (Maurer optimization test).

Administration of triforine to mice, rats and dogs in repeated-dose toxicity studies (4-week and 13-week studies in mice, 4-week, 13-week and 2-year studies in rats and 13-week and 2-year studies in dogs) resulted in haemolytic anaemia and associated effects.

In a 4-week study of toxicity in mice, triforine was administered in the diet at a concentration of 0, 200, 1000 or 5000 ppm (equal to 0, 39.0, 195.8 and 982.1 mg/kg bw per day for males and 0, 45.2, 237.0 and 1284.3 mg/kg bw per day for females, respectively). The NOAEL was 1000 ppm (equal to 195.8 mg/kg bw per day), on the basis of mild haemolytic anaemia in mice of both sexes, slightly reduced body weight gain in males and increased relative liver weight in females at 5000 ppm (equal to 982.1 mg/kg bw per day).

In a 13-week study of toxicity in mice designed solely to determine the high dose for use in longer-term studies, triforine was administered in the diet at a concentration of 0 or 7000 ppm (equal to 1354 mg/kg bw per day for males and 2239 mg/kg bw per day for females). Evidence of mild haemolytic anaemia and moderately increased spleen and liver weights were seen in treated animals.

In a 4-week study of toxicity in rats, triforine was administered in the diet at a concentration of 0, 500, 2500 or 12 500 ppm (equal to 0, 49.7, 238.2 and 1233.7 mg/kg bw per day for males and 0, 48.5, 233.2 and 1180.8 mg/kg bw per day for females, respectively). A NOAEL was not identified in this study, as the incidence and severity of haemosiderin deposition in the spleen were increased in females of all dose groups.

In a 13-week study of toxicity in rats, triforine was administered in the diet at a concentration of 0 or 20 000 ppm (equal to mean achieved doses of 1630 mg/kg bw per day for males and 1945 mg/kg bw per day for females). Treated animals showed mild haemolytic anaemia and increased spleen and liver weights.

In a 3-month study of toxicity in rats, triforine was administered in the diet at concentrations providing doses of 0, 10, 100 and 1000 mg/kg bw per day. The NOAEL was 10 mg/kg bw per day for males and females, based on haemolytic anaemia and increased liver weights at 100 mg/kg bw per day and above.

In a 13-week study of toxicity in rats, triforine was administered in the diet at a concentration of 0, 2500, 7000 or 20 000 ppm (equal to 0, 162.7, 453.6 and 1315.3 mg/kg bw per day for males and 0, 174.1, 491.4 and 1451.4 mg/kg bw per day for females, respectively). A NOAEL was not identified, as the incidence of marked haemosiderin deposition in the spleen was increased in female rats at 2500 ppm (equal to 174.1 mg/kg bw per day), the lowest dose tested.

In a 14-week study of toxicity in rats, triforine was administered in the diet at a concentration of 0, 100 or 500 ppm (equal to 0, 6.0 and 30.4 mg/kg bw per day for males and 0, 6.9 and 34.0 mg/kg bw per day for females, respectively). The NOAEL was 500 ppm (equal to 30.4 mg/kg bw per day), the highest dose tested.

In a 90-day study of toxicity and neurotoxicity in rats, triforine was administered in the diet at a concentration of 0, 200, 2000 or 20 000 ppm (equal to 0, 13, 133 and 1344 mg/kg bw per day for males and 0, 15, 150 and 1540 mg/kg bw per day for females, respectively). The NOAEL for neurotoxicity was 20 000 ppm (equal to 1344 mg/kg bw per day), the highest dose tested. The NOAEL for all other effects was 200 ppm (equal to 13 mg/kg bw per day), based on evidence of haemolytic anaemia and changes in kidney and liver at 2000 ppm (equal to 133 mg/kg bw per day) and above.

In a 13-week study of toxicity in dogs, triforine was administered in the diet at a concentration of 0, 3500, 10 000 or 30 000 ppm (equal to 0, 83, 230 and 690 mg/kg bw per day for males and 0, 85, 240 and 730 mg/kg bw per day for females, respectively). A NOAEL was not identified, as signs of haemolytic anaemia were observed at all doses.

In a further 13-week study of toxicity in dogs, triforine was administered in the diet at a concentration of 0, 100, 600 or 3500 ppm (equal to 0, 3.6, 22.6 and 121.0 mg/kg bw per day for males and 0, 3.4, 21.3 and 120.7 mg/kg bw per day for females, respectively). The NOAEL was 100 ppm (equal to 3.4 mg/kg bw per day), on the basis of increased haemosiderin deposits in the liver, spleen and bone marrow at 600 ppm (equal to 21.3 mg/kg bw per day) and above.

In a 2-year study of toxicity in dogs, triforine was administered in the diet at a concentration of 0, 10, 40, 100 or 1000 ppm (equal to 0, 0.23, 0.93, 2.39 and 22.50 mg/kg bw per day for males and 0, 0.25, 0.99, 2.56 and 23.60 mg/kg bw per day for females, respectively). The NOAEL was 100 ppm (equal to 2.39 mg/kg bw per day), based on evidence of haemolytic anaemia, increased erythropoiesis and haemosiderin deposition in the liver and bone marrow at 1000 ppm (equal to 22.50 mg/kg bw per day).

As the pattern of changes in the 13-week and 2-year dog studies was similar, the overall NOAEL was 100 ppm (equal to 3.4 mg/kg bw per day). The overall LOAEL was 600 ppm (equal to 21.3 mg/kg bw per day).

In a 105-week carcinogenicity study in mice, triforine was administered in the diet at a concentration of 0, 70, 700 or 7000 ppm (equal to 0, 11.4, 117 and 1204 mg/kg bw per day for males and 0, 15.9, 161 and 1570 mg/kg bw per day for females, respectively). The NOAEL for systemic toxicity was 70 ppm (equal to 11.4 mg/kg bw per day), based on a slight decrease in body weight gain and changes in large intestine in males at 700 ppm (equal to 117 mg/kg bw per day) and above. In males, higher incidences of hepatocellular adenoma and carcinoma at 7000 ppm were within the historical control ranges and not associated with an increase in the incidence of preneoplastic changes. In females, incidences of alveolar/bronchiolar adenoma, carcinoma and adenoma plus carcinoma at 7000 ppm were statistically significantly increased; the incidence of adenoma was slightly higher than the historical control range, whereas the incidence of carcinoma was within the historical control range. The NOAEL for carcinogenicity was 700 ppm (equal to 161 mg/kg bw per day), based on an increased incidence of lung tumours (predominantly adenomas) in females at 7000 ppm (equal to 1570 mg/kg bw per day).

In a non-GLP-compliant 2-year study of carcinogenicity in rats, triforine was administered in the diet at a concentration of 0, 25, 125, 625 or 3125 ppm (equal to mean achieved doses of 0, 1.2, 6.2, 31.2 and 158.7 mg/kg bw per day for males and 0, 1.5, 7.8, 38.6 and 195.4 mg/kg bw per day for females, respectively). The NOAEL for toxicity was 625 ppm (equal to 31.2 mg/kg bw per day), based on evidence of haemolytic anaemia at 3125 ppm (equal to 158.7 mg/kg bw per day). Triforine was not carcinogenic in this study.

In a subsequent 2-year GLP-compliant study of toxicity and carcinogenicity in rats, triforine was administered in the diet at a concentration of 0, 200, 2000 or 20 000 ppm (equal to 0, 10.3, 101 and 1038 mg/kg bw per day for males and 0, 13.1, 136 and 1436 mg/kg bw per day for females, respectively). The NOAEL for toxicity was 200 ppm (equal to 10.3 mg/kg bw per day), based on evidence of haemolytic anaemia and related changes at 2000 ppm (equal to 101 mg/kg bw per day) and above. Triforine was not carcinogenic in this study.

The Meeting concluded that triforine is carcinogenic in female mice, but not in male mice or male or female rats.

Triforine was tested for genotoxicity in an adequate range of assays, both in vitro and in vivo. It did not induce gene mutation in bacterial and mammalian cell lines, and it did not induce unscheduled DNA synthesis or DNA repair in rat hepatocytes in vitro. Structural chromosomal aberrations were inducible in vitro, but not in vivo, in a mouse bone marrow micronucleus test.

The Meeting concluded that triforine is unlikely to be genotoxic in vivo.

In view of the lack of genotoxicity in vivo, the absence of carcinogenicity in male mice and male and female rats and the fact that an increased incidence of lung tumours (predominantly adenomas) was observed only in female mice at the highest dose tested, the Meeting concluded that triforine is unlikely to pose a carcinogenic risk to humans from the diet.

In a two-generation reproductive toxicity study in rats, triforine was administered in the diet at a concentration of 0, 500, 3000 or 20 000 ppm (equal to 0, 39.4, 252.5 and 1768.3 mg/kg bw per day for males and 0, 54.6, 323.3 and 2209.2 mg/kg bw per day for females, respectively). The NOAEL for reproductive toxicity was 20 000 ppm (equal to 1768.3 mg/kg bw per day), the highest dose tested. The NOAEL for parental toxicity was 500 ppm (equal to 39.4 mg/kg bw per day), based on reduced weight gain, increased spleen weight and haemosiderin deposition in the spleen at 3000 ppm (equal to 252.5 mg/kg bw per day) and above. The NOAEL for offspring toxicity was 500 ppm (equal to 39.4 mg/kg bw per day), based on reduced preweaning body weight gain at 3000 ppm (equal to 252.5 mg/kg bw per day) and above.

In a study of developmental toxicity in rats dosed at 0, 200, 500 or 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. There was no evidence of teratogenicity.

In a developmental toxicity study in which Himalayan rabbits were dosed at 0, 5, 25 or 125 mg/kg bw per day, the NOAEL for maternal toxicity was 25 mg/kg bw per day, based on a reduction in

feed consumption and body weight at 125 mg/kg bw per day during the first days of treatment. The NOAEL for embryo and fetal toxicity was 125 mg/kg bw per day, the highest dose tested.

In a second developmental toxicity study in which New Zealand White rabbits were dosed at 0, 6, 30 or 150 mg/kg bw per day, the NOAEL for maternal toxicity was 30 mg/kg bw per day, based on reductions in feed consumption and body weight gain at 150 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 150 mg/kg bw per day, the highest dose tested.

In a third developmental toxicity study in which New Zealand White rabbits were dosed at 0 or 1000 mg/kg bw per day, a NOAEL for maternal toxicity and embryo/fetal toxicity was not identified, based on reductions in body weight gain and feed consumption in dams, a slight reduction in fetal weights and a delay in ossification in fetuses at 1000 mg/kg bw per day, the only dose tested. There was no evidence of teratogenicity.

The Meeting concluded that triforine is not teratogenic.

The Meeting concluded that triforine is not neurotoxic, based on the 90-day study in rats described previously.

In a 28-day dietary study in mice and rats, no immunotoxic effects were seen up to the highest dose tested (1115 mg/kg bw per day in both species).

The Meeting concluded that triforine is not immunotoxic.

Following a 28-day administration of triforine to mice at a dietary concentration of 7000 ppm (equal to 1555.3 mg/kg bw per day) and to rats at a dietary concentration of 20 000 ppm (equal to 1956.9 mg/kg bw per day), it was concluded that triforine does not have any marked stimulatory or inhibitory effect on hepatic xenobiotic metabolism and does not produce hepatic peroxisome proliferation in either species.

Toxicological data on metabolites and/or degradates

No metabolites or degradates have been identified in plants.

Human data

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

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

Toxicological evaluation

The Meeting established an ADI of 0–0.03 mg/kg bw, derived from an overall NOAEL of 3.4 mg/kg bw per day from studies of toxicity in dogs. A safety factor of 100 was applied. The margin of exposure between the upper bound of the ADI and the LOAEL for lung tumours in female mice is greater than 50 000.

The Meeting established an ARfD of 0.3 mg/kg bw based on the NOAEL of 25 mg/kg bw per day for reduced body weight gain and reduced feed intake in dams in the first days after dosing in a rabbit developmental toxicity study. The Meeting considered the early reduction in feed intake at 25 mg/kg bw per day in this study as not relevant because it was transient, not associated with reduced body weight gain and not observed at 30 or 150 mg/kg bw per day in a second rabbit study. A safety factor of 100 was applied.

Levels relevant to risk assessment of triforine

Species	Study	Effect	NOAEL	LOAEL
Mouse	Two-year study of toxicity and carcinogenicity[a]	Toxicity	70 ppm, equal to 11.4 mg/kg bw per day	700 ppm, equal to 117 mg/kg bw per day
		Carcinogenicity	700 ppm, equal to 161 mg/kg bw per day	7 000 ppm, equal to 1 570 mg/kg bw per day
Rat	Two-year studies of toxicity and carcinogenicity[a,b]	Toxicity	200 ppm, equal to 10.3 mg/kg bw per day	2 000 ppm, equal to 101 mg/kg bw per day
		Carcinogenicity	20 000 ppm, equal to 1 038 mg/kg bw per day[c]	–
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	20 000 ppm, equal to 1 768.3 mg/kg bw per day[c]	–
		Parental toxicity	500 ppm, equal to 39.4 mg/kg bw per day	3 000 ppm, equal to 252.5 mg/kg bw per day
		Offspring toxicity	500 ppm, equal to 39.4 mg/kg bw per day	3 000 ppm, equal to 252.5 mg/kg bw per day
	Developmental toxicity study[d]	Maternal toxicity	1 000 mg/kg bw per day[c]	–
		Embryo and fetal toxicity	1 000 mg/kg bw per day[c]	–
Rabbit	Developmental toxicity study[d]	Maternal toxicity	25 mg/kg bw per day	125 mg/kg bw per day
		Embryo and fetal toxicity	125 mg/kg bw per day[c]	–
Dog	Thirteen-week and 2-year studies of toxicity[a,b]	Toxicity	100 ppm, equal to 3.4 mg/kg bw per day	600 ppm, equal to 21.3 mg/kg bw per day

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

Estimate of acceptable daily intake (ADI)

0–0.03 mg/kg bw

Estimate of acute reference dose (ARfD)

0.3 mg/kg bw

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

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

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

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid; absorption is ~80% at low dose and 10–20% at high dose
Dermal absorption	No data
Distribution	Widely distributed
Potential for accumulation	None
Rate and extent of excretion	Rapid; at low dose, ~75% within 24 hours, mainly via urine; at high dose, 77–85% via faeces within 168 hours
Metabolism in animals	Extensively metabolized; cleavage of side-chain, followed by oxidation and conjugation with glucuronic acid or glutathione
Toxicologically significant compounds in animals and plants	Triforine
Acute toxicity	
Rat, LD_{50}, oral	> 5 000 mg/kg bw
Rat, LD_{50}, dermal	> 2 000 mg/kg bw
Rat, LC_{50}, inhalation	> 5.12 mg/L
Rabbit, dermal irritation	Not irritating
Rabbit, ocular irritation	Not irritating
Guinea-pig, dermal sensitization	Not sensitizing (Maurer optimization test)
Short-term studies of toxicity	
Target/critical effect	Haematopoietic system / haemolytic anaemia
Lowest relevant oral NOAEL	3.4 mg/kg bw (dog)
Lowest relevant dermal NOAEL	1 100 mg/kg bw, highest dose tested (rat)
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Haematopoietic system / haemolytic anaemia
Lowest relevant NOAEL	10.3 mg/kg bw per day (rat)
Carcinogenicity	Lung tumours in female mice; unlikely to pose a carcinogenic risk to humans from the diet
Genotoxicity	
	Unlikely to be genotoxic in vivo
Reproductive toxicity	
Target/critical effect	No reproductive effect
Lowest relevant parental NOAEL	39.4 mg/kg bw per day
Lowest relevant offspring NOAEL	39.4 mg/kg bw per day
Lowest relevant reproductive NOAEL	1 768.3 mg/kg bw per day, highest dose tested
Developmental toxicity	
Target/critical effect	None
Lowest relevant maternal NOAEL	25 mg/kg bw per day (rabbit)

Lowest relevant embryo/fetal NOAEL	125 mg/kg bw per day, highest dose tested (rabbit)
Neurotoxicity	
Acute neurotoxicity NOAEL	No data
Subchronic neurotoxicity NOAEL	1 344 mg/kg bw per day, highest dose tested
Developmental neurotoxicity NOAEL	No data
Other toxicological studies	
Immunotoxicity NOAEL	1 115 mg/kg bw per day, highest dose tested
Medical data	
	No adverse health effects reported in workers at triforine manufacturing plants

Summary

	Value	Study	Safety factor
ADI	0–0.03 mg/kg bw	Thirteen-week and 2-year studies of toxicity (dog)	100
ARfD	0.3 mg/kg bw	Developmental toxicity study (rabbit)	100

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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.
3. Evaluation of the toxicity of pesticide residues in food. Report of the Second Joint Meeting of the FAO Committee on Pesticides in Agriculture and the WHO Expert Committee on Pesticide Residues. FAO Meeting Report, No. PL/1965/10; WHO/Food Add./26.65, 1965.
4. Evaluation of the toxicity of pesticide residues in food. FAO Meeting Report, No. PL/1965/10/1; WHO/Food Add./27.65, 1965.
5. Evaluation of the hazards to consumers resulting from the use of fumigants in the protection of food. FAO Meeting Report, No. PL/1965/10/2; WHO/Food Add./28.65, 1965.
6. Pesticide residues in food. Joint report of the FAO Working Party on Pesticide Residues and the WHO Expert Committee on Pesticide Residues. FAO Agricultural Studies, No. 73; WHO Technical Report Series, No. 370, 1967.
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This volume contains toxicological monographs that were prepared by the 2014 Joint FAO/WHO Meeting on Pesticide Residues (JMPR), which met in Rome on 16–25 September 2014.

The monographs in this volume summarize the safety data on 10 pesticides that could leave residues in food commodities. These pesticides are aminocyclopyrachlor, cyflumetofen, dichlobenil, flufenoxuron, imazamox, mesotrione, metrafenone, myclobutanil, pymetrozine and triforine. 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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