Pesticide residues in food — 2018

Joint FAO/WHO Meeting on Pesticide Residues

EVALUATIONS 2018

Part II — Toxicological

Pesticide residues in food – 2018

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

Berlin, Germany, 18–27 September 2018

The summaries and evaluations contained in this book are, in most cases, based on unpublished proprietary data submitted for the purpose of the JMPR assessment. A registration authority should not grant a registration on the basis of an evaluation unless it has first received authorization for such use from the owner who submitted the data for JMPR review or has received the data on which the summaries are based, either from the owner of the data or from a second party that has obtained permission from the owner of the data for this purpose.

Pesticide residues in food - 2018: toxicological evaluations / 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, Berlin, Germany, 18–27 September 2018

ISBN 978-92-4-165533-0

© World Health Organization and Food and Agriculture Organization of the United Nations, 2019

Some rights reserved. This work is available under the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 IGO licence (CC BY-NC-SA 3.0 IGO; https://creativecommons.org/licenses/by-nc-sa/3.0/igo/).

Under the terms of this licence, you may copy, redistribute and adapt the work for non-commercial purposes, provided the work is appropriately cited, as indicated below. In any use of this work, there should be no suggestion that the World Health Organization (WHO) or the Food and Agriculture Organization of the United Nations (FAO) endorse any specific organization, products or services. The use of the WHO or FAO logo is not permitted. If you adapt the work, then you must license your work under the same or equivalent Creative Commons licence. If you create a translation of this work, you should add the following disclaimer along with the suggested citation: "This translation was not created by the World Health Organization (WHO) or the Food and Agriculture Organization of the United Nations (FAO). WHO and FAO are not responsible for the content or accuracy of this translation. The original English edition shall be the binding and authentic edition".

Any mediation relating to disputes arising under the licence shall be conducted in accordance with the mediation rules of the World Intellectual Property Organization http://www.wipo.int/amc/en/mediation/rules.

Suggested citation. Pesticide residues in food - 2018: toxicological evaluations / 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, Berlin, Germany, 18–27 September 2018. Geneva: World Health Organization and Food and Agriculture Organization of the United Nations; 2019. Licence: CC BY-NC-SA 3.0 IGO.

Cataloguing-in-Publication (CIP) data. CIP data are available at http://apps.who.int/iris.

Sales, rights and licensing. To purchase WHO publications, see http://apps.who.int/bookorders. To submit requests for commercial use and queries on rights and licensing, see http://www.who.int/about/licensing.

Third-party materials. If you wish to reuse material from this work that is attributed to a third party, such as tables, figures or images, it is your responsibility to determine whether permission is needed for that reuse and to obtain permission from the copyright holder. The risk of claims resulting from infringement of any third-party-owned component in the work rests solely with the user.

WHO Photographs. WHO photographs are copyrighted and are not to be reproduced in any medium without obtaining prior written permission. Requests for permission to reproduce WHO photographs should be addressed to: http://www.who.int/about/licensing/copyright_form/en/

The designations employed and the presentation of the material in this publication do not imply the expression of any opinion whatsoever on the part of WHO or FAO concerning the legal or development status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. Dotted lines on maps represent approximate border lines for which there may not yet be full agreement.

The mention of specific companies or of certain manufacturers' products, whether or not these have been patented, does not imply that they are endorsed or recommended by WHO or FAO in preference to others of a similar nature that are not mentioned. Errors and omissions excepted, the names of proprietary products are distinguished by initial capital letters.

All reasonable precautions have been taken by WHO and FAO to verify the information contained in this publication. However, the published material is being distributed without warranty of any kind, either expressed or implied. The responsibility for the interpretation and use of the material lies with the reader. In no event shall WHO and FAO be liable for damages arising from its use.

This publication contains the collective views of an international group of experts and does not necessarily represent the decisions or the policies of WHO or FAO.

TABLE OF CONTENTS

Page

List of participants iv

Abbreviations used.......... viii

Introduction.......... xii

Toxicological monographs and monograph addenda 1
- Chlorfenapyr 3
- Ethiprole* 11
- Fenpicoxamid* 57
- Fluxapyroxad.......... 107
- Imazalil** 129
- Kresoxim-methyl** 225
- Lambda-cyhalothrin 279
- Mandestrobin* 295
- Mandipropamid 407
- Norflurazon* 421
- Pydiflumetofen* 475
- Pyraclostrobin 585
- Pyriofenone* 651
- Tioxazafen* 711

Annex 1. Reports and other documents resulting from previous Joint Meetings of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group on Pesticide Residues 769

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

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

Berlin, 18–27 September 2018

List of participants

Mr Davide Arcella, Evidence Management Unit (DATA), European Food Safety Authority, Via Carlo Magno 1/a, I-43126 Parma, Italy *(WHO Expert)*

Ms Janis Baines, Canberra, Australian Capital Territory, Australia *(WHO Expert)*

Professor Alan R. Boobis, Centre for Pharmacology & Therapeutics, Department of Medicine, Faculty of Medicine, Imperial College London, Hammersmith Campus, Ducane Road, London W12 0NN, United Kingdom *(WHO Expert)*

Ms Marloes Busschers, Senior Regulatory Toxicologist, Charles River, PO Box 3476, 5203 DL, ’s-Hertogenbosch, the Netherlands *(WHO Expert)*

Dr Carl E. Cerniglia, Director, Division of Microbiology, National Center for Toxicological Research, HFT-250, United States Food and Drug Administration, 3900 NCTR Road, Jefferson, Arkansas 72079, United States of America (USA) *(WHO Expert)*

Dr Julian Cudmore, Chemistry Team, Chemicals Regulation Division, Health & Safety Executive, Room 1E, Mallard House, Kings Pool, 3 Peasholme Green, York YO1 7PX, United Kingdom *(FAO Expert)*

Dr Esther de Jong, Board for the Authorisation of Plant Protection Products and Biocides, Bemmekomseweg 41, 6717 LL Ede, the Netherlands *(WHO Expert)*

Dr Ian Dewhurst, Sunnycroft, Leavening, North Yorkshire YO17 9SA, United Kingdom *(WHO Expert)*

Dr Michael DiNovi, Supervisory Chemist, Center for Food Safety and Applied Nutrition, United States Food and Drug Administration, 5001 Campus Drive, HFS-255, College Park, Maryland 20704, USA *(WHO Expert)*

Dr Michael Doherty, Office of Pesticide Programs, Health Effects Division, United States Environmental Protection Agency, Room 10347, One Potomac Yard, 2777 South Crystal Drive, Arlington, Virginia 22202, USA *(FAO Chairman)*

Professor Eloisa Dutra Caldas, Pharmaceutical Sciences Department, College of Health Sciences, University of Brasilia, Campus Universitario Darci Ribeiro, Brazil *(FAO Expert)*

Dr David Eastmond, Department of Molecular, Cell and Systems Biology, 2109 Biological Sciences Building, University of California, Riverside, California 92521, USA *(WHO Expert)*

Dr Jochen Heidler, Residues and Analytical Methods Unit, Department of Pesticide Safety, Federal Institute for Risk Assessment, Max-Dohrn-Strasse 8-10, D-10589 Berlin, Germany *(FAO Expert)*

Dr Paul Humphrey, Residues and Trade Section, Scientific Assessment and Chemical Review Program, Australian Pesticides and Veterinary Medicines Authority, 18 Worland Street, Symonston, Australian Capital Territory 2609, Australia *(FAO Expert)*

Mr Makoto Irie, Agricultural Chemicals Office, Plant Products Safety Division, Food Safety and Consumer Affairs Bureau, Ministry of Agriculture, Forestry and Fisheries, 1-2-1 Kasumigaseki, Chiyoda-ku, Tokyo 100-8950, Japan *(FAO Expert)*

Dr Debabrata Kanungo, Chairman, Scientific Panel on Residues of Pesticides, Food Safety and Standards Authority of India, Nityakshetra 294/Sector-21D, Faridabad 121005, India *(WHO Expert)*

Dr April Kluever, Toxicologist, Division of Food Contact Notifications, Center for Food Safety and Applied Nutrition, United States Food and Drug Administration, 4300 River Road, HFS-275, College Park, Maryland 20740, USA *(WHO Expert)*

Dr Jean-Charles Leblanc, French Agency for Food, Environmental and Occupational Health and Safety, 14 rue Pierre et Marie Curie, 94701 Maisons-Alfort, France *(WHO Expert)*

Dr Mi-Gyung Lee, Department of Food Science & Biotechnology, College of Natural Science, Andong National University, 1375 Gyeongdong-ro, Andong-si, Gyeongbuk 760-749, Republic of Korea *(FAO Expert)*

Ms Kimberley Low, Health Evaluation Directorate, Pest Management Regulatory Agency, Health Canada, Sir Charles Tupper Building, 2720 Riverside Drive, Ottawa, Ontario, Canada K1A 0K9 *(WHO Expert)*

Mr David Lunn, Principal Adviser (Residues), Plant, Food & Environment Directorate, Ministry for Primary Industries, PO Box 2526, Wellington, New Zealand *(FAO Rapporteur)*

Dr Dugald MacLachlan, Department of Agriculture and Water Resources, Australian Government, GPO Box 858, Canberra, Australian Capital Territory 2601, Australia *(FAO Expert)*

Ms Karin Mahieu, Department of Food Safety, Centre for Nutrition Prevention and Health Services, National Institute for Public Health and the Environment (RIVM), PO Box 1, 3720 BA Bilthoven, the Netherlands *(FAO Expert)*

Dr Farag Mahmoud Malhat, Pesticide Residues, Central Agricultural Pesticide Laboratory, Environmental Pollution Department, 7-Nadi El-Saad Street, Dokki, Giza 12618, Egypt *(FAO Expert)*

Professor Angelo Moretto, Department of Biomedical and Clinical Sciences, University of Milan, Director, International Centre for Pesticides and Health Risk Prevention (ICPS), ASST Fatebenefratelli Sacco, Via GB Grassi 74, 20157 Milan, Italy *(WHO Expert)*

Dr Lars Niemann, Department of Pesticide Safety, Federal Institute for Risk Assessment, Max-Dohrn-Strasse 8-10, D-10589 Berlin, Germany *(WHO Expert)*

Dr Canping Pan, Department of Applied Chemistry, College of Science, China Agricultural University, Yuanminyuan Western Road No. 2, Beijing 2100193, China *(FAO Expert)*

Dr Chris Schyvens, Health Assessment Team, Scientific Assessment and Chemical Review Program, Australian Pesticides and Veterinary Medicines Authority, 18 Wormald Street, Symonston, Australian Capital Territory 2609, Australia *(WHO Expert)*

Dr Prakashchandra V. Shah, Chief, Chemistry, Inerts and Toxicology Assessment Branch, Registration Division (MDTS 7505P), Office of Pesticide Programs, United States Environmental Protection Agency, 1200 Pennsylvania Avenue NW, Washington, DC 20460, USA *(WHO Expert)*

Mr Christian Sieke, Residues and Analytical Methods Unit, Department of Pesticide Safety, Federal Institute for Risk Assessment, Max-Dohrn-Strasse 8-10, D-10589 Berlin, Germany *(FAO Expert)*

Ms Monique Thomas, Pest Management Regulatory Agency, Health Canada, 2720 Riverside Drive, Ottawa, Ontario, Canada K1A 0K9 *(FAO Expert)*

Dr Luca Tosti, International Centre for Pesticides and Health Risk Prevention (ICPS), ASST Fatebenefratelli Sacco, Polo Universitario, Padiglione 17, Via GB Grassi 74, 20157 Milan, Italy *(WHO Expert)*

Ms Julie Van Alstine, Risk Assessment Branch 6, Health Effects Division, Office of Pesticide Programs, United States Environmental Protection Agency, MC 7509P, Washington, DC 20460, USA *(FAO Expert)*

Ms Trijntje Van der Velde-Koerts, Centre for Nutrition, Prevention and Health Services (VPZ), National Institute for Public Health and the Environment (RIVM), Antonie van Leeuwenhoeklaan 9, PO Box 1, 3720 BA Bilthoven, the Netherlands *(FAO Expert)*

Dr Takahiro Watanabe, National Institute of Health Sciences, Ministry of Health, Labour and Welfare, Kawasaki, Japan *(FAO Expert)*

Dr Gerrit Wolterink, Centre for Nutrition, Prevention and Health Services (VPZ), National Institute for Public Health and the Environment (RIVM), Antonie van Leeuwenhoeklaan 9, 3720 BA Bilthoven, the Netherlands *(WHO Expert)*

Dr Yukiko Yamada, Ministry of Agriculture, Forestry and Fisheries, 1-2-1 Kasumigaseki, Chiyoda-ku, Tokyo 100-8950, Japan *(FAO Expert)*

Dr Guibiao Ye, Institute for the Control of Agrochemicals, Ministry of Agriculture, Maizidian 22, Chaoyang District, Beijing 100125, China *(FAO Expert)*

Dr Midori Yoshida, Commissioner, Food Safety Commission, Cabinet Office, Akasaka Park Building, 22nd Floor, 5-2-20 Akasaka Minato-ku, Tokyo 107-6122, Japan *(WHO Expert)*

Dr Katsuhiko Yoshizawa, Mukogawa Women's University, 6-46 Ikebiraki-cho, Nishinomiya, Hyogo 663-8558, Japan *(WHO Expert)*

Dr Jürg Zarn, Federal Food Safety and Veterinary Office, Schwarzenburgstrasse 155, CH-3003 Bern, Switzerland *(WHO Expert)*

Secretariat

Ms Emanuela Aquilini, Plant Production and Protection Division, Food and Agriculture Organization of the United Nations, Viale delle Terme di Caracalla, 00153 Rome, Italy *(FAO JMPR Secretariat)*

Mr Kevin Bodnaruk, West Pymble, New South Wales 2073, Australia *(FAO Editor)*

Ms Grazia Chiu, Plant Production and Protection Division, Food and Agriculture Organization of the United Nations, Viale delle Terme di Caracalla, 00153 Rome, Italy *(FAO JMPR Secretariat)*

Ms Myoengsin Choi, Food Standards Officer, Joint FAO/WHO Food Standards Programme, Food and Agriculture Organization of the United Nations, Viale delle Terme di Caracalla, 00153 Rome, Italy *(Codex Secretariat)*

Dr Vittorio Fattori,[1] Agriculture and Consumer Protection Department, Food and Agriculture Organization of the United Nations, Viale delle Terme di Caracalla, 00153 Rome, Italy *(JECFA FAO Secretariat)*

Dr Jeevan Khurana, Usingen 61250, Germany *(FAO Editor)*

Dr Xiongwu Qiao, Shanxi Academy of Agricultural Sciences, 81 Longcheng Street, Taiyuan, Shanxi 030031, China *(CCPR Chairman)*

Dr Markus Lipp,[1] Agriculture and Consumer Protection Department, Food and Agriculture Organization of the United Nations, Viale delle Terme di Caracalla, 00153 Rome, Italy *(JECFA FAO Secretariat)*

Ms Marla Sheffer, Orleans, Ontario, Canada K1E 2K5 *(WHO Editor)*

Dr Philippe Verger, Department of Food Safety and Zoonoses (FOS), World Health Organization, 1211 Geneva 27, Switzerland *(WHO JMPR Secretariat)*

[1] Participated in discussion on the topic of the harmonization of residue definitions.

Ms Yong Zhen Yang, Plant Production and Protection Division, Food and Agriculture Organization of the United Nations, Viale delle Terme di Caracalla, 00153 Rome, Italy *(FAO JMPR Secretariat)*

Abbreviations used

ACOVA	covariance analysis
Acox1	acyl-coenzyme A oxidase 1
AD	administered dose
ADI	acceptable daily intake
ADME	absorption, distribution, metabolism and excretion
AFC	antibody-forming cell
AhR	aryl hydrocarbon receptor
ALP	alkaline phosphatase
ALT	alanine aminotransferase
APTT	activated partial thromboplastin time
AR	accumulation ratio; androgen receptor
ARfD	acute reference dose
AST	aspartate aminotransferase
ATP	adenosine-5′-triphosphate
AUC	area under the concentration–time curve
$AUC_{120\ h}$	area under the concentration–time curve at 120 hours
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
AUC_{last}	area under the concentration–time curve at the last time point
BMD	benchmark dose
$BMDL_{10}$	lower confidence limit on the benchmark dose for a 10% response
BQ	benzyloxyquinoline *O*-debenzylation
BrdU	5-bromo-2′-deoxyuridine
BROD	7-benzyloxyresorufin *O*-debenzylase; 7-benzyloxyresorufin *O*-debenzylation
bw	body weight
C	concentration
CAR	constitutive androstane receptor
CAS	Chemical Abstracts Service
CHL	Chinese hamster lung
CHO	Chinese hamster ovary
CI	confidence interval
Cipro	ciprofibrate
CITCO	6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde-*O*-(3,4-dichlorobenzyl) oxime
C_{max}	maximum concentration
CoA	coenzyme A
CPA	cyclophosphamide
CPN	chronic progressive nephropathy
CPS	cyclophosphamide monohydrate
CYP	cytochrome P450
D	dose
DEN	*N*-nitrosodiethylamine
DMSO	dimethyl sulfoxide
DN	dose-normalized
DNA	deoxyribonucleic acid
dUTP	deoxyuridine triphosphate
EBSS	Earle's Balanced Salt Solution
EC_{50}	median effective concentration
ECOD	7-ethoxycoumarin *O*-deethylation; 7-ethoxycoumarin *O*-deethylase
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EMD	*N*-ethylmorphine *N*-demethylation; *N*-ethylmorphine *N*-demethylase
EMS	ethyl methane sulfonate

eq	equivalent
Eq	equivalent
equiv	equivalent
ER	estrogen receptor
EROD	ethoxyresorufin *O*-deethylation; ethoxyresorufin *O*-deethylase
F	female
F	bioavailability
F_0	parental generation
F_1	first filial generation
F_2	second filial generation
FAO	Food and Agriculture Organization of the United Nations
FOB	functional observational battery
GD	gestation day
GGT	gamma-glutamyl transferase
GI	gastrointestinal
GLP	good laboratory practice
GST	glutathione *S*-transferase
GST-P	placental form of glutathione *S*-transferase
hAR	human androgen receptor
HCT	haematocrit
HDL	high-density lipoprotein
HDW	haemoglobin distribution width
H&E	haematoxylin and eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hERα	human estrogen receptor alpha
HGB	haemoglobin
HPLC	high-performance liquid chromatography
HPRT	hypoxanthine–guanine phosphoribosyltransferase
HTCP	hydroxylated 2,4,6-trichlorophenol
IC_{50}	median inhibitory concentration
IgM	immunoglobulin M
ILSI	International Life Sciences Institute
ip	intraperitoneal
IPCS	International Programme on Chemical Safety
IU	international units
JMPR	Joint FAO/WHO Meeting on Pesticide Residues
KE	key event
LAH	lauric acid 12-hydroxylation; lauric acid ω-hydroxylase
LC_{50}	median lethal concentration
LC-MS	liquid chromatography–mass spectrometry
LC-MS/MS	liquid chromatography–tandem mass spectrometry
LD	lactation day
LD_{50}	median lethal dose
LDH	lactate dehydrogenase
LiHep	lithium heparin
LKE	liver key event
LLNA	local lymph node assay
LLQ	lower level of quantification
LOAEL	lowest-observed-adverse-effect level
LOD	limit of detection
LOEL	lowest-observed-effect level
LOQ	limit of quantification
LUC	large unstained cell counts
M	male
MCH	mean corpuscular (or cell) haemoglobin

MCHC	mean corpuscular (or cell) haemoglobin concentration
MCV	mean corpuscular volume
MDA	malondialdehyde
MNPCE	micronucleated polychromatic erythrocytes
MOA	mode of action
MPE	mean phototoxic effect
mRNA	messenger ribonucleic acid
MRT	mean residence time
MTF	morphological transformation frequency
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MTD	maximum tolerated dose
n	number
NA	not applicable; not available
NAD^+	nicotinamide adenine dinucleotide (oxidized)
NADH	nicotinamide adenine dinucleotide (reduced)
NC	not calculable
NCE	normochromatic erythrocyte
NE	not examined
NOAEC	no-observed-adverse-effect concentration
NOAEL	no-observed-adverse-effect level
NQ	non-quantifiable
nr	not reported
NR1I3	nuclear receptor subfamily 1, group I, member 3
NRU	neutral red uptake
OECD	Organisation for Economic Co-operation and Development
P	probability
PB	phenobarbital
PCE	polychromatic erythrocyte
PCNA	proliferating cell nuclear antigen
PCO	CN^--insensitive palmitoyl coenzyme A oxidation
PCoA	peroxisomal acyl-coenzyme A oxidase
PEG	polyethylene glycol
PH-tioxazafen	[oxadiazole-3-^{13}C, phenyl-UL-^{14}C]tioxazafen
PIF	photo-irritancy factor
PND	postnatal day
PPAR	peroxisome proliferator–activated receptor
PPARα	peroxisome proliferator–activated receptor alpha
PPARγ	peroxisome proliferator–activated receptor gamma
ppm	parts per million
PROD	pentoxyresorufin *O*-depentylation; 7-pentoxyresorufin *O*-depentylase
PT	prothrombin time
PTU	6-propyl-2-thiouracil
PWG	pathology working group
PXR	pregnane X receptor
QSAR	quantitative structure–activity relationship
R_{ac}	accumulation ratio
RBC	red blood cell
RCC	relative cell count
RDW	red cell distribution width
RICC	relative increase in cell count
RNA	ribonucleic acid
RPE	relative plating efficacy
S9	9000 × *g* supernatant fraction of rat liver homogenate
SAR	structure–activity relationship

SCE	sister chromatid exchange
SD	standard deviation; Sprague Dawley
SDH	sorbitol dehydrogenase
SER	smooth endoplasmic reticulum
SPF	specific pathogen–free
sRBC	sheep red blood cell
t	time
$t_{½}$	terminal half-life
$t_{½ \mathrm{el}}$	elimination half-life
T_3	triiodothyronine
T_4	thyroxine
TBG	thyroxine binding globulin
tBIL	total bilirubin
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TCDF	2,3,7,8-tetrachlorodibenzofuran
TCP	trichlorophenol
TCPM	trichlorophenol methanol
TCPOBOP	1,4-bis[2-(3,5-dichloropyridyloxy)]benzene
TDAR	T cell–dependent antibody response
TH-tioxazafen	[oxadiazole-5-^{13}C, thiophene-2-^{14}C]tioxazafen
tk	thymidine kinase
TK	thymidine kinase
TKE	thyroid key event
T_{max}	time to reach maximum concentration
TRH	thyrotropin-releasing hormone
Trog	troglitazone
TRR	total radioactive residues
TSH	thyroid stimulating hormone
TTC	threshold of toxicological concern
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labelling
U	units; uniformly labelled
UDP	uridine diphosphate
UDPGT	uridine diphosphate glucuronosyltransferase
UDS	unscheduled DNA synthesis
UGT	uridine diphosphate glucuronosyltransferase
UGT4	thyroxine–uridine diphosphate glucuronosyltransferase
UL	uniformly labelled
USA	United States of America
USEPA	United States Environmental Protection Agency
UVA	ultraviolet A
V_d	volume of distribution
V_{ss}	volume of distribution at steady state
v/v	volume per volume
WBC	white blood cell
WHO	World Health Organization
w/v	weight per volume

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 Berlin, Germany, on 18–27 September 2018.

Seven of the substances evaluated by the WHO Core Assessment Group (ethiprole, fenpicoxamid, mandestrobin, norflurazon, pydiflumetofen, pyriofenone and tioxazafen) were evaluated for the first time. Two compounds (imazalil and kresoxim-methyl) were re-evaluated within the periodic review programme of the Codex Committee on Pesticide Residues (CCPR). Follow-up evaluations were conducted on the remaining five compounds (chlorfenapyr, fluxapyroxad, lambda-cyhalothrin, mandipropamid and pyraclostrobin). 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 FAO as *FAO Plant Production and Protection Paper 234*. That report contains comments on the compounds considered, acceptable daily intakes and acute reference doses established by the WHO Core Assessment Group and maximum residue levels established by the FAO Panel of Experts. Monographs on residues prepared by the FAO Panel of Experts are published as a companion volume, as *Evaluations 2018, 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 2018 Joint Meeting. A special acknowledgement is made to those experts and to the experts of the Joint Meeting who reviewed early drafts of these working papers.

These monographs were prepared based on the evaluation of the original studies and the dossier provided by the sponsor(s) of the compound, of the relevant published scientific literature and of the data submitted by Codex members. When found consistent with the data of the original study, the monographs may contain parts of the text and tables of the dossier submitted by the sponsor(s), but not the conclusions of the sponsor(s). These monographs and their conclusions are based on an independent review of the available data and do not constitute an endorsement of the position of the sponsor(s).

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

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

TOXICOLOGICAL MONOGRAPHS
AND MONOGRAPH ADDENDA

CHLORFENAPYR (addendum)

First draft prepared by
April Neal-Kluever[1] and Alan R. Boobis[2]

[1] Center for Food Safety and Applied Nutrition, United States Food and Drug Administration, College Park, Maryland, United States of America (USA)
[2] Centre for Pharmacology and Therapeutics, Department of Medicine, Imperial College London, London, United Kingdom

Explanation........3
Evaluation for acceptable intake........3
1. Biochemical aspects........3
1.1 Biotransformation........3
(a) In vitro metabolism of chlorfenapyr........3
(b) Updated metabolic scheme for chlorfenapyr........6
2. Toxicological studies........7
2.1 Special studies........7
(a) Studies on metabolites........7
3. Observations in humans........7
Comments........9
Toxicological evaluation........9
References........10

Explanation

Chlorfenapyr was evaluated in 2012 by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR), which established an acceptable daily intake (ADI) of 0–0.03 mg/kg body weight (bw) and an acute reference dose (ARfD) of 0.03 mg/kg bw.

Studies with the metabolite tralopyril were reviewed by the 2013 Meeting, and a potency factor of 10 was established for a comparison of exposure to tralopyril with both the ADI and the ARfD of chlorfenapyr.

Following a request for maximum residue levels by the Codex Committee on Pesticide Residues, chlorfenapyr was placed on the agenda of the present Meeting, which assessed additional toxicological information on chlorfenapyr available since the last review.

A study on the in vitro metabolism of chlorfenapyr was submitted. An updated literature search was performed, and studies relevant to the assessment of chlorfenapyr were evaluated.

Evaluation for acceptable intake

1. Biochemical aspects

1.1 Biotransformation

(a) In vitro metabolism of chlorfenapyr

In an in vitro study (Funk-Weyer & Kemper, 2017) that was conducted in compliance with good laboratory practice (GLP), radiolabelled chlorfenapyr (phenyl or pyrrole radiolabel; Fig. 1) was incubated with Sprague Dawley rat liver hepatocytes, the 9000 × *g* supernatant (S9) fraction from rat liver homogenate or rat liver microsomes at a concentration of 1 µmol/L (hepatocytes) or 10 µmol/L (subcellular fractions). Samples from male and female rats were mixed in an approximately 1:1 ratio prior to addition of the radiolabelled compound. Experiments were performed in triplicate. Cell viability (hepatocyte incubations) was assessed using a luminescent cell viability assay. After 0, 10, 30, 60 and 180 minutes (hepatocytes) or 0, 30, 60, 180 and 360 minutes (subcellular fractions),

ethanol was added to the incubation mixtures to stop the reaction. The samples were processed and then analysed by high-performance liquid chromatography (HPLC). Comparison of retention times, co-chromatography experiments and HPLC with mass spectrometry (HPLC-MS) experiments with reference compounds were used for the identification of parent compound and metabolites.

Fig. 1. Chlorfenapyr radiolabels: phenyl-U-^{14}C and pyrrole-2-^{14}C

[Phenyl-U-^{14}C]Chlorfenapyr

Specific activity: 7.64 MBq/mg
Radiochemical purity: 99.7%
Chemical purity: 100.0%

[Pyrrole-2-^{14}C]Chlorfenapyr

Specific activity: 5.28 MBq/mg
Radiochemical purity: 99.3%
Chemical purity: 98.2%

Source: Funk-Weyer & Kemper (2017)

Positive control chemicals were used to demonstrate the sensitivity of the assay to detect phase I and phase II metabolic reaction products. Appropriate negative control chemicals were used for each assay and both radiolabels.

The viability of the hepatocytes after a 180-minute incubation with unlabelled chlorfenapyr (BAS 306 I; batch no. L76-56; purity 99.6%) at 5 or 10 µmol/L was less than or equal to 53% of the viability of the cells incubated without the test item, whereas the viability was 121% at a concentration of 1 µmol/L. Therefore, a chlorfenapyr concentration of 1 µmol/L was chosen for the incubation experiments. The viability of the hepatocytes after a 180-minute incubation with radiolabelled chlorfenapyr at 1 µmol/L was 74% (phenyl label) and 105% (pyrrole label) compared with the viability without the test item. The viability studies after exposure to unlabelled or radiolabelled chlorfenapyr were performed in triplicate.

Recoveries of the applied radioactivity in the in vitro assays with chlorfenapyr were investigated for the hepatocyte, microsome and S9 experiments. For hepatocytes, the recovery in the supernatants was 71.9–95.2%. For microsomes, the recovery in the supernatants was 66.1–78.1%. The sum recovery (supernatant, extract(s) and remaining pellet) was generally around 90% (the lowest value was 85.1%). For the S9 fraction, the recovery in the supernatants was 67.3–79.2%. The sum recovery was generally around 90% (the two lowest values were 81.8%).

Chlorfenapyr was detected at all time points for hepatocytes, microsomes and S9 fraction. The concentration of chlorfenapyr generally decreased with duration of incubation.

Tralopyril was identified in almost all test systems and for both radiolabels, and its concentration as a percentage of the applied radioactivity increased with time (Table 1). There was one exception: tralopyril was not detected in the hepatocyte system after a 180-minute incubation with [pyrrole-2-^{14}C]chlorfenapyr, presumably as a result of further metabolism. The data suggest that tralopyril was formed directly from parent chlorfenapyr, as no other peaks or metabolites were detected at earlier time points. However, the existence of a transient intermediary metabolite cannot be discounted. For the phenyl label, the concentration of tralopyril increased from 2.57% to 14.89% of the applied radioactivity (hepatocytes), from 3.02% to 9.74% of the applied radioactivity

(microsomes) and from 2.75% to 8.63% of the applied radioactivity (S9 fraction); for the pyrrole label, the concentration of tralopyril increased from 1.73% to 5.30% of the applied radioactivity (hepatocytes), from 1.94% to 11.55% of the applied radioactivity (microsomes) and from 1.93% to 6.04% of the applied radioactivity (S9 fraction).

Table 1. Time course of the metabolism of chlorfenapyr (identified components)

Test system	Incubation time (min)	Chlorfenapyr[a] (% AR)	Tralopyril[a] (% AR)	Metabolite M-5[a] (% AR)
Phenyl label				
Hepatocytes	0	86.36	2.57	–
	10	90.42	4.04	–
	30	91.62	6.05	–
	60	88.42	9.75	–
	180	68.38	14.89	–
Microsomes	0	84.31	3.02	–
	30	85.85	3.36	–
	60	82.11	4.49	–
	180	80.43	6.11	–
	360	75.42	9.74	0.35
S9 fraction	0	81.73	2.75	–
	30	84.53	3.62	–
	60	83.66	4.49	–
	180	81.05	6.91	–
	360	79.84	8.63	0.48
Pyrrole label				
Hepatocytes	0	91.15	1.73	–
	10	87.81	3.08	–
	30	89.61	3.98	–
	60	93.21	5.30	–
	180	65.46	–	–
Microsomes	0	87.86	1.94	–
	30	86.08	3.92	–
	60	84.00	5.70	–
	180	79.54	8.86	–
	360	71.58	11.55	0.72

Test system	Incubation time (min)	Chlorfenapyr[a] (% AR)	Tralopyril[a] (% AR)	Metabolite M-5[a] (% AR)
S9 fraction	0	89.11	1.93	–
	30	87.54	3.17	–
	60	87.21	4.01	–
	180	85.82	5.20	–
	360	85.79	6.04	–

AR: applied radioactivity; S9: 9000 × *g* supernatant fraction from rat liver homogenate
[a] Sum % AR (mean value of triplicates) of respective supernatant and extract(s).
Source: Funk-Weyer & Kemper (2017)

Another metabolite was detected in the chromatograms and was determined to be metabolite M-5 (reg. no. 4110561; CL325195). Its identity was confirmed via comparison of the retention times in the HPLC chromatogram and in the chromatogram of the HPLC-MS analysis and via tandem mass spectrometry (MS/MS) analysis. This metabolite was found only in very low concentrations (0.35–0.72% of the applied radioactivity) at the longest incubation time with microsomes and the S9 fraction.

Additional compounds were found at the longest incubation time (180 minutes) in hepatocytes for the pyrrole label. Most likely they are only some of the multiple metabolites that are formed from tralopyril or metabolite M-5 (Funk-Weyer & Kemper, 2017).

(b) Updated metabolic scheme for chlorfenapyr

Based on the results of the in vitro study by Funk-Weyer & Kemper (2017), the sponsor provided an updated scheme for the metabolism of chlorfenapyr (Fig. 2).

Fig. 2. Metabolic pathways for chlorfenapyr

Chlorfenapyr
(BAS 306 I)

Tralopyril

Further metabolites

Metabolite M-5

Source: BASF (2017)

2. Toxicological studies

No toxicological studies were submitted for the current evaluation.

The sponsor provided the results of a literature search. Six online databases were searched with various terms describing chlorfenapyr and/or tralopyril. Emphasis was placed on records providing information about additional, new, unknown or potentially contradictory effects or data that might have an impact on the hazard assessment end-points or the hazard characterization and that, in addition, have a high grade of reliability (i.e. grade 1 or 2 based on the “Klimisch” scoring system).

In total, 32 studies were retrieved, of which 20 were considered relevant to the chlorfenapyr risk assessment. Reliability scores for these records ranged between 2 and 4. Only records with a score of 1, 2 or 3 (12 records) were considered potentially useful to the hazard identification or hazard characterization by the sponsor. However, upon review, it was determined that some records provided only summary information or did not provide new evidence relevant to the assessment (five records). None of the remaining seven studies (all case reports; see section 3 below) was relevant to the toxicological assessment.

2.1 Special studies

(a) Studies on metabolites

Six metabolites were identified in residue data that were not addressed by previous Meetings. These metabolites are CL152837 (M-4), CL325195 (M-5), CL322250 (M-5A), CL152835 (M-6), CL325157 (M-6A) and CL152832 (M-7A).

The sponsor provided the results of quantitative structure–activity relationship (QSAR) assessments (Derek Nexis and Organisation for Economic Co-operation and Development [OECD] QSAR Toolbox version 4.2). In both analyses, the metabolites were considered negative for genotoxicity alerts. The OECD QSAR Toolbox identified all six metabolites as Class III according to the Cramer classification method.

3. Observations in humans

Seven case reports identified in the literature search described above in section 2 described accidental or intentional ingestion or injection (one case) of chlorfenapyr formulations (Table 2). Chlorfenapyr exposure was not confirmed in any of these case reports via analytical methods. The patients died in all but one case. In general, patients exhibited extreme diaphoresis, rhabdomyolysis, neurological dysfunction and cardiac arrest prior to death. In a few cases, the neurological effects were characterized through imaging techniques, and white matter lesions were observed. These lesions are consistent with the white matter effects observed in animal toxicity studies following repeated doses of chlorfenapyr or tralopyril (Foss, 1994; Mellert, 2004). In several cases, a latency period was observed after ingestion, during which the patient did not exhibit signs or symptoms of toxicity. The latency period lasted roughly 2 weeks. Once signs or symptoms manifested, the clinical courses were similar. Sign or symptom onset, severity and latency between exposure and death appeared dose dependent where estimates of intake were possible.

Table 2. Summary of case-studies on chlorfenapyr poisonings[a]

Sex; age	Exposure	Dose estimate (mg/kg bw)	Route	Clinical observations	Outcome	Reference
Male; 55 years	250 mL, 10% chlorfenapyr	420	Ingestion	No latency before onset	Death 5 days after	Choi et al.

Sex; age	Exposure	Dose estimate (mg/kg bw)	Route	Clinical observations	Outcome	Reference
old	solution			Diaphoresis, renal failure, rhabdomyolysis, confusion, coma, fever, cardiac arrest	exposure	(2010)
Female; 28 years old	Formulation not reported	NA	Ingestion	Five-day latency before onset Progressive weakness and limb pain, swaying, drowsiness, paralysis, demyelination (MRI), coma, fever	Death 10 days after exposure	Tharaknath et al. (2013)
Male; 80 years old	200 mL, 10% chlorfenapyr solution	330	Ingestion	Three-day latency before onset Abdominal pain, diaphoresis, acute pancreatitis	Death 11 days after exposure	Huang & Lin (2015)
Female; 44 years old	10% chlorfenapyr solution (immediately expectorated)	NA	Ingestion	Fourteen-day latency before onset Bilateral leg weakness, urinary incontinence, hypoaesthesia below T11 dermatome, paraplegia, leukoencephalopathy (MRI)	Survival with unresolved paraplegia	Baek et al. (2016)
Female; 41 years old	20 mL, formulation not reported	NA	Ingestion	Fourteen-day latency before onset Nausea, weakness, drowsiness, fever, cerebral swelling and low-density white matter lesions (CT), diaphoresis, cardiac arrest	Death 14 days after exposure	Kang et al. (2014)
Male; 49 years old	200 mL, 6% chlorfenapyr solution[b]	200	Ingestion	No latency before onset Diaphoresis, dizziness, weakness, abdominal pain, tachypnoea, bilateral symmetric white matter lesions (MRI), diffuse cerebral dysfunction (EEG), fever, cardiac arrest	Death 14 days after exposure	Kwon et al. (2012)
Male; 74 years old	20 mL, formulation not reported	NA	Intraperitoneal injection	No latency before onset Diaphoresis, bowel ischaemia and sepsis, fever, cardiac arrest	Death 12 days after exposure	Lee et al. (2013)

bw: body weight; CT: computer tomography; EEG: electroencephalogram; MRI: magnetic resonance imaging; NA: not available

[a] Doses were estimated using a default body weight of 60 kg and the conversion of 1% solution = 1 g/1000 mL.

[b] Exposure was to “a combined product of chlorfenapyr and clothianidine”.

Comments

Biochemical aspects

Tralopyril was shown to be the main phase I metabolite in a GLP-compliant in vitro study on the metabolism of chlorfenapyr in rats (Funk-Weyer & Kemper, 2017). In the context of the data previously reviewed by JMPR, this study provides assurance that rats exposed to chlorfenapyr are also exposed systemically to tralopyril. It was not possible to estimate quantitative exposure to this metabolite in vivo from this study.

Toxicological data

No new information on the toxicity of chlorfenapyr or tralopyril was submitted to the 2018 Meeting, nor was any new information identified in the open literature.

Toxicological data on metabolites and/or degradates

Six metabolites were identified in residue studies that were not addressed by previous Meetings and were of potential relevance for the residue definition for risk assessment. These metabolites are CL152837 (M-4), CL325195 (M-5), CL322250 (M-5A), CL152835 (M-6), CL325157 (M-6A) and CL152832 (M-7A). No toxicological data were submitted for these metabolites, but they did not show any alerts for genotoxicity. For chronic toxicity, the threshold of toxicological concern (TTC) approach can be applied (Cramer class III).

Human data

Several case reports were identified in the scientific literature that described human poisonings after self-reported ingestion or, in one case, intra-abdominal injection of chlorfenapyr solutions. Neurological signs and symptoms (diaphoresis, dizziness, weakness, fever, paralysis) were common and progressed in severity until death by cardiac arrest in all but one case (Choi et al., 2010; Kwon et al., 2012; Lee et al., 2013; Tharaknath et al., 2013; Kang et al., 2014; Huang & Lin, 2015; Baek et al., 2016). In some cases, imaging techniques were used to characterize white matter lesions in the patients, which appeared consistent with the effects noted after chlorfenapyr or tralopyril exposure in rats (Foss, 1994; Mellert, 2004). In cases where an estimate of dose was feasible, lethal doses appeared to range from 200 to 420 mg/kg bw (compared with the rat median lethal dose [LD_{50}] of 441 mg/kg bw; Lowe, 1993). The Meeting noted that the ARfD provided a margin of four orders of magnitude relative to these estimated lethal doses.

The human clinical data suggest that, compared with rats, humans are of similar or greater sensitivity to the acute effects of chlorfenapyr, and the toxicological consequences to the nervous system are very similar. There is no information available on the comparative metabolism of chlorfenapyr in rats and humans.

Toxicological evaluation

The Meeting concluded that no revision of the ADI or ARfD was necessary.

The Meeting also concluded that six metabolites identified in residue studies – CL152837 (M-4), CL325195 (M-5), CL322250 (M-5A), CL152835 (M-6), CL325157 (M-6A) and CL152832 (M-7A) – were toxicologically not relevant at currently estimated dietary exposures.

References

Baek BH, Kim SK, Yoon W, Heo TW, Lee YY, Kang HK (2016). Chlorfenapyr-induced toxic leukoencephalopathy with radiologic reversibility: a case report and literature review. Korean J Radiol. 17(2):277–80.

BASF (2017). Chlorfenapyr (BAS 306 I). 'Joint Meeting on Pesticide Residues' (JMPR). Toxicology: Monograph Supplement. BASF DocID 2017/1136246. BASF Corporation, Research Triangle Park, NC, USA; and BASF SE, Crop Protection Division, Limburgerhof, Germany. Submitted to WHO by BASF.

Choi UT, Kang GH, Jang YS, Ahn H (2010). Fatality from acute chlorfenapyr poisoning. Clin Toxicol. 48(5):458–9.

Foss JA (1994). A one-year dietary neurotoxicity study with AC 303,630 in rats. Unpublished report no. CK-451-002. Argus Research Laboratories Inc., Horsham, PA, USA. Submitted to WHO by BASF.

Funk-Weyer D, Kemper C (2017). In-vitro metabolism of ^{14}C-BAS 306 I in rats. Unpublished report no. 729076. Crop Protection Ecology and Environmental Analytics, BASF SE, Limburgerhof, Germany. Submitted to WHO by BASF.

Huang H-S, Lin C-C (2015). Chlorfenapyr intoxication: a fatal case with acute pancreatitis. In: XXXV International Congress of the European Association of Poisons Centres and Clinical Toxicologists (EAPCCT), 26–29 May 2015, St Julian's, Malta. Clin Toxicol. 53(4):331. doi:10.3109/15563650.2015.1024953.

Kang C, Kim DH, Kim SC, Kim DS (2014). A patient fatality following the ingestion of a small amount of chlorfenapyr. J Emerg Trauma Shock. 7(3):239–41.

Kwon JS, Kim HY, Han HJ, Kim JY, Park JH (2012). A case of chlorfenapyr intoxication with central nervous system involvement. J Clin Toxicol. 2(8):147. doi:10.4172/2161-0495.1000147.

Lee J, Lee JH, Baek JM, Lee DS, Park IY, Won JM et al. (2013). Toxicity from intra-abdominal injection of chlorfenapyr. Case Rep Emerg Med. 2013: Article ID 425179. doi:10.1155/2013/425179.

Lowe CA (1993). Oral LD_{50} study in albino rats with AC 303,630 technical. Unpublished report no. CK-411-001. American Cyanamid Co., Princeton, NJ, USA. Submitted to WHO by BASF.

Mellert W (2004). Subchronic toxicity study in Sprague Dawley rats: administration via the diet over 3 months and recovery period of 4 weeks. Unpublished report no. 50C0238/0321. BASF Aktiengesellschaft, Ludwigshafen/Rhein, Germany. Submitted to WHO by Janssen PMP on behalf of BASF.

Tharaknath VR, Prabhakar YVS, Kumar KS, Babu NK (2013). Clinical and radiological findings in chlorfenapyr poisoning. Ann Indian Acad Neurol. 16(2):252–4.

ETHIPROLE

First draft prepared by
Marloes Busschers[1] and Jürg Zarn[2]

[1] Charles River Laboratories, 's-Hertogenbosch, the Netherlands
[2] Federal Food Safety and Veterinary Office (FSVO), Bern, Switzerland

Explanation 11
Evaluation for acceptable intake 12
1. Biochemical aspects 12
 1.1 Absorption, distribution and excretion 12
  (a) Main experiment 12
  (b) Pharmacokinetics 13
  (c) Tissue kinetics 14
  (d) Biliary excretion 14
 1.2 Biotransformation 15
2. Toxicological studies 15
 2.1 Acute toxicity 15
 2.2 Short-term studies of toxicity 15
  (a) Oral administration 15
  (b) Dermal application 25
  (c) Exposure by inhalation 25
 2.3 Long-term studies of toxicity and carcinogenicity 26
 2.4 Genotoxicity 34
 2.5 Reproductive and developmental toxicity 34
  (a) Multigeneration studies 34
  (b) Developmental toxicity 36
 2.6 Special studies 39
  (a) Acute neurotoxicity 39
  (b) Subchronic neurotoxicity 42
  (c) Mechanistic studies 43
  (d) Immunotoxic effects 44
  (e) Studies on metabolites 44
3. Observations in humans 46
Comments 46
Toxicological evaluation 50
References 53

Explanation

Ethiprole (Fig. 1) is the common name approved by the International Organization for Standardization for 5-amino-1-(2,6-dichloro-α,α,α-trifluoro-*p*-tolyl)-4-ethylsulfinylpyrazole-3-carbonitrile (International Union of Pure and Applied Chemistry), with the Chemical Abstracts Service number 181587-01-9.

Ethiprole is a non-systemic insecticide of the fiproles (phenylpyrazoles) group. It acts by interfering with the flow of chloride ions through the γ-aminobutyric acid–regulated chloride channel, thereby disrupting the central nervous system activity of insects.

Ethiprole 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) and were conducted in accordance with relevant national or international test guidelines, unless

otherwise specified. No additional information from a literature search was identified that complemented the toxicological information submitted for the current assessment.

Fig. 1. Structure of ethiprole (company codes: RPA 107382 and AE 0316423)

Note: Asterisk (*) represents position of the ^{14}C label.

Evaluation for acceptable intake

1. Biochemical aspects

1.1 Absorption, distribution and excretion

The absorption, distribution and excretion of ethiprole were studied in rats following the administration of [phenyl-U-^{14}C]ethiprole in a single oral low dose or a single oral high dose, the administration of a single oral low daily dose of non-radiolabelled ethiprole repeated for 14 days followed by administration of a radioactive dose, and in a biliary excretion study. In these studies, a single radiolabelled position was used because there was evidence to show that the molecule cleaved only minimally during the biotransformation process (McCorquodale & Anderson, 1999).

(a) Main experiment

The absorption, distribution and excretion of total radioactivity were determined following a single oral administration of [phenyl-U-^{14}C]ethiprole (batch no. 2CFS94, purity 99.7% [non-radiolabelled material]; and batch no. GRX 485A, purity 99.3% [radiolabelled material]) to two groups of five male and five female Sprague Dawley rats at 5 or 1000 mg/kg body weight (bw). A third group (five males and five females) received a single oral administration of [phenyl-U-^{14}C]ethiprole at 5 mg/kg bw following 14 daily oral administrations of non-radiolabelled ethiprole (5 mg/kg bw). Urine and faeces were collected quantitatively up to 168 hours post-dosing from all animals. The animals were humanely killed at 7 days post-dosing, and selected tissues were retained in order to investigate the tissue residues of [phenyl-U-^{14}C]ethiprole.

At the low dose, results for the cumulative recovery of total radioactivity were consistent with the findings from a pilot experiment. The majority of the dose was recovered within the first 48 hours post-dosing, with a mean of about 86% observed for both males and females. The predominant route of elimination was via faeces, with means of 67% (males) and 55% (females) of the administered dose excreted by this route by 168 hours post-dosing. Urinary excretion accounted for means of 24% and 36% of the administered dose in males and females, respectively. By the end of the 168-hour collection period, the overall mean recoveries in males and females were 92% and 94%, respectively.

Following administration of [phenyl-U-^{14}C]ethiprole to male and female rats at the high dose, excretion of total radioactivity was slower than that observed following administration at the low dose, with mean recoveries of about 77% and 64% observed by 48 hours post-dosing. The major route of elimination was again via faeces, with a mean of about 88% of the dose observed for males and females

after 168 hours. Urinary excretion accounted for means of about 3% and 5% in males and females, respectively. By the end of the 168-hour collection period, the overall mean recoveries in male and female rats were about 92% and 93%, respectively.

Results for the recovery of total radioactivity following the administration of [phenyl-U-^{14}C]ethiprole to male and female rats at the low dose after the repeated oral administration of non-radiolabelled ethiprole were similar to those obtained following the single administration of [phenyl-U-^{14}C]ethiprole at the low dose. The predominant route of elimination was via faeces, with means of about 71% (males) and 56% (females) of the dose excreted by this route. Urinary excretion accounted for means of about 23% and 35% of the dose for males and females, respectively. Excretion of the dose was rapid, with means of about 82% (males) and 86% (females) of the radioactivity recovered within the first 48 hours post-dosing. Mean recoveries of about 94% were observed in both males and females by the end of the 168-hour collection period.

Following the administration of [phenyl-U-^{14}C]ethiprole at both doses, low concentrations of radioactivity were observed in most tissues. The highest concentrations were found in the tissues associated with metabolism (liver and kidney). Concentrations of total radioactivity in whole blood were higher in females than in males both for the single low dose and following repeated administration of non-radiolabelled ethiprole. However, this difference was not evident at the high dose.

(b) Pharmacokinetics

The pharmacokinetics of [phenyl-U-^{14}C]ethiprole was investigated following oral administration. Two groups of five male and five female Sprague Dawley rats received a single oral dose of [phenyl-U-^{14}C]ethiprole at 5 or 1000 mg/kg bw. The levels of total radioactivity were measured in whole blood samples collected at intervals up to 168 hours post-dosing.

At the low dose, concentrations of total radioactivity in whole blood increased rapidly to mean peak concentrations of 2.11 and 1.63 µg equivalents (equiv)/g in males and females, respectively, at 8 hours post-dosing. Mean concentrations declined in a biphasic manner, with concentrations decreasing rapidly to 1.47 µg equiv/g (males) and 1.03 µg equiv/g (females) at 24 hours post-dosing. Thereafter, the rate of decline slowed, with low concentrations of radioactivity in whole blood from males and females (0.09 and 0.17 µg equiv/g, respectively) observed at 168 hours post-dosing.

At the high dose, concentrations of radioactivity in whole blood increased gradually to mean peak concentrations of 41.7 µg equiv/g in males at 24–48 (mean 33.6) hours post-dosing and 29.8 µg equiv/g in females at 48 hours post-dosing. Following each peak, concentrations decreased slowly to means of 2.9 µg equiv/g (males) and 3.0 µg equiv/g (females) at 168 hours post-dosing.

The mean peak concentrations in whole blood, C_{max}, were observed at 8 and 24–48 hours post-dosing (= T_{max}) in both sexes at the low and high doses, respectively. Within dose groups, T_{max} estimates demonstrated minimal variability. Whole blood concentrations of [phenyl-U-^{14}C]ethiprole had mean elimination half-lives ($t_{½\ el}$) of between 44.33 and 49.18 hours, except in low-dose females (113.98 hours), for which interanimal variation was more pronounced. This parameter was not, however, considered to change significantly as a function of dose.

Estimates for C_{max} and both area under the concentration–time curve (AUC) parameters increased with increasing dose of [phenyl-U-^{14}C]ethiprole. The mean $AUC_{(0-t)}$ was 94.88 and 79.38 µg equiv·h/mL for males and females, respectively, at the low dose and 2620 and 2240 µg equiv·h/mL for males and females, respectively, at the high dose. $AUC_{(0-\infty)}$ and C_{max} demonstrated similar trends. This increase was not dose proportional, as a 200-fold increase in dose resulted in fold increases (males and females, respectively) of 28 and 22 for $AUC_{(0-\infty)}$, 28 and 28 for $AUC_{(0-t)}$ and 20 and 18 for C_{max}. There was no consistent effect of dose on $t_{½\ el}$ in this study.

(c) Tissue kinetics

The tissue kinetics of [phenyl-U-^{14}C]ethiprole was investigated in male and female Sprague Dawley rats following a single oral administration of [phenyl-U-^{14}C]ethiprole at 5 or 1000 mg/kg bw. Four male and four female rats were killed at each of 8, 24 and 48 hours post-dosing at the low dose and 48, 72 and 96 hours post-dosing at the high dose, and selected tissues, organs and biological fluids were retained for the determination of total radioactivity.

At the low dose of 5 mg/kg bw, the highest tissue concentrations were observed in glandular tissues (adrenal, pancreas and thyroid) and those associated with metabolism (kidney and liver). High concentrations of radioactivity were also observed in the renal fat. Peak concentrations were observed for all tissues at 8 hours post-dosing (the first sampling time). The mean percentages of dose associated with the liver and kidneys at this time were approximately 12% and 1% (males) and 11% and 1% (females), respectively. Following each peak, concentrations of radioactivity decreased in all tissues.

At the high dose of 1000 mg/kg bw, peak concentrations of radioactivity were observed at 48 hours post-dosing (the first sampling time). The pattern of tissue distribution was similar to that observed at the low dose, with the highest concentrations observed in glandular tissues (adrenal, pancreas and thyroid) and those associated with metabolism (kidney and liver). High concentrations of radioactivity were also observed in the renal fat. The mean percentages of dose associated with the liver and kidneys at this time were approximately 1% and less than 1% for both sexes, respectively.

(d) Biliary excretion

During the bile duct cannulation experiments, two groups of five male and five female Sprague Dawley rats fitted with bile duct cannulae received a single oral administration of [phenyl-U-^{14}C]ethiprole at either 5 or 1000 mg/kg bw. Samples of urine, faeces and bile were collected from each animal at intervals up to 96 hours post-dosing.

At the low dose of 5 mg/kg bw, the major route of excretion was via bile, with means of about 67% (males) and 52% (females) of the dose recovered by 96 hours post-dosing. Excretion via urine and faeces over the same period accounted for, respectively, about 11% and 11% (males) and about 30% and 11% (females) of the administered dose. Absorption of the administered dose was rapid. The low level of excretion via faeces, approximately 11% (both sexes) of the dose, indicated that at least about 89% of an oral dose of [phenyl-U-^{14}C]ethiprole at the low dose was absorbed, with the bulk of this absorbed within 24 hours post-dosing.

By the end of the study period, at 96 hours post-dosing, the mean recovery of total radioactivity from bile, urine, faeces and cage wash accounted for about 92% and about 96% of the dose in males and females, respectively. Recovery of total radioactivity from the remaining carcasses was low, accounting for means of 1.49% and 3.49% of the dose in males and females, respectively, suggesting that low residues remained in the tissues. Recovery in all samples by the end of the study period represented means of 93.9% and 99.1% of the administered dose in males and females, respectively.

At 1000 mg/kg bw, the major route of excretion was via faeces, with means of 86.0% (males) and 79.3% (females) of the dose recovered by 96 hours post-dosing. Excretion via urine and bile over the same period accounted for, respectively, about 1.1% and 8.9% (males) and about 1.5% and 7.0% (females) of the administered dose. Absorption of the administered dose was low. Excretion via faeces of 86.0% (males) and 79.3% (females) of the dose indicated that it was likely that only about 14% (males) and 20% (females) of an oral dose of [phenyl-U-^{14}C]ethiprole at the high dose were absorbed, with the bulk of this absorbed within 48 hours post-dosing. By the end of the study period, at 96 hours post-dosing, the mean recovery of total radioactivity from bile, urine, faeces and cage wash accounted for about 97% of the administered dose for males and about 88% for females. Recovery of total radioactivity from the remaining carcasses was low, accounting for means of 0.36% and 5.78% of the dose in males and females, respectively, suggesting that low residues remained in the tissues. Recovery in all samples by the end of the study period represented means of about 97% and 94% of the administered dose in males and females, respectively (McCorquodale & Anderson, 1999).

1.2 Biotransformation

The biotransformation of ethiprole in rats was investigated in the same study (McCorquodale & Anderson, 1999).

Analysis of the excreted radioactivity indicated that orally administered [phenyl-U-^{14}C]ethiprole was extensively metabolized. The pattern of metabolites in urine was similar in male and female rats following administration of the low dose. The major components were the polar glucuronide conjugate of hydroxy-MB 45897 and the sulfinic acid RPA 104615, as well as the less polar (non-conjugated) MB 45897 and, in female urine, the carboxylic acid RPA 112705. A similar pattern of metabolites was observed after repeated low doses or a single high dose. The excreted radioactive components could be identified to a proportion of approximately 65% or 80% of the administered radioactivity at the low or high dose.

Three primary parallel metabolic pathways could be derived from the metabolites observed, followed by further reactions, including conjugate formation:

1) Hydrolysis of the nitrile group of ethiprole to form the amide RPA 112916.
2) Reduction of the sulfoxide group of ethiprole to form the sulfide RPA 107566. A subsequent alkyl oxidation results in the carboxylic acid RPA 112716.
3) Oxidation of the sulfoxide group of ethiprole to form the major metabolite (i.e. the sulfone RPA 097973), followed by further metabolic reactions:
   - The sulfone RPA 097973 can be alkyl hydroxylated to generate RPA 114345, which is subsequently metabolized by alkyl oxidation to form RPA 112705 and by conjugation with sulfuric acid. Further metabolic steps can follow.
   - An oxidative dealkylation of the sulfone RPA 097973 forms the sulfuric acid RPA 104615. Replacement of the sulfuric acid group by a hydroxy group generates the intermediate hydroxy-MB 45897, which subsequently can be stabilized as the sulfate or glucuronide conjugate.
   - The nitrile group of the sulfone RPA 097973 can be further hydrolysed to the amide (step 1), resulting in RPA 112917.

The proposed metabolic pathway of ethiprole in the rat is shown in Fig. 2.

2. Toxicological studies

2.1 Acute toxicity

The result of an acute toxicity study with ethiprole is summarized in Table 1. No studies were submitted on the acute dermal or inhalation toxicity of ethiprole or on its potential for ocular or dermal irritation or dermal sensitization.

2.2 Short-term studies of toxicity

(a) Oral administration

Mice

Ethiprole (batch no. CDR9706; purity 93.0%) was administered in the diet to groups of 15 male and 15 female C57B1/6N mice for at least 28 days at a dietary concentration of 0, 50, 250, 1000 or 2500 parts per million (ppm) (equal to 0, 9.3, 47.4, 186.2 and 458.0 mg/kg bw per day for males and 0, 11.8, 57.9, 234.4 and 513.0 mg/kg bw per day for females, respectively) to establish suitable doses for an 80-week carcinogenicity study. Mortality and clinical signs were checked daily. Body weight and feed consumption were measured weekly throughout the study. Clinical chemistry was performed towards the end of the dosing phase. All animals were subjected to necropsy, selected organs were weighed and designated tissues were sampled and examined microscopically. In addition, total cytochrome P450 content in the liver was determined.

Fig. 2. Proposed metabolic pathway of ethiprole in rats

RPA 112916
RPA 112917
RPA 114345
RPA 107382
RPA 097973
Sulphate conjugate of RPA 114345
RPA 112705
RPA 107566
RPA 104615
Hydroxy M+B 45897
Sulphate of hydroxy M+B 45897
RPA 112716
Sulphinic acid derivative of RPA 104615
RPA 097920/M+B 45897
Glucuronide of hydroxy M+B 45897

Tentatively identified by co-chromatography with a reference standard.
[ ] Postulated intermediate.
Source: McCorquodale & Anderson (1999)

Table 1. Study of acute toxicity of ethiprole

Species	Strain	Sex	Route	Purity (%)	Result	Reference
Rat	Wistar	M + F	Oral	93.0%[a]	LD_{50} > 7 080 mg/kg bw	Steiblen (1997)

bw: body weight; F: females; LD_{50}: median lethal dose; M: male
[a] Batch no. CDR9706.

The highest dietary concentration of 2500 ppm caused the death or required the premature termination of almost all animals and was considered to have exceeded the maximum tolerated dose. Ethiprole occasionally induced increased motor activity in males and females at 2500 ppm. The body weight gains of animals at 2500 ppm were comparable to those of controls. At 2500 ppm, statistically significantly lower feed consumption was noted in males and females during the first week of treatment. Decreases in mean total bilirubin concentrations were seen in males from 50 ppm onwards and in females from 250 ppm onwards. Higher mean aminotransferase activities were seen in males at 1000 and 2500 ppm. Ethiprole induced a dose-related increase in total liver cytochrome P450 content in males at 1000 and 2500 ppm (123% and 142% of controls, respectively). In the liver, dose-related increases in absolute and relative weights were observed at 250, 1000 and 2500 ppm in males (115%, 140% and 170% of control values for absolute, respectively; $P < 0.01$) and in females (115%, 143% and 210% of control values for absolute, respectively; $P < 0.01$). The 10% increased absolute liver weight in females at 50 ppm was not statistically significant. Liver enlargement and/or pale colour were observed in most animals at 1000 and 2500 ppm at gross necropsy. At 250 and 1000 ppm, histopathological examination revealed centrilobular to panlobular hepatocellular hypertrophy in both sexes and hepatocyte microvacuolation (characterized by the cytoplasmic accumulation of small vacuoles in the hepatocytes) and necrosis in males.

In the absence of other corroborative changes, the minimally higher liver weight without histopathological changes in females at 50 ppm and the lower total bilirubin in males at 50 ppm were not considered to be toxicologically relevant. The no-observed-adverse-effect level (NOAEL) was 50 ppm (equal to 9.3 mg/kg bw per day), based on increased liver weights and histopathological changes in the liver at 250 ppm (equal to 47.4 mg/kg bw per day) (Dange, 1999).

Rats

In a 28-day toxicity study, ethiprole (batch no. CDR9706; purity 93%) was administered to groups of 10 male and 10 female Wistar (RJ: WI (IOPS AF)) rats in the diet at a concentration of 0, 20, 100, 500 or 2500 ppm (equal to 0, 1.8, 9.2, 46.1 and 219.3 mg/kg bw per day for males and 0, 2.0, 9.6, 46.3 and 220.2 mg/kg bw per day for females, respectively). Mortality and clinical signs were checked daily. Body weight and feed consumption were measured weekly throughout the study. Neurotoxicity assessment and ophthalmic examinations were performed on all animals during the acclimatization period and during week 3 of dosing. Clinical chemistry, haematology and urine analysis were performed towards the end of the dosing phase. In addition, a blood sample was collected during week 4 for triiodothyronine (T_3), thyroxine (T_4) and thyroid stimulating hormone (TSH) analysis. All animals were subjected to necropsy, selected organs were weighed and designated tissues were sampled and examined microscopically.

At the top dose of 2500 ppm, there were signs of overt toxicity, with the premature deaths of two males; however, ethiprole induced no treatment-related clinical signs. The body weight gains at 2500 ppm were reduced compared with control values throughout the study in males (final body weight 92% of control value) and only during the first week of treatment in females (final body weight 100% of control value). At 2500 ppm, statistically significantly lower feed consumption was noted in males during the first 2 weeks and in females during the first week. Increases in prothrombin time were noted in males at 500 and 2500 ppm (120% and 140% of control values, respectively; $P < 0.001$). Decreased prothrombin times observed in females at 20, 100 and 500 ppm were considered to be incidental and unrelated to treatment, as they were not dose related and/or not biologically relevant. A tendency towards higher platelet counts was noted in males and females at 2500 ppm (124% and 117% in males

and females, respectively). Higher mean alanine aminotransferase activity was observed in males at 2500 ppm (464% of control value; $P < 0.001$). Mean total protein concentrations were higher in females at 500 and 2500 ppm (116% and 113% of control values; $P < 0.001$). Slight, but statistically significant, increases were also observed in males at 2500 ppm (107% of control value; $P < 0.01$) and in females at 100 ppm (107% of control value; $P < 0.05$). Lower mean albumin concentration was noted in males at 2500 ppm (91% of control value; $P < 0.001$). Mean total cholesterol concentrations were higher in males at 2500 ppm (212% of control value; $P < 0.001$) and in females at 500 and 2500 ppm (169% and 247% of control values; $P < 0.001$). In females, these changes were associated with higher mean triglyceride concentrations at 500 and 2500 ppm (184% and 349% of control values, respectively; $P < 0.001$).

Ethiprole induced a statistically significant imbalance of thyroid hormones in animals treated at 500 and 2500 ppm, resulting in higher levels of TSH and T_3 and lower levels of T_4 in both sexes (Table 2). Although statistically not significant, the groups begin to separate into sensitive and less sensitive subpopulations at 100 ppm.

Table 2. Twenty-eight-day study in the rat: mean T_3, T_4 and TSH plasma levels (week 4)

	0 ppm	20 ppm	100 ppm	500 ppm	2 500 ppm
Males					
No. of animals examined	9–10	9	9–10	10	9
T_3 (mmol/L)	0.439	0.410	0.447	0.447	0.608*
T_4 (mmol/L)	31.7	29.2	26.3	22.5***	15.2***
TSH (ng/mL)	2.75	2.14	3.64	6.93**	9.88***
Females					
No. of animals examined	9–10	7–9	8–10	9–10	9–10
T_3 (mmol/L)	0.407	0.430	0.438	0.355	0.605**
T_4 (mmol/L)	17.9	21.9	21.6	15.1	15.9
TSH (ng/mL)	1.18	1.23	2.72	5.18***	4.51**

ppm: parts per million; T_3: triiodothyronine; T_4: thyroxine; TSH: thyroid stimulating hormone; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ (Mann-Whitney U test [TSH] or Dunnett's test [T_3, T_4])
Source: Dange (2001a)

In the liver, dose-related increases in both absolute and relative weights were observed at 500 and 2500 ppm in males (135% and 198% of control values for absolute weight, respectively; $P < 0.001$) and in females at 100, 500 and 2500 ppm (approximately 120%, 196% and 262% of control values for absolute weight, respectively; $P < 0.001$). In the thyroid, dose-related increases in both absolute and relative weights were observed at 500 and 2500 ppm in males (141% and 153% of control values for absolute weight, respectively; $P < 0.001$ and $P < 0.01$, respectively) and in females (140% and 160% of control values for absolute weight, respectively; $P < 0.01$ and $P < 0.001$, respectively). In the adrenals, higher absolute and relative weights were observed in males at 500 and 2500 ppm (116% and 123% of control values for absolute weight, respectively; $P < 0.01$ and $P < 0.001$, respectively) and in females at 100, 500 and 2500 ppm (123%, 115% and 135% of control values for absolute weight, respectively; $P < 0.01$, not statistically significant and $P < 0.001$, respectively) (Table 3).

At the histopathological examination, the liver with centrilobular to panlobular hepatocellular hypertrophy, the thyroid with follicular cell hypertrophy and the adrenals with vacuolation of the cortex were found to be the target organs in animals at 500 and/or 2500 ppm. The kidney was also affected at 2500 ppm, with both sexes showing tubular golden brown pigment. At 100 ppm, one female was observed with vacuolation of the adrenal cortex; the incidence was low, but, based on the dose–effect relationship, 100 ppm should be considered the minimal effect dose for this finding.

Table 3. Twenty-eight-day study in the rat: effects of ethiprole on liver, thyroid and adrenal weights

		0 ppm	20 ppm	100 ppm	500 ppm	2 500 ppm
Males						
Number of animals		10	9	10	10	8
Terminal body weight (g)		338.6	339.9	332.8	334.2	310.4*
Liver weight	Absolute (g)	10.3	10.1	10.3	13.9***	20.4***
	Relative to body (%)	3.03	2.96	3.10	4.16***	6.58***
	Relative to brain (%)	529	515	529	714***	1 080***
Thyroid weight	Absolute (g)	0.017	0.018	0.019	0.024***	0.02***
	Relative to body (%)	0.004 9	0.005 3	0.005 8	0.007 3***	0.008 4**
	Relative to brain (%)	0.861	0.914	0.985	1.25**	1.39**
Adrenal weight	Absolute (g)	0.057	0.052	0.054	0.066**	0.070***
	Relative to body (%)	0.017	0.015	0.016	0.020**	0.022***
	Relative to brain (%)	2.92	2.65	2.76	3.39*	3.69***
Females						
Number of animals		10	9	10	7	10
Terminal body weight (g)		201.4	212.3	215.2	221.1	210.3
Liver weight	Absolute (g)	5.98	6.27	7.20***	11.7***	15.6***
	Relative to body (%)	2.85	2.96	3.35***	5.31***	7.43***
	Relative to brain (%)	323	345	387**	631***	851***
Thyroid weight	Absolute (g)	0.015	0.016	0.014	0.021**	0.024***
	Relative to body (%)	0.007 2	0.007 6	0.006 7	0.009 5*	0.011 3***
	Relative to brain (%)	0.807	0.889	0.771	1.13**	1.30***
Adrenal weight	Absolute (g)	0.060	0.066	0.074**	0.069	0.081***
	Relative to body (%)	0.029	0.031	0.035*	0.031	0.039***
	Relative to brain (%)	3.26	3.64	3.96*	3.68	4.42***

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$
Source: Dange (2001a)

The NOAEL was 20 ppm (equal to 1.8 mg/kg bw per day), based on adrenal effects (increased weight with slight increase in vacuolation) and slight, non-statistically significant effects on thyroid hormones at 100 ppm (equal to 9.2 mg/kg bw per day) (Dange, 2001a).

It should be noted that no adrenal vacuolation was reported in the 90-day toxicity study in the rat, which used doses of 0, 5, 20, 500 and 2500 ppm (see below).

In a 90-day toxicity study, ethiprole (batch no. CDR9706; purity 93%) was administered continuously via the diet to groups of 10 male and 10 female Wistar (RJ: WI (IOPS AF)) rats at a dietary concentration of 0, 5, 20, 500 or 2500 ppm (equal to 0, 0.296, 1.17, 30.5 and 155 mg/kg bw per day for males and 0, 0.373, 1.50, 37.6 and 188 mg/kg bw per day for females, respectively). Mortality and clinical signs were checked daily. Body weight was measured weekly, and feed consumption was measured daily throughout the study. Ophthalmic examinations were performed on all animals during the acclimatization period and during week 4 of dosing. Clinical chemistry, haematology and urine analysis were performed towards the end of the dosing phase. In addition, a blood sample was collected

during weeks 2, 6 and 13 for T_3, T_4 and TSH analysis. All animals were subjected to necropsy, selected organs were weighed and designated tissues were sampled and examined microscopically.

One female at 500 ppm and seven males at 2500 ppm were found dead. All these mortalities occurred between days 19 and 90 and were considered treatment related. One male and one female at 500 ppm and one male at 2500 ppm were moribund and were terminated. Clinical signs were observed at 2500 ppm and consisted of piloerection, increased and/or reduced motor activity and irritability to touch. These signs were observed on only very few occasions during the study. In addition, most of the males that died at 2500 ppm exhibited a general pallor before death. Body weights were significantly lower than those of controls at 2500 ppm during the first 5 weeks of dosing for males and only during the first week of treatment for females. At 2500 ppm, significantly lower feed consumption was noted in males during the first 3 weeks and in females during the first week. Prothrombin times were higher in males at 500 and 2500 ppm. In addition, a tendency towards higher platelet counts was noted at 2500 ppm in males (128% of control value; not statistically analysed) and in females (122% of control value; $P < 0.05$).

Higher mean total protein concentrations were noted in males at 500 and 2500 ppm (108% and 121% of control values, respectively; $P < 0.01$ and not statistically analysed, respectively) and in females at the same doses (119% and 125% of control values, respectively; $P < 0.01$). Higher mean total cholesterol concentrations were observed in females at 500 and 2500 ppm (214% and 333% of control values, respectively; $P < 0.01$). These changes were associated with higher mean triglyceride concentrations (137% and 141% of control values, respectively; not statistically significant and $P < 0.05$, respectively). In addition, a tendency towards slightly higher calcium and potassium concentrations and slightly lower chloride concentrations was noted at 500 and 2500 ppm.

Ethiprole induced an imbalance of thyroid hormones in animals treated at 500 and 2500 ppm, resulting in higher levels of TSH and T_3 and lower levels of T_4 (Table 4).

No treatment-related urine analysis changes were observed at any dose in either sex.

Statistically significant increases in liver and thyroid weights were observed at 500 and 2500 ppm. In the liver, dose-related increases in both absolute and relative weights were observed at 500 and 2500 ppm in males (157% and 257% of control values for absolute weight, respectively; $P < 0.01$ and not statistically analysed, respectively) and in females (196% and 292% of control values for absolute weight, respectively; $P < 0.01$). In the thyroid, dose-related increases in both absolute and relative weights were observed at 500 and 2500 ppm in males (148% and 162% of control values for absolute weight, respectively; $P < 0.01$) and females (144% and 156% of control values for absolute weight, respectively; $P < 0.01$).

At termination, histopathological examination revealed treatment-related microscopic findings in the liver, thyroid and kidney at 500 and/or 2500 ppm. In the liver, centrilobular hepatocyte hypertrophy attributable to treatment was noted in both sexes at 500 and 2500 ppm. Changes were generally more pronounced in females. Mild to moderate amounts of golden brown intracytoplasmic pigment were also observed in the majority of females at 2500 ppm.

In the thyroid, follicular cell hypertrophy/hyperplasia was noted in almost all examined animals of both sexes administered 500 or 2500 ppm. In addition, slight follicular epithelial hypertrophy/hyperplasia was seen in a single male at both 20 and 5 ppm, and mild follicular epithelial hypertrophy/hyperplasia was observed in two males at 20 ppm. However, these findings at these lower doses were not considered to be toxicologically relevant in view of the low incidence involved, the slight/mild nature of the change and the absence of corroborative changes in thyroid weights or thyroid hormone levels. In the kidney, an increased degree of cortical tubular brown pigment was reported in all surviving males and females at 2500 ppm.

The NOAEL was 20 ppm (equal to 1.17 mg/kg bw per day), based on mortality, changes in thyroid hormone levels, changes in clinical chemistry parameters and increased liver and thyroid weights associated with microscopic changes in these target organs at 500 ppm (equal to 30.5 mg/kg bw per day) (Dange & Foulon, 2002a).

Table 4. Ninety-day study in the rat: effects of ethiprole on mean T_3, T_4 and TSH plasma levels

Interval	Parameter	0 ppm	5 ppm	20 ppm	500 ppm	2 500 ppm
Males						
Week 2	No. of animals examined	10	10	10	10	10
	T_3 (mmol/L)	0.78	0.72	0.80	0.77	0.89
	T_4 (mmol/L)	36.2	36.5	37.7	28.6**	20.3**
	TSH (ng/mL)	0.57	0.65	0.62	1.13**	1.17**
Week 6	No. of animals examined	10	10	10	10	7
	T_3 (mmol/L)	0.74	0.63	0.71	0.85	0.71
	T_4 (mmol/L)	39.3	37.7	37.6	30.5**	21.3**
	TSH (ng/mL)	0.71	0.72	0.86	1.56**	2.13**
Week 13	No. of animals examined	8	7	8	8	4
	T_3 (mmol/L)	0.66	0.75	0.66	0.83	1.09*
	T_4 (mmol/L)	36.8	36.3	37.5	29.8*	22.2**
	TSH (ng/mL)	0.83	0.80	0.74	1.50**	1.63*
Females						
Week 2	No. of animals examined	10	10	10	10	10
	T_3 (mmol/L)	0.81	0.81	0.89	0.81	0.79
	T_4 (mmol/L)	28.3	29.1	28.1	25.5	22.0
	TSH (ng/mL)	0.48	0.42	0.48	0.76**	0.97**
Week 6	No. of animals examined	10	10	10	9	10
	T_3 (mmol/L)	0.77	0.72	0.85	0.96	1.16**
	T_4 (mmol/L)	31.4	34.6	32.9	24.1**	23.6**
	TSH (ng/mL)	0.52	0.52	0.48	0.82**	1.35**
Week 13	No. of animals examined	5	8	7	5	10
	T_3 (mmol/L)	0.73	0.76	0.91	1.08	1.26**
	T_4 (mmol/L)	23.5	26.3	29.8	22.1	22.9
	TSH (ng/mL)	0.64	0.64	0.62	0.81	1.44**

ppm: parts per million; T_3: triiodothyronine; T_4: thyroxine; TSH: thyroid stimulating hormone; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$

Source: Dange & Foulon (2002a)

Dogs

In a 90-day toxicity study, ethiprole (batch no. CDR9706; purity 93%) was administered continuously via the diet to groups of four male and four female beagle dogs at a dietary concentration of 0, 30, 90 or 200 ppm (equal to 0, 1.0, 3.2 and 7.6 mg/kg bw per day for males and 0, 1.1, 3.6 and 8.5 mg/kg bw per day for females, respectively). Mortality and clinical signs were checked daily. Body weight and feed consumption were measured weekly throughout the study. Ophthalmic examinations were performed on all animals during the acclimatization period and during week 12 of dosing. Clinical chemistry, haematology and urine analysis were performed in weeks 6 and 13. In addition, a blood sample was collected during weeks 2 and 13 for T_3, T_4 and TSH analysis. All animals were subjected to necropsy, selected organs were weighed and designated tissues were sampled and examined microscopically.

At 200 ppm, one moribund female was prematurely terminated on day 24. Clinical signs were limited to this female and consisted of bloody faeces, diarrhoea, soiled fur, prostration, tremors and increased salivation. This female had the highest ethiprole intake over weeks 1–3 (10.8 mg/kg bw per day, compared with 5, 7 and 8.4 mg/kg bw per day for the other three females in that group).

Treatment-related decreases in body weight and body weight gain were observed at 90 and 200 ppm in males. In males, differences were statistically significant only at 200 ppm and included lower mean body weight from day 36 onwards and decreases in mean body weight gain at weeks 2, 4 and 5 and overall for weeks 1–12. At 90 ppm, mean body weight was 88% of control values by day 85, and overall body weight gain (weeks 1–12) was 57% of that of controls. In females, although no apparent effect was observed on mean body weight at 200 ppm, a lower body weight gain was observed frequently (statistically significant at weeks 1 and 4) (Table 5). At 200 ppm, statistically significantly lower feed consumption was noted in males during the first 5 weeks.

Table 5. Ninety-day study in the dog: effects of ethiprole on body weight and body weight gain

	0 ppm	30 ppm	90 ppm	200 ppm
Males				
Body weight (g)				
Day 01	7 385	7 227	7 079	7 015
Day 36	8 396	8 209	7 703	6 903*
Day 43	8 526	8 362	7 779	6 895*
Day 50	8 728	8 567	7 903	6 992*
Day 58	8 813	8 686	7 903	6 952**
Day 64	8 966	8 823	7 997	6 951**
Day 71	9 200	9 050	8 175	7 050**
Day 78	9 279	9 095	8 183	6 946**
Day 85	9 464	9 207	8 279	6 971**
Body weight gain (g/day)				
Overall (weeks 1–12)	24.4	23.8	13.8	−0.6**
Females				
Body weight (g)				
Day 01	5 493	5 614	5 657	5 708
Day 08	5 548	5 671	5 632	5 510
Day 43	6 109	6 276	6 186	6 162 (n = 3)
Day 50	6 190	6 338	6 275	6 198 (n = 3)
Day 58	6 325	6 494	6 341	6 146 (n = 3)
Day 64	6 431	6 584	6 485	6 408 (n = 3)
Day 71	6 650	6 800	6 625	6 400 (n = 3)
Day 78	6 681	6 878	6 710	6 555 (n = 3)
Day 85	6 785	7 100	6 825	6 716 (n = 3)
Body weight gain (g/day)				
Overall (weeks 1–12)	15.3	17.1	13.8	8.2 (n = 3)

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$
Source: Bigot (2002)

Statistically significant increases were observed in alkaline phosphatase activities at 200 ppm in females in weeks 7 and 13. The same tendency was noted at 200 ppm in males in week 13 only, but it did not reach statistical significance. Ethiprole did not induce any changes in T_3 or T_4 at any dose in either sex; TSH could not be measured, probably because of the frozen storage period of the plasma samples (7–10.5 months).

In the thymus, lower absolute and relative weights were noted in males at 90 and 200 ppm (80% and 32% of control values, respectively; not statistically significant and $P < 0.05$, respectively). These effects were considered to be related to lower terminal body weights. In male sexual organs, lower absolute and relative weights were observed at 90 and 200 ppm (65% and 50% of control values in the epididymis, 59% and 44% of control values in the testis and 28% and 17% of control values in the prostate, respectively) (Table 6).

At 30 ppm, lower absolute organ weights were also noted in the prostate (41% of control value; $P < 0.01$), in the epididymis (66% of control value) and in the testis (69% of control value) (Table 6). Taking into account that these effects were not associated with any untoward histological changes and that in the subsequent 1-year study (Chevalier, 2001) in beagle dogs, no similar changes were observed up to 90 ppm, the lower sexual organ weights noted at 30 ppm were considered not to be toxicologically relevant.

In the brain, a higher relative weight was noted at 200 ppm in males (130% of control value; $P < 0.05$) (Table 6). This effect was considered to be related to lower terminal body weights. At termination, small testes and small prostate were seen in one male at 200 ppm. No treatment-related macroscopic changes were observed at 30 or 90 ppm in males or at any dose in females.

Table 6. Ninety-day study in the dog (males): effects of ethiprole on brain, thymus, epididymis, testis and prostate weights

		0 ppm	**30 ppm**	**90 ppm**	**200 ppm**
Number of animals examined		4	4	4	4
Terminal body weight (kg)		9.5	9.2	8.3	6.9**
Brain weight	Absolute (g)	76.2	78.2	77.5	75.2
	Relative to body (%)	0.825	0.850*	0.950	1.075*
	Relative to brain (%)	–	–	–	–
Thymus weight	Absolute (g)	8.95	11.524	7.10	2.90*
	Relative to body (%)	0.095	0.123	0.080	0.040
	Relative to brain (%)	11.8	14.8	9.15	3.70
Epididymis weight	Absolute (g)	2.87	1.90	1.85	1.45
	Relative to body (%)	0.027	0.017	0.020	0.017
	Relative to brain (%)	3.75	2.45	2.33	1.93
Testes weight	Absolute (g)	13.32	9.22	7.82*	5.85**
	Relative to body (%)	0.145	0.100	0.092	0.082
	Relative to brain (%)	17.4	11.8*	10.1*	7.73**
Prostate weight	Absolute (g)	6.10	2.52**	1.67**	1.05**
	Relative to body (%)	0.065	0.027**	0.022*	0.015**
	Relative to brain (%)	7.98	3.28**	2.15**	1.40**

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$
Source: Bigot (2002)

In the liver, slight to mild centrilobular hepatocyte hypertrophy attributable to treatment was noted in two males at 90 ppm and in one male at 200 ppm. Glycogen depletion was noted in one male and one female treated at 200 ppm. In the thymus, atrophy was noted in two males at 90 ppm and in four males and two females treated at 200 ppm. In the sexual organs of males, immaturity was reported in all males, including controls, but the severity of this finding was more pronounced at 90 and 200 ppm (Table 7).

Table 7. Ninety-day study in the dog: selected microscopic findings at termination

	Males				Females			
	0 ppm	**30 ppm**	**90 ppm**	**200 ppm**	**0 ppm**	**30 ppm**	**90 ppm**	**200 ppm**
Liver								
Number examined	4	4	4	4	4	4	4	3
Hepatocyte hypertrophy – centrilobular becoming generalized	0	0	2	1	0	0	0	0
Glycogen depletion	0	0	0	1	0	0	0	1
Thymus								
Number examined	4	4	4	4	4	4	4	3
Atrophy	1	0	2	4	0	0	0	2
Epididymis								
Number examined	4	4	4	4	–	–	–	–
No sperm	0	0	1	2	–	–	–	–
Few sperm	1	2	2	1	–	–	–	–
Testis								
Number examined	4	4	4	4	–	–	–	–
Immaturity	4	4	4	4	–	–	–	–
Prostate								
Number examined	4	4	4	4	–	–	–	–
Immaturity	0	1	3	4	–	–	–	–

ppm: parts per million
Source: Bigot (2002)

The NOAEL was 30 ppm (equal to 1.0 mg/kg bw per day), based on reduced body weight gain and decreased thymus weight with atrophy at 90 ppm (equal to 3.2 mg/kg bw per day) (Bigot, 2002).

In a 1-year toxicity study, ethiprole (batch no. CDR 9706; purity 93%) was administered to groups of five male and five female beagle dogs in the diet at a concentration of 0, 9, 30 or 90 ppm (equal to 0, 0.27, 0.70 and 2.73 mg/kg bw per day for males and 0, 0.22, 0.76 and 2.51 mg/kg bw per day for females, respectively) for at least 52 weeks. Mortality and clinical signs were checked daily. Body weight was measured during the acclimatization period and weekly thereafter. Feed consumption was measured daily throughout the study. Ophthalmic examinations were performed on all animals during the acclimatization period and during week 12 of dosing. Clinical chemistry, haematology and urine analysis were performed towards the end of the dosing phase. In addition, a blood sample was

collected during weeks 2 and 12 for T_3, T_4 and TSH analysis. All animals were subjected to necropsy, selected organs were weighed and designated tissues were sampled and examined microscopically.

The only treatment-related findings in this study were slight decreases in the overall body weight gain in both males and females at 90 ppm (−19% and −23% in males and females, respectively) (Table 8). At 9 ppm, one male presented with emaciation from week 15, with body weight loss noted from week 12 (−200 g compared with week 11) until week 16. Thereafter, the body weight of this dog was stable (around 7.5 kg), reaching 7.9 kg at the end of the treatment period. No variation in feed consumption was noted. In the absence of signs of emaciation in the 30 ppm group, the relationship to treatment was doubtful, and the body weight loss in this male was probably unrelated to the test substance.

Table 8. One-year study in the dog: effects of ethiprole on body weight and body weight gain

	0 ppm	**9 ppm**	**30 ppm**	**90 ppm**
Males				
Body weight (kg)				
Week 1	7.4 ± 0.53	7.3 ± 0.54	7.6 ± 0.29	7.3 ± 0.55
Week 13	8.7 ± 1.16	8.7 ± 0.89	10.0 ± 0.39	9.0 ± 0.57
Week 52	11.0 ± 1.71	10.1 ± 2.11	12.2 ± 1.06	10.0 ± 1.28
Body weight gain (g/day)				
Overall (weeks 1–52)	3.7	2.9	4.8	3.0
Females				
Body weight (kg)				
Week 1	6.7 ± 0.60	6.6 ± 0.51	6.6 ± 0.61	6.6 ± 0.66
Week 13	8.6 ± 0.80	8.8 ± 0.55	8.5 ± 0.93	7.8 ± 0.54
Week 52	9.6 ± 1.11	10.3 ± 1.84	9.9 ± 1.26	8.9 ± 1.00
Body weight gain (g/day)				
Overall (weeks 1–53)	3.1	3.9	3.4	2.4

ppm: parts per million
Source: Chevalier (2001)

The NOAEL was 30 ppm (equal to 0.70 mg/kg bw per day), based on overall reduced body weight gain at 90 ppm (equal to 2.51 mg/kg bw per day) (Chevalier, 2001).

(b) Dermal application

No studies were submitted.

(c) Exposure by inhalation

No studies were submitted.

2.3 Long-term studies of toxicity and carcinogenicity

The long-term toxicity and carcinogenic potential of ethiprole were evaluated in a 78-week study in the mouse and a 104-week study in the rat. Both studies included a 52-week long-term toxicity phase.

Mice

In a chronic toxicity and carcinogenicity study, ethiprole (batch no. CDR 9706; purity 93%) was administered to groups of 50 male and 50 female C57BL/6J mice for at least 78 weeks at a dietary concentration of 0, 10, 50, 150 or 300 ppm (equal to 0, 1.7, 8.6, 25.6 and 50.8 mg/kg bw per day for males and 0, 1.7, 12.5, 36.3 and 73.5 mg/kg bw per day for females, respectively). For evaluation of toxic effects, 10 satellite males and 10 satellite females per group were terminated after a 1-year treatment. Throughout the study, clinical signs and mortality were checked daily, and careful examination was carried out weekly to assess possible neurotoxic effects. Palpation of possible masses was carried out every 4 weeks from weeks 4 to 52 and every 2 weeks thereafter. Body weight and feed consumption were measured at weekly intervals during the first 13 weeks of the study and every 4 weeks thereafter. Haematological investigations (differential white cell count) were carried out in week 52 and at the end of the study (principal animals). Before the termination of satellite animals in week 52, blood was taken for the determination of liver enzyme activities (aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase). At the end of the appropriate scheduled treatment period (week 52 or 78), all animals were terminated and submitted to a macroscopic postmortem examination. Designated organs were weighed. A microscopic examination was performed on all samples of tissues from satellite animals, from principal animals of the control and high-dose groups, and from principal animals that died or were killed prematurely during the study.

Mortality was slightly higher in females of the 300 ppm group (11/50) compared with the control group (4/50). There were no treatment-related clinical signs noted at any dose during either phase of the study. No signs of neurotoxic effects were observed, and there was no evidence that treatment produced an increase in the incidence or reduction in the onset time of palpable tumours. The body weight gain was similar in the controls and the treated groups for the males, but was slightly lower for the females given 50, 150 or 300 ppm during the first 4 weeks of the treatment period, without correlation with variations in feed consumption. As the lower gain was slight, transient, of low magnitude, not dose related, recorded only in females and not observed in the 28-day study in mice at much higher doses, it was not considered to be of toxicological relevance. After week 4, the body weight changes were similar to those of the controls. No effects on feed consumption or differential white cell count were observed.

Increases of greater than 115% (117–122%) of control values were noted in absolute and relative liver weights in males dosed at 300 ppm after 52 and 78 weeks. These increases were, however, without correlative histopathological changes, and they were therefore considered to be of doubtful toxicological importance.

There were no treatment-related non-neoplastic findings at any dose after treatment for either 52 or 78 weeks. The non-neoplastic findings observed in all organs and tissues were recognized as those that are commonly recorded in ageing mice of this strain and age and were regarded as being of no toxicological importance. After 78 weeks of treatment, the only neoplastic change was an increase (P = 0.0133) in the incidence of hepatocellular adenomas in females at 300 ppm (6/50 versus 0/50 in controls). The incidence and time of onset of the liver neoplasms were similar in all groups. All neoplasms were recorded in animals at termination, suggesting that treatment with the test substance did not shorten the time of onset of this lesion. In addition, the incidence and time of onset of the hepatocellular carcinomas were similar in all groups (Table 9).

Other (without any dose–response relationship) observed neoplastic lesions found in the haemolymphoreticular system (as assessed from the microscopic examination of the liver and lymphoid organs) were diagnosed as malignant lymphoma (lymphocytic, pleiomorphic or heterogenous [mixed cell] types) and histiocytic sarcoma.

Table 9. Mouse carcinogenicity study: effect of ethiprole on incidence of hepatic neoplastic lesions

	0 ppm	10 ppm	50 ppm	150 ppm	300 ppm
Males					
Hepatocellular adenoma	5/49	5/50	4/50	1/50	1/50
Hepatocellular carcinoma	0/49	3/50	1/50	0/50	1/50
Total hepatocellular adenoma + carcinoma	5/49	8/50	5/50	1/50	2/50
Females					
Hepatocellular adenoma	0/50	2/50	1/50	2/50	6/50
Hepatocellular carcinoma	0/50	0/50	0/50	0/50	0/50
Total hepatocellular adenoma + carcinoma	0/50	2/50	1/50	2/50	6/50

ppm: parts per million
Source: Richard (2002)

The NOAEL for toxicity was 150 ppm (equal to 36.3 mg/kg bw per day), based on a decrease in survival rate in females at 300 ppm (equal to 73.5 mg/kg bw per day). The NOAEL for carcinogenicity was 150 ppm (equal to 36.3 mg/kg bw per day), based on an increase in the incidence of hepatocellular adenomas in females at 300 ppm (equal to 73.5 mg/kg bw per day) (Richard, 2002).

Rats

In a chronic toxicity and carcinogenicity study, ethiprole (batch no. CDR9706; purity 93%) was administered in the diet to groups of 60 male and 60 female Wistar (RJ: WI (IOPS AF)) rats at a dietary concentration of 0, 5, 20, 75 or 250 ppm (equal to 0, 0.22, 0.85, 3.21 and 10.8 mg/kg bw per day for males and 0, 0.29, 1.17, 4.40 and 14.7 mg/kg bw per day for females, respectively). Additional groups were similarly dosed: groups of 10 males and 10 females were used for the interim kill at 52 weeks (all dose groups), and groups of 15 males and 15 females were used for a 90-day recovery phase after 52 weeks of exposure (control and high dose only). Mortality and clinical signs were checked daily. Body weight and feed consumption were measured weekly for the first 13 weeks and every 4 weeks thereafter. Ophthalmic examinations were performed on all animals during the acclimatization period and at 1 and 2 years. Clinical chemistry, haematology determinations and urine analysis were performed during months 6, 12, 15, 18 and 24 on selected animals. In addition, a blood sample was collected during weeks 2, 9, 24 and 52 and during weeks 2, 6 and 13 of the recovery period for T_3, T_4 and TSH analysis. All remaining animals of the chronic, recovery and carcinogenicity phases were subjected to necropsy, selected organs were weighed and designated tissues were sampled and examined microscopically.

No treatment-related changes in the mortality rate or clinical signs were observed. The body weight changes in males and females at 75 and 250 ppm were slightly increased compared with controls, especially during the first year of treatment. The overall feed consumption was generally higher in males and lower in females, but without a dose–response relationship.

Prothrombin times were higher in males and lower in females at 75 and 250 ppm. However, the effect in males was only greater than a 10% increase compared with controls at 6 months in the 250 ppm group (126%); in females, the effects were slight (87–92% of control values), with no consistent dose–response relationship. In addition, at 250 ppm in females, the mean platelet counts were statistically significantly higher on months 12 (125% of control value) and 24 (135% of control value). After the recovery period, no relevant differences were noted between control and high-dose animals, indicating that these effects were reversible.

In females at 250 ppm, mean total cholesterol concentration was higher at months 6 (144% of control value; $P < 0.001$), 12 (155% of control value; $P < 0.001$), 18 (184% of control value; $P < 0.01$) and 24 (133% of control value; not statistically significant).

Mean total bilirubin concentrations were lower at 75 and 250 ppm in both sexes (63–76% and 50–61% of control values in males, respectively, and 56–67% and 40–53% of control values in females, respectively; dose related) and at 20 ppm in males only at 18 months (69% of control value). After the 3-month recovery period, no relevant changes were noted between controls and the high-dose animals, indicating that these effects were reversible.

Ethiprole induced an imbalance of thyroid hormones, resulting in higher levels of TSH and/or lower levels of T_4 in males and females at 75 and 250 ppm (Table 10). During the recovery period, TSH and T_4 returned to control levels within 2 weeks of the withdrawal of treatment at 250 ppm. The imbalance of TSH and T_4 was most likely secondary to a treatment-induced increase in the metabolic activity of the liver, causing an increased biliary clearance of T_4 and, consequently, an overstimulation of the thyroid due to increased levels of TSH.

At 52 weeks, treatment-related organ weight changes were noted in the liver and thyroid. There were statistically significantly increased liver weights at 250 ppm in both sexes (130% and 119% of control values for absolute and relative liver weights in males, respectively; and 145% and 146% of control values for absolute and relative liver weights in females, respectively). The increased liver weights at 75 ppm in females were slight (112% and 114% of control values for absolute and relative weights, respectively). The thyroid weights were increased in males at 75 ppm (136% and 121% of control values for absolute and relative [not statistically significant] weights) and at 250 ppm in both sexes (168% and 150% of control values for absolute and relative weights in males, respectively; and 120% and 124% of control values for absolute and relative weights in females, respectively). No treatment-related changes in organ weights were noted in the reversibility groups.

At the end of the carcinogenicity phase, treatment-related organ weight changes were noted in the liver and thyroid. There was a statistically significantly increased liver weight at 250 ppm in both sexes (123% and 125% of control values for absolute and relative weights in males, respectively; and 138% and 137% of control values for absolute and relative weights in females, respectively). The increased liver weight at 75 ppm in females was slight (113% and 114% of control values for absolute and relative weights, respectively). The thyroid weight was increased at 75 ppm in both sexes (138% and 136% of control values for absolute and relative weights in males, respectively; and 128% and 133% of control values for absolute and relative weights in females, respectively) and at 250 ppm in both sexes (121% of control values for males and 135–136% of control values for females, for both absolute and relative weights), reaching statistical significance in females only. No treatment-related changes in organ weights were noted in the reversibility groups.

At 52 weeks, treatment-related changes were noted in the liver and thyroid. In the liver, treatment-related centrilobular hepatocyte hypertrophy was noted in all females at 250 ppm. This change, characterized by an appreciable increase in the size of hepatocytes in the area of the central vein, was generally graded slight to mild and is considered to represent a minor adaptive change. In the thyroid, follicular cell hypertrophy was noted in animals of both sexes dosed at 20 ppm and above. This change, which was characterized by an increase in height of the follicular epithelial cells accompanied by a decrease in the follicular size and in colloid content, was graded slight to moderate. There was evidence of a dose–effect relationship in both sexes, more prominently in females than in males. In addition, colloid mineralization was observed in a small proportion of males (2/9) and females (4/10) at 250 ppm (Table 11).

At the end of the reversibility phase after 52 weeks of treatment, the centrilobular hepatocyte hypertrophy was fully reversible. In the thyroid gland, the colloid mineralization and slight follicular cell hypertrophy were still present, with some indications that reversibility was in progress.

Histopathological examination at the termination of the carcinogenicity phase revealed a number of treatment-related non-neoplastic findings in the liver: bile duct hyperplasia and sclerosis and focal sinusoidal dilatation. These changes were noted in many animals, with the incidence and severity

Table 10. Rat chronic toxicity and carcinogenicity study: effects of ethiprole on mean T_3, T_4 and TSH plasma levels

		0 ppm	5 ppm	20 ppm	75 ppm	250 ppm
Males						
No. of animals examined		23–25	10	10	10	24–25
Week 2	TSH (ng/mL)	6.28	6.30	6.58	6.06	9.01***
	T_4 (ng/mL)	35.2	34.1	36.5	34.1	32.5*
	T_3 (ng/mL)	0.675	0.661	0.770	0.691	0.699
Week 9	TSH (ng/mL)	6.48	6.98	6.79	7.07	7.32
	T_4 (ng/mL)	27.7	27.6	28.9	27.2	24.1**
	T_3 (ng/mL)	0.352	0.412	0.421	0.391	0.372
Week 24	TSH (ng/mL)	6.51	6.50	7.15	5.72	8.87***
	T_4 (ng/mL)	23.7	24.5	26.7	25.6	25.6
	T_3 (ng/mL)	0.260	0.287	0.372**	0.339	0.402***
Week 52	TSH (ng/mL)	6.66	5.96	6.76	5.56	7.12
	T_4 (ng/mL)	24.0	22.5	23.0	23.0	22.3
	T_3 (ng/mL)	0.420	0.389	0.397	0.376	0.469
Females						
No. of animals examined		24–25	10	9–10	10	23–25
Week 2	TSH (ng/mL)	5.05	5.45	5.09	5.49	8.07***
	T_4 (ng/mL)	29.1	28. 9	26.6	27.3	25.5
	T_3 (ng/mL)	0.656	0.759	0.660	0.692	0.646
Week 9	TSH (ng/mL)	4.75	4.91	5.22	5.69*	7.09***
	T_4 (ng/mL)	24.6	25.6	23.0	22.3	21.1*
	T_3 (ng/mL)	0.503	0.531	0.478	0.473	0.483
Week 24	TSH (ng/mL)	4.64	5.50	5.21	4.95	7.21***
	T_4 (ng/mL)	22.7	23.1	19.3	19.1	19.5
	T_3 (ng/mL)	0.410	0.415	0.397	0.392	0.472
Week 52	TSH (ng/mL)	5.09	4.70	5.49	5.91	7.30**
	T_4 (ng/mL)	21.9	19.9	23.4	16.8***	18.9*
	T_3 (ng/mL)	0.548	0.527	0.600	0.472	0.538

ppm: parts per million; T_3: triiodothyronine; T_4: thyroxine; TSH: thyroid stimulating hormone; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$
Source: Dange & Foulon (2002b)

being slightly higher in females at 250 ppm. Also in the liver, eosinophilic and basophilic "tigroid" foci of cellular alteration were noted in many animals from all groups. The incidence of both types (and the severity of "tigroid" foci) was slightly higher in males dosed at 250 ppm in comparison with controls. In females, the incidence of "tigroid" altered hepatocyte foci was lower in animals dosed at 250 ppm, with no effect on the incidence of eosinophilic foci of cellular alteration. In the thyroid, colloid mineralization was the predominant finding in rats of both sexes at 250 ppm and in females at 75 ppm. Females at 250 ppm also showed significant diffuse follicular hypertrophy. These changes are consistent with minor, chronic stimulation. In the kidneys, chronic progressive nephropathy was noted

in most animals from all groups. The severity, but not the incidence, was slightly higher in rats of both sexes at 250 ppm. In addition, the incidence of arteritis/periarteritis in the kidney was marginally higher than in controls in females at 250 ppm. This change may be related to the increased severity of chronic progressive nephropathy.

Table 11. Rat chronic toxicity and carcinogenicity study: non-neoplastic microscopic findings at week 52 (chronic phase) and week 104 (carcinogenicity phase)

			Dietary concentration (ppm)									
			Males					Females				
Organ	**Finding**		**0**	**5**	**20**	**75**	**250**	**0**	**5**	**20**	**75**	**250**
52 weeks												
Liver	Number examined		10	10	10	10	9	10	10	10	10	10
	Centrilobular hypertrophy		0	1	0	0	0	0	0	1	0	10
Thyroid	Number examined		10	10	10	10	9	10	10	10	10	10
	Follicular cell hypertrophy		0	0	1	3	5	0	0	2	3	9
	Mineralization, colloid		0	0	0	0	2	0	0	0	0	4
Ovaries	Number examined		–	–	–	–	–	10	0	0	2	10
	Mineralization, unilateral		–	–	–	–	–	1	0	0	0	0
	Sertoliform hyperplasia, unilateral		–	–	–	–	–	1	0	0	0	0
	Sertoliform hyperplasia, bilateral		–	–	–	–	–	0	0	0	0	0
	Cyst(s), unilateral		–	–	–	–	–	2	0	0	0	3
	Cyst(s), bilateral		–	–	–	–	–	3	0	0	2	1
104 weeks												
Liver	Number examined		60	60	60	60	60	60	60	60	60	60
	Hyperplasia, bile ducts	Slight	23	33	29	26	30	16	22	15	24	18
		Mild	14	1	4	14	8	3	1	2	5	14
		Moderate	3	0	1	0	1	0	0	0	0	1
		Total	40	34	34	40	39	19	23	17	29	33
	Sclerosis, bile ducts	Slight	18	29	26	28	33	9	13	6	17	22
		Mild	11	9	10	11	9	1	0	0	0	7
		Moderate	0	0	0	0	0	0	0	1	0	0
		Total	29	38	36	39	42	10	13	7	17	29
	Dilatation, sinusoidal, focal	Slight	4	2	9	8	5	14	13	14	17	28
		Mild	0	0	0	0	0	1	0	0	0	2
		Total	4	2	9	8	5	15	13	14	17	30
	Cellular alteration, basophilic, “tigroid”, focal	Slight	30	33	38	33	37	30	30	27	32	17
		Mild	2	2	3	6	10	10	13	8	4	4
		Moderate	0	0	0	0	3	2	0	0	2	0
		Marked	0	0	0	0	1	0	0	0	1	0
		Total	32	35	41	39	51	42	43	35	39	21

Organ	Finding		Males 0	Males 5	Males 20	Males 75	Males 250	Females 0	Females 5	Females 20	Females 75	Females 250
	Cellular alteration, eosinophilic type, focal	Slight	23	20	16	16	32	22	32	24	17	29
		Mild	3	3	3	4	8	5	3	6	6	2
		Moderate	0	0	1	0	0	0	0	2	0	0
		Total	26	23	20	20	40	27	35	32	23	31
Thyroid	Number examined		60	60	59	60	59	59	59	60	60	60
	Mineralization, colloid	Slight	4	4	4	9	12	0	1	3	5	16
		Mild	3	2	0	2	6	0	0	0	2	16
		Moderate	1	1	1	3	11	0	0	0	2	10
		Marked	1	0	0	0	0	0	0	0	0	0
		Total	9	7	5	14	29	0	1	3	9	42
	Hypertrophy follicular, diffuse	Slight	1	0	2	3	3	0	0	0	3	14
		Mild	1	1	2	0	0	0	0	0	1	6
		Total	2	1	4	3	3	0	0	0	4	20
Kidneys	Number examined		60	60	60	60	60	60	60	60	60	60
	Nephropathy, chronic, progressive	Slight	33	32	33	36	28	37	36	30	28	26
		Mild	10	8	9	6	16	1	2	2	9	11
		Moderate	6	3	5	7	10	3	1	2	1	5
		Marked	0	0	1	1	3	0	0	0	3	2
		Severe	0	1	0	0	0	0	0	0	1	3
		Total	49	44	48	50	57	41	39	34	42	47
	Arteritis/ periarteritis	Slight	0	0	1	2	0	0	1	0	2	2
		Mild	0	0	0	0	1	0	0	0	1	2
		Moderate	0	0	0	0	0	0	1	0	0	2
		Total	0	0	1	2	1	0	2	0	3	6
Lungs	Number examined		60	60	60	60	60	60	59	60	60	59
	Alveolar macrophages, focal	Slight	22	22	24	18	21	17	18	19	24	29
		Mild	3	4	1	1	3	6	3	4	3	6
		Moderate	2	0	0	0	0	0	0	0	0	1
		Total	21	26	25	19	30	23	21	23	27	36
Ovaries	Number examined		–	–	–	–	–	60	60	60	60	60
	Atrophy		–	–	–	–	–	9	10	17	16	7
	Hyperplasia, tubular, focal		–	–	–	–	–	12	15	17	10	13
	Hyperplasia, sertoliform, focal		–	–	–	–	–	28	29	28	24	29
	Cyst(s), unilateral		–	–	–	–	–	25	29	25	29	16
	Cyst(s), bilateral		–	–	–	–	–	28	29	31	29	32

Dietary concentration (ppm)

ppm: parts per million
Source: Dange & Foulon (2002b)

At 52 weeks, there was no evidence of a treatment-related increased incidence of neoplastic changes, nor were there any treatment-related neoplastic changes in the recovery groups.

In Table 12, the numbers of neoplastic findings in animals that died prematurely and animals that survived until scheduled termination in the carcinogenicity phase are combined. Neoplastic findings in the target organs (liver and thyroid) in the animals that died prematurely were as follows: Thyroid follicular cell adenoma was seen in four males dosed at 250 ppm, with no similar lesion in controls. Focal follicular cell hyperplasia was seen in one control male, one 5 ppm male, four 250 ppm males and one 250 ppm female. In the liver, hepatocellular adenoma was seen in one male dosed at 250 ppm. In the subcutis, adipose tissue lipoma was observed in three males at 250 ppm. In the ovaries, sex cord stromal tumours were found in females dosed at 5, 75 and 250 ppm (0, 1, 0, 4 and 3 in the 0, 5, 20, 75 and 250 ppm groups, respectively).

Table 12. Rat chronic toxicity and carcinogenicity study: microscopic neoplastic findings in animals that died prematurely and at scheduled termination

		Dietary concentration (ppm)									
		Males					Females				
Organ	**Finding**	**0**	**5**	**20**	**75**	**250**	**0**	**5**	**20**	**75**	**250**
Liver	Number examined	60	60	60	60	60	60	60	60	60	60
	Hepatocellular adenoma	0	0	0	0	3	0	0	0	1	0
Thyroid	Number examined	60	60	59	60	59	59	59	60	60	60
	Hyperplasia, follicular cell, focal	2	1	0	1	5	0	1	0	1	2
	Adenoma, follicular cell	0	0	0	0	4	0	0	0	0	2
	Carcinoma, follicular cell	0	0	0	0	0	0	0	1	1	0
	Total with focal proliferative lesions	2	1	0	1	8	0	1	1	2	4
Subcutis	Number examined	60	60	60	60	60	60	60	60	60	60
	Lipoma	0	0	1	1	5	0	0	0	1	1
Ovaries	Number examined	–	–	–	–	–	60	60	60	60	60
	One benign granulosa cell tumour	–	–	–	–	–	0	0	1	1	1
	One benign thecoma	–	–	–	–	–	2	0	0	0	0
	One sertoliform adenoma	–	–	–	–	–	2	4	1	4	7
	Two sertoliform adenomas	–	–	–	–	–	0	1	0	2	2
	One benign sex cord stromal tumour	–	–	–	–	–	0	0	1	0	0
	Total sex cord stromal tumours	–	–	–	–	–	4	5	3	7	10

ppm: parts per million
Source: Dange & Foulon (2002b)

At the scheduled termination, thyroid follicular cell adenoma was noted in two females in the 250 ppm group. In the liver, hepatocellular adenoma was noted in two males at 250 ppm and in one female at 75 ppm. In the subcutis, adipose tissue lipoma was observed in one male at 20 ppm, one male and one female at 75 ppm, and two males and one female at 250 ppm. In the ovaries, sex cord stromal tumours were found in all groups, but with a dose-related increase (4, 4, 3, 3 and 7 in the 0, 5, 20, 75 and 250 ppm groups, respectively).

A variety of spontaneous tumours were noted in animals from all groups, with no evidence of an effect of treatment. Tumours were commonly encountered (10 or more in a group) in the pituitary gland, thyroid gland (C-cells) and mammary gland (females). The spectrum was consistent with the range of spontaneous lesions encountered in ageing rats of this strain.

Overall, there was an increase in several tumours at 250 ppm: hepatocellular adenoma in males, subcutaneous lipomas in males and thyroid adenomas in males and females, which were in general outside the incidence in concurrent controls and the laboratory historical control data (Table 13) and therefore were considered treatment related. The increase in ovary sex cord stromal tumours was considered equivocal.

Table 13. Summary of background data for the selected tumours, compiled from the original study and the testing laboratory's historical control data

Tumour type	Study control group data	Testing laboratory's historical control data (2000–2003)
Subcutaneous lipoma		
Males		
Mean % incidence (no. of tumours / no. of rats examined / no. of studies)	n/a	1.8 (7 / 400 / 7)
Study range: maximum % (no. of tumours / no. of rats examined in that study)	0 (0 / 60)	3.3 (2 / 60)
Females		
Mean % incidence (no. of tumours / no. of rats examined / no. of studies)	n/a	1.8 (4 / 401 / 7)
Study range: maximum % (no. of tumours / no. of rats examined in that study)	0 (0 / 60)	2.0 (1 / 49)
Hepatocellular adenoma		
Males		
Mean % incidence (no. of tumours / no. of rats examined / no. of studies)	n/a	2.0 (8 / 405 / 7)
Study range: maximum % (no. of tumours / no. of rats examined in that study)	0 (0 / 60)	3.3 (2 / 60)
Females		
Mean % incidence (no. of tumours / no. of rats examined / no. of studies)	n/a	1.5 (6 / 405 / 7)
Study range: maximum % (no. of tumours / no. of rats examined in that study)	0 (0 / 60)	5.0 (3 / 60)
Ovary sex cord stromal tumours (total)		
Females		
Mean % incidence (no. of tumours / no. of rats examined / no. of studies)	n/a	1.0 (4 / 405 / 7)
Study range: maximum % (no. of tumours / no. of rats examined in that study)	2.4 (4 / 60)	3.7 (2 / 55)

F: female; M: male; n/a: not applicable

The slightly increased incidence of follicular cell hypertrophy observed at 12 months in males and females was not observed at 24 months and was therefore considered to be within biological variation because of the low control incidence.

The NOAEL for toxicity was 20 ppm (equal to 0.85 mg/kg bw per day), based on observed effects in the thyroid and/or liver (histopathological changes, increased organ weights and/or altered thyroid hormone or bilirubin levels) at 75 ppm (equal to 3.21 mg/kg bw per day). Increased incidences of tumours (subcutaneous lipoma in males, hepatocellular adenoma in males and thyroid adenomas in males and females) were noted at 250 ppm (equal to 10.8 mg/kg bw per day). The slight increase in ovary sex cord tumours at 250 ppm (equal to 14.7 mg/kg bw per day) was considered equivocal. The NOAEL for carcinogenicity was 75 ppm (equal to 3.21 mg/kg bw per day) (Dange & Foulon, 2002b).

2.4 Genotoxicity

Ethiprole is not mutagenic to bacterial or mammalian cells in vitro and is not clastogenic in human lymphocytes in vitro. It is not clastogenic and does not induce DNA repair in vivo. Ethiprole shows no genotoxic potential either in vitro or in vivo (Table 14).

Table 14. Overview of genotoxicity with ethiprole[a]

End-point	Test object	Concentration	Batch no., purity	Results	Reference
In vitro					
Bacterial gene mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537 and *Escherichia coli* WP2 *uvr*A	1st experiment: 8, 40, 200, 1 000 and 5 000 µg/plate in DMSO (± S9) 2nd: 156.25 (with S9 only), 312.5, 625, 1 250, 2 500 and 5 000 µg/plate in DMSO (± S9)	CDR9706, 93%	Negative	Ballantyne (1998)
Mammalian cytogenetic test	Human lymphocytes	Up to 800 µg/mL in DMSO (± S9)	OP9750149, 93%	Negative	Marshall (1998)
Mammalian cell gene mutation	Mouse lymphoma L5178Y $TK^{+/-}$	15.625–500 µg/mL in DMSO (± S9)	CDR9706, 93%	Negative	Fellows (1999)
In vivo					
Micronucleus test in mice	CD-1 mice	500, 1 000 and 2 000 mg/kg bw in 0.5% methylcellulose	CDR9706, 93%	Negative	Burman (1999)
Unscheduled DNA synthesis	Han Wistar rat (liver)	2 000 mg/kg bw in 0.5% methylcellulose	CDR9706, 91.5%	Negative	Howe (2001)

bw: body weight; DMSO: dimethyl sulfoxide; DNA: deoxyribonucleic acid; S9: 9000 × *g* supernatant fraction from rat liver homogenate; *TK*: thymidine kinase

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

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

In a two-generation study of reproductive toxicity, four groups of 30 male and 30 female CD (Sprague Dawley) rats were given diets containing ethiprole (batch no. CDR9706; purity 92.7%) at a concentration of 0, 10, 75 or 500 ppm (equal to 0, 0.66, 4.8 and 32 mg/kg bw per day for males and 0, 0.78, 5.8 and 37 mg/kg bw per day for females, respectively, based on the 10-week premating feed intake in parental generation [F_0] animals).

Male and female rats were administered ethiprole in the feed for 10 weeks (premating period). Body weights and feed consumption were recorded weekly, and clinical signs were recorded at least once daily. Vaginal cytology was evaluated for the last 3 weeks of the prebreeding period. Animals were then randomly mated (one male with one female) within treatment groups for a 2-week mating period to produce the F_1 generation, with exposure continuing. F_0 males were necropsied after the delivery period, with histological evaluation of reproductive and other organs and andrological assessments (reproductive organ weights, epididymal sperm number, motility and morphology, testicular homogenization-resistant spermatid head counts, daily sperm production and efficiency of daily sperm production). The dams were allowed to rear their young to day 21 postpartum. F_1 litters were culled to 10 pups on postnatal day (PND) 4 and weaned on PND 21. At weaning, up to three weanlings of each sex per litter were necropsied, and 30 of each sex per dose were selected as F_1 parents of the F_2 generation. F_0 females were then necropsied, with measurement of organ weights, stage of estrus at necropsy, enumeration of ovarian primordial follicles, and histopathology of reproductive and other selected organs. Selected F_1 weanlings were administered ethiprole in the diet for a 10-week prebreeding exposure period; they were then mated for a 2-week period, as described above. The dams were allowed to rear their young to day 21 postpartum. On day 21 postpartum, each litter was weaned, and up to three weanlings of each sex per litter were necropsied. F_1 males were necropsied after the delivery period, with histopathology and andrological assessments (as described above). At weaning of the F_2 litters, parental F_1 females were then necropsied, with histopathology, as described above, and F_2 weanlings, up to three of each sex per litter, were necropsied.

There were no treatment-related deaths or clinical signs. At the start of the premating period, the body weights of F_1 animals at 500 ppm were significantly reduced (−13% for males and −17% for females, compared with controls), and body weight gains were reduced (−13%) in 500 ppm F_0 females in the premating period. Feed consumption was significantly lower for variable time points in F_0 males at 75 and 500 ppm and in F_0 females at 500 ppm.

At necropsy, F_0 parental absolute and relative liver weights (> +30% in males and > +70% in females) and thyroid weights (+28% in males and > +17% in females) and F_0 maternal adrenal weights (+11%) were significantly increased at 500 ppm. At 75 ppm, absolute and relative liver weights of F_0 females were slightly (+14%), but statistically significantly, increased. In F_1 animals, absolute and relative liver weights (> +26% in males and > +70% in females) and thyroid weights (> +32% in males and > +11% in females) were increased at 500 ppm. At 75 ppm, liver weights of F_1 females were slightly (+16% and +20% for absolute and relative weights, respectively), but statistically significantly, increased. At 500 ppm, histopathological findings were observed in the liver (hepatocyte cytoplasmic alteration in F_1 males, hypertrophy in F_0 and F_1 females) and thyroid (follicular cell hypertrophy in F_1 males and in F_0 and F_1 females). In the absence of concomitant histopathological findings, the increased liver weights at 75 ppm and increased adrenal weights at 500 ppm are considered not toxicologically relevant.

In general, JMPR considers increased liver weight with histopathological hypertrophy as an adaptive response in the absence of other liver effects. However, the liver weights were more than 170% of control values in both F_0 and F_1 females. Based on the rather large weight increase and the lack of blood parameter assessments, the liver effects at 500 ppm in females are considered adverse.

F_1 and F_2 pup body weights per litter were statistically significantly reduced at 500 ppm on PNDs 4, 7, 14 and 21. At necropsy at weaning, F_1 male and female pup body weights were statistically significantly reduced at 500 ppm. Absolute measured organ weights for F_1 pups were reduced at 500 ppm (gaining statistical significance for thymus, spleen, paired kidneys and brain, but not for liver); relative organ weights of spleen and kidneys were reduced, whereas relative liver and brain weights were increased. There were no treatment-related gross necropsy findings in either the F_1 males or the F_1 females at weaning.

During the postweaning/prebreeding exposure period of the selected F_1 offspring, the age at acquisition of female vaginal opening was statistically significantly delayed by approximately 2 days at 500 ppm. F_1 male preputial separation was delayed by approximately 3 days when expressed as age at acquisition or adjusted age at acquisition. Delay in acquisition of puberty for F_1 females was

associated with statistically significantly reduced body weights at acquisition at 500 ppm. These minimal effects on reproductive development in the F_1 offspring triggered measurement of anogenital distance in F_2 offspring at birth (PND 0), which showed no differences among groups.

At necropsy at weaning, F_2 male and female pup body weights were statistically significantly reduced at 500 ppm. Most likely due to reduced body weights, all absolute organ weights for F_2 pups were also reduced at 500 ppm. Relative organ weights for both sexes were either increased or unaffected (again, due to body weight reductions) at 500 ppm. There were no treatment-related gross necropsy findings in either the F_2 males or the F_2 females at weaning.

The NOAEL for parental toxicity was 75 ppm (equal to 4.8 mg/kg bw per day), based on reduced body weight and body weight gain and effects on liver and thyroid (histopathological and increased organ weights) at 500 ppm (equal to 32 mg/kg bw per day).

The NOAEL for offspring toxicity was 75 ppm (equal to 4.8 mg/kg bw per day), based on reduced body weight with associated delays in acquisition of puberty at 500 ppm (equal to 32 mg/kg bw per day).

The NOAEL for reproductive toxicity was 500 ppm (equal to 32 mg/kg bw per day), the highest dose tested (Tyl, Myers & Marr, 2001).

(b) Developmental toxicity

Rats

A dose range–finding developmental toxicity study was performed in groups of pregnant rats administered ethiprole by gavage at a dose of 0, 7.5, 25, 50 or 150 mg/kg bw per day from GD 6 to GD 20 (Foulon, 1999a). The results of this study were not remarkably different from those of the main developmental toxicity study described below.

In the main developmental toxicity study, ethiprole (batch no. CDR 9706; purity 92.7%) in 0.5% aqueous methylcellulose 400 was administered daily by gavage to groups of 23–25 mated Sprague Dawley (Crl: CD (SD) BR) rats from gestation day (GD) 6 to GD 20 at 0, 3, 10 or 30 mg/kg bw per day. All animals were examined twice daily except once daily on weekends, on public holidays and on GD 20. Body weight was determined on day 0 and on alternate days from GD 6 to GD 20. Feed consumption was determined during the intervals GDs 1–6, 6–8, 8–10, 10–12, 12–14, 14–16, 16–18 and 18–21. The fetuses were delivered by caesarean section on GD 21. The females were subjected to gross pathological examination. The gravid uterine weight was recorded. The dams were evaluated for the number of corpora lutea and the number and status of implantations (resorptions, dead and live fetuses). Live fetuses were removed from the uteri, counted, weighed, sexed and examined externally. Half of the fetuses were fixed in Bouin's solution and processed by scalpel and /or razor blade sectioning for visceral malformations. The other half of the fetuses were processed for skeletal and cartilage findings.

No deaths occurred in the study. Adverse maternal effects were noted at 30 mg/kg bw per day through body weight loss from GD 6 to GD 8 (Table 15) and reduced feed consumption from GD 6 to GD 12 (approximately 88% of control values).

The general conditions of females at 3 and 10 mg/kg bw per day were similar to those of controls. Macroscopic examination of females revealed increased mean liver weight at 10 and 30 mg/kg bw per day (111% and 115% of control values, respectively), and an accentuated lobular pattern was observed in 5/25 females at 30 mg/kg bw per day, compared with none in any of the other groups.

There were no significant effects of treatment on gestational parameters, including early and late resorptions, fetal death status and percentage of male fetuses. Analysis of mean fetal weights (calculated by sex and sexes combined) revealed no significant differences between treated and control groups. At 30 mg/kg bw per day, a few fetal findings were observed: increased incidences of enlarged thymus in male and female fetuses, of a slight ossification delay for a few bones (7th cervical centrum, 1st metatarsal and dumbbell at thoracic centrum) and of the anomalies at the 13th ribs (no, a short or a

discontinuous costal cartilage). No treatment-related malformations were observed. Minimal increased incidences in anomalies at the 4th and 7th costal cartilages and the thoracic centrum cartilage were observed at 30 mg/kg bw per day. At 10 and 30 mg/kg bw per day, one fetus per dose showed the presence of 25 presacral vertebrae, compared with none in the control and low-dose groups (Table 16).

Table 15. Developmental toxicity study in the rat: selected mean maternal body weights and maternal body weight change intervals

	0 ppm	3 ppm	10 ppm	30 ppm
Number of animals observed	25	23	23	25
Maternal body weight (g)				
Pretreatment: day 0	267.0	267.6	267.1	267.7
Pretreatment: day 6	301.0	299.4	301.1	300.6
Treatment day 8	307.0	305.0	304.5	300.0
Treatment day 21	438.8	439.9	443.7	430.5
Maternal body weight change (g)				
Pretreatment: days 0–6	34.1	31.8	34.0	32.9
Treatment days 6–8	6.0	5.6	3.4	−0.6***
Treatment days 8–10	10.7	10.1	11.3	9.7
Corrected body weight change[a]	65.8	64.0	66.8	56.8
Gravid uterine weight (g)	106.1	108.3	109. 8	106. 0
Liver weight (g)	14.2	14.8 (104%)	16.4*** (111%)	18.8*** (115%)

ppm: parts per million; ***: $P < 0.001$
[a] Corrected body weight change = (body weight on GD 21 − body weight on GD 0) − (gravid uterine weight).
Source: Foulon (2000)

Table 16. Developmental toxicity study in the rat: selected visceral and skeletal findings

	Number (%) of fetuses affected				Number (%) of litters affected			
	0 mg/kg bw per day	**3 mg/kg bw per day**	**10 mg/kg bw per day**	**30 mg/kg bw per day**	**0 mg/kg bw per day**	**3 mg/kg bw per day**	**10 mg/kg bw per day**	**30 mg/kg bw per day**
Number of fetuses/litters examined	180	168	169	176	24	23	23	24
Visceral examinations								
Variations								
Thymus (unilateral/bilateral): enlarged	7 (3.8)	2 (1.2)	6 (3.6)	16 (9.3)	5 (20.8)	2 (8.7)	4 (17.4)	11 (44.0)
Skeletal examinations								
Variations								
7th cervical centrum: unossified/normal cartilage	6 (3.2)	4 (2.2)	14 (7.9)	13 (6.8)	5 (20.0)	3 (13.0)	7 (30.4)	9 (36.0)

	Number (%) of fetuses affected				Number (%) of litters affected			
	0 mg/kg bw per day	3 mg/kg bw per day	10 mg/kg bw per day	30 mg/kg bw per day	0 mg/kg bw per day	3 mg/kg bw per day	10 mg/kg bw per day	30 mg/kg bw per day
14th thoracic rib (unilateral)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.6)	0 (0.0)	0 (0.0)	0 (0.0)	1 (4.0)
Thoracic centrum: dumbbell/normal cartilage	6 (3.2)	6 (3.5)	12 (7.6)	18 (9.6)	4 (16.0)	6 (26.1)	7 (30.4)	14 (56.0)
1st metatarsal (bilateral): unossified/normal cartilage	11 (5.6)	25 (14.0)	24 (13.8)	55 (29.1)	6 (24.0)	11 (47.8)	11 (47.8)	19 (76.0)
3rd and 4th proximal phalanges of forepaws (bilateral): unossified/normal cartilage	1 (0.6)	2 (1.3)	3 (1.6)	4 (2.4)	1 (4.0)	2 (8.7)	2 (8.7)	3 (12.0)
Anomalies								
13th rib(s) (unilateral/bilateral): short/discontinuous costal cartilage or costal cartilage absent	4 (2.1)	1 (0.6)	3 (1.7)	9 (5.0)	3 (12.0)	1 (4.3)	2 (8.7)	6 (24.0)
7th costal cartilages (bilateral) not attached to the sternum	0 (0.0)	0 (0.0)	0 (0.0)	2 (0.9)	0 (0.0)	0 (0.0)	0 (0.0)	2 (8.0)
4th costal cartilage (unilateral): misshapen	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	1 (4.0)
Presence of 25 presacral vertebrae	0 (0.0)	0 (0.0)	1 (0.6)	1 (0.5)	0 (0.0)	0 (0.0)	1 (4.3)	1 (4.0)
Thoracic centrum: bipartite and/or dumbbell or bipartite cartilage	2 (1.1)	2 (0.6)	2 (1.3)	5 (2.5)	2 (8.0)	1 (4.3)	2 (8.7)	5 (20.0)

bw: body weight
Source: Foulon (2000)

The NOAEL for maternal toxicity was 10 mg/kg bw per day, based on the slight decrease in body weight at GDs 6–8, decreased feed consumption and liver effects at 30 mg/kg bw per day.

The NOAEL for embryo/fetal toxicity was 10 mg/kg bw per day, based on the increased incidence of enlarged thymus and ossification delays in several bones at 30 mg/kg bw per day (Foulon, 2000).

Rabbits

A dose range–finding study was performed in which groups of eight pregnant rabbits were administered ethiprole by gavage at a dose of 0, 0.25, 0.75, 2 or 5 mg/kg bw per day from GD 6 to GD 28 (Foulon, 1999b). The results of this study were not remarkably different from those of the main developmental toxicity study described below.

In the main developmental toxicity study, groups of 30 artificially inseminated New Zealand white rabbits were exposed to ethiprole (batch no. CDR-9706; purity 92.7%) by gavage from GD 6 to GD 28. The doses given were 0, 0.5, 2.0 and 4.0 mg/kg bw per day in suspension in aqueous solution

of 0.5% methylcellulose 400. Two additional groups, a second control group and a low-dose group (0.25 mg/kg bw per day), were added to the study after a high abortion rate was observed at 4.0 mg/kg bw per day. The lower-dose group was added to ensure that at termination, there would be at least 20 pregnant females in each of the three treated groups. The additional control group was added to ensure that the newly added dose group (0.25 mg/kg bw per day) would have concurrent controls for statistical comparisons.

Maternal body weights were recorded for all females on GDs 0, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26 and 29. Feed consumption was also measured daily from GD 1 to GD 29 for all females. Clinical observations were recorded daily. At scheduled termination, on GD 29, gravid uterine weight and number of ribs were recorded, and the dams were evaluated for number of corpora lutea and number and status of implantations (resorptions, dead and live fetuses). Live fetuses were removed from the uteri, counted, weighed and examined externally. The heads of fetuses from approximately half of each litter were immersed in Bouin fluid, and the internal structures were examined after fixation. The bodies of all fetuses were dissected for soft tissue anomalies. All the fetuses were sexed. Fetuses were eviscerated, skinned and fixed in absolute ethanol before staining. A subsequent skeletal examination was performed.

Three control rabbits and two rabbits dosed at 0.25 mg/kg bw per day died owing to gavage errors during the study. None of the deaths was considered to be related to treatment with ethiprole. No other unscheduled deaths occurred during the study. In the 2.0 mg/kg bw per day dose group, two females aborted (on GD 25 and GD 28) after body weight loss and reduced feed intake. Eleven females in the 4.0 mg/kg bw per day dose group aborted between GD 21 and GD 27. In all cases, this occurred after a severe reduction of feed intake since the start of dosing and a body weight loss. At necropsy, 10 out of these 11 females had a pale and/or accentuated lobular pattern of liver. Body weight changes and feed consumption at 2.0 and 4.0 mg/kg bw per day were statistically significantly reduced for many intervals during the dosing period, when compared with control values.

The mean number of late resorptions was slightly increased at 4.0 mg/kg bw per day. All the other litter parameters did not show any statistically significant treatment-related effects. Fetal weights were considered unaffected by treatment. No malformations at external, visceral or skeletal examination were considered to reflect any abnormal development linked to treatment, except for two fetuses at 4.0 mg/kg bw per day with a diaphragmatic hernia. Incomplete ossification was noted for several bones (pubis, metacarpal and/or middle phalanges) at 2.0 and 4.0 mg/kg bw per day. Increased incidences of enlarged fontanelles and the presence of 27th presacral vertebrae were also observed at 2.0 and 4.0 mg/kg bw per day. The single incidence of a 27th presacral vertebrae at 0.5 mg/kg bw per day is considered to be within the biological range (Table 17).

The NOAEL for maternal toxicity was 0.5 mg/kg bw per day, based on excessive maternal toxicity (abortion, decreased body weight and reduced feed consumption) at 2.0 mg/kg bw per day.

The NOAEL for embryo/fetal toxicity was 0.5 mg/kg bw per day, based on increased incidences of ossification delays in several bones (metacarpal, phalanges, pubis), enlarged fontanelles and the presence of 27 presacral vertebrae at 2.0 mg/kg bw per day (Foulon, 2002).

2.6 Special studies

(a) Acute neurotoxicity

The acute neurotoxicity of ethiprole (batch no. CD9706; purity 93.0%) was investigated in groups of 10 male and 10 female Crl:CD BR rats administered a single dose of 0, 100, 500 or 2000 mg/kg bw by oral gavage. Observations made at 4 hours, 7 days and 14 days after dosing included a functional observational battery (FOB) and motor activity assessment. At the end of the study period, all animals were killed and the tissues fixed by whole body perfusion. Examination was confined to designated tissues of the nervous system, which were subsequently examined microscopically.

Table 17. Developmental toxicity study in the rabbit: selected visceral and skeletal findings

Observation	Number (%) of fetuses affected						Number (%) of litters affected					
	0 mg/kg bw per day	**0 mg/kg bw per day**	**0.25 mg/kg bw per day**	**0.50 mg/kg bw per day**	**2.0 mg/kg bw per day**	**4.0 mg/kg bw per day**	**0 mg/kg bw per day**	**0 mg/kg bw per day**	**0.25 mg/kg bw per day**	**0.50 mg/kg bw per day**	**2.0 mg/kg bw per day**	**4.0 mg/kg bw per day**
Number of fetuses/litters examined	178	169	191	171	136	80	23	23	26	24	20	13
Number of heads/litters (heads) examined	83	80	90	78	63	38	22	23	26	23	20	12
Visceral examinations												
Malformations												
Diaphragmatic hernia	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (1.6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (15.4)
Skeletal examinations												
Variations												
Anterior and/or posterior fontanelle(s): enlarged	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (4.3)	1 (1.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (15.0)	1 (7.7)
Hyoid centrum: unossified	1 (0.7)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.0)	4 (6.2)	1 (4.3)	0 (0.0)	0 (0.0)	0 (0.0)	1 (5.0)	2 (15.4)
Presence of 27 presacral vertebrae	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)	5 (2.6)	3 (3.3)	0 (0.0)	0 (0.0)	0 (0.0)	1 (4.2)	2 (10.0)	2 (15.4)
1st metacarpal: incomplete ossification or unossified	18 (9.8)	19 (11.4)	20 (12.1)	17 (10.6)	39 (27.6)	31 (35.4)	6 (26.1)	12 (52.2)	13 (50.0)	11 (45.8)	13 (65.0)	8 (61.5)

Observation	Number (%) of fetuses affected						Number (%) of litters affected					
	0 mg/kg bw per day	0 mg/kg bw per day	0.25 mg/kg bw per day	0.50 mg/kg bw per day	2.0 mg/kg bw per day	4.0 mg/kg bw per day	0 mg/kg bw per day	0 mg/kg bw per day	0.25 mg/kg bw per day	0.50 mg/kg bw per day	2.0 mg/kg bw per day	4.0 mg/kg bw per day
Forepaws: 4th or/and 5th middle phalanges: unossified												
Pubis (unilateral/ bilateral): incomplete ossification	4 (3.1)	3 (1.2)	4 (2.5)	2 (1.0)	14 (8.2)	11 (11.0)	3 (13.0)	2 (8.7)	4 (15.4)	1 (4.2)	6 (30.0)	4 (30.8)

bw: body weight
Source: Foulon (2002)

There were no treatment-related clinical signs or changes in body weight noted at any dose. Decreased feed consumption was noted among females treated at 2000 mg/kg bw. Behavioural changes were confined to the day of treatment. Behavioural observations at 2000 mg/kg bw included licking and chewing behaviours, decreased activity and rearing counts in the arena, increased forelimb grip strength, decreased landing foot splay, decreased rectal temperature, and a lower level of locomotor activity when assessed in the Coulbourn system. At 500 mg/kg bw, behavioural observations were increased forelimb grip strength, decreased landing foot splay, decreased rectal temperature (in females only) and a lower level of locomotor activity when assessed in the Coulbourn system. Females were also observed in the arena as chewing and with lower activity and rearing counts. At 100 mg/kg bw, behavioural observations were limited to decreased landing foot splay in both males and females. Females also had a lower level of locomotor activity when assessed in the Coulbourn system. Females were also observed in the arena as chewing. All changes appeared to be fully reversible, as behavioural parameters, rectal temperature and activity counts were normal at later evaluation times. There was no evidence of neuropathology in this study.

No NOAEL was identified. The lowest-observed-adverse-effect level (LOAEL) was 100 mg/kg bw, the lowest dose tested, based on decreased landing foot splay (both sexes) and a lower level of activity compared with controls (females only) on the day of dosing only (Palmer, 2001a).

In a second acute neurotoxicity study, ethiprole (batch no. CDR 9706; purity 93.0%) was administered to groups of 10 male and 10 female Crl:CD BR rats as a single oral dose (by gavage) of 0, 10, 25, 35 or 250 mg/kg bw. All of these animals were subjected to an FOB prior to treatment, at 4 hours after dosing and at 7 and 14 days after treatment. Motor activity of each animal was also quantitatively assessed at the same intervals. Throughout the study, clinical signs, body weights and feed consumption were monitored. At the end of the observation period, all animals were killed. There were no further investigations.

There were no clinical signs of reaction to treatment following dosing. There were no differences in body weights, feed conversion ratios or feed consumption between treated and control groups. The FOB changes associated with treatment were confined to the day of treatment. There were no differences between groups 7 and 14 days after treatment. At 250 mg/kg bw, 4 hours after dosing, observations included lower levels of arousal, higher incidence of closure of eyes, and lower activity and rearing counts among females. Forelimb and hindlimb grip strengths were increased in males, and females showed a decreased hindlimb splay. Locomotor activity was statistically significantly decreased in females. At 35 mg/kg bw, very limited changes were observed: indications of a lower level of arousal and a higher incidence of closure of the eyes. No effects on motor activity or FOB assessments were seen at 10 or 25 mg/kg bw.

The NOAEL for acute neurotoxicity was 25 mg/kg bw, based on the lower level of arousal and a higher incidence of closure of the eyes at 35 mg/kg bw on the day of dosing only (Palmer, 2001b).

(b) Subchronic neurotoxicity

In a subchronic assessment of the potential neurotoxicity of ethiprole (batch no. CDR 9706; purity 93%), groups of 10 male and 10 female Crl:CD BR rats were given ethiprole by dietary administration at a concentration of 0, 20, 100 or 400 ppm (equal to 0, 1.4, 7.2 and 28.7 mg/kg bw per day for males and 0, 1.7, 8.4 and 33.0 mg/kg bw per day for females, respectively) for 13 weeks. All of these animals were subjected to an FOB prior to treatment and after 4, 8 and 13 weeks of treatment. Motor activity of each animal was also quantitatively assessed at the same intervals. Throughout the study, clinical signs, body weights and feed consumption were monitored. At the end of the treatment period, all animals were killed and the tissues fixed by whole body perfusion. Brain, thyroid and liver were weighed, but relative weights were not calculated. Examination was confined to designated tissues of the nervous system, which were subsequently examined microscopically in five males and five females per group in control and high-dose animals.

At 400 ppm, findings were limited to statistically significantly increased liver weights (122% and 165% of control values for males and females, respectively) and thyroid weights (144% and 135% of control values for males and females, respectively). At 100 ppm, only the thyroid weights among males were statistically significantly increased (124% and 114% of control values for males and females, respectively), whereas the liver weights showed a 104% and 128% increase relative to controls for males and females, respectively. There were no signs of toxicity at 20 ppm or effects noted in the behavioural or neuropathology assessment for any treatment group. At 20 ppm, the thyroid and liver weights were not statistically significantly increased (thyroid: 119% and 106% of control values for males and females, respectively; liver: 97% and 116% of control values for males and females, respectively).

The NOAEL for systemic toxicity was 20 ppm (equal to 1.4 mg/kg bw per day), based on a statistically significant increase in thyroid weight in males at 100 ppm (equal to 7.2 mg/kg bw per day). No signs of neurotoxicity were observed at 400 ppm (equal to 28.7 mg/kg bw per day), the highest dose tested (Palmer, 2001c).

In the public literature, two studies were found on the reproductive and neurobehavioural effects of maternal exposure in F_1 generation mice: Tanaka & Inomata (2017) and Tanaka, Suzuki & Inomata (2018).

In Tanaka & Inomata (2017), ethiprole (lot no. KPL1496; purity 99.5%) was administered to groups of 10 male and 10 female mice at a dietary concentration of 0%, 0.0003%, 0.0009% or 0.0027% (equal to 0, 0.48, 1.47 and 4.56 mg/kg bw per day for males and 0, 0.58, 1.75 and 4.96 mg/kg bw per day for females, respectively, based on feed intake during premating). F_0 animals were treated from 5 weeks of age onwards; at 9 weeks of age, each female was mated with one male for 5 days. Males were subsequently killed just after mating (at 9 weeks of age), and females were killed after weaning of the offspring. Ethiprole administration was continued in one male and one female offspring from each litter until the end of the study, when the selected offspring were 11 weeks of age. Body weight, exploratory behaviour, offspring parameters, and functional and behavioural developmental parameters were measured.

In Tanaka, Suzuki & Inomata (2018), the study design was similar to that described in Tanaka & Inomata (2017), except for slightly different doses, and mated females were used. Ethiprole treatment was stopped at weaning, and the study proceeded with one male and one female offspring from each litter until 11 weeks of age after weaning.

The authors reported several effects on functional and behavioural parameters and suggested that these could be caused by irreversible effects on the central nervous systems of the pups, due to exposure of the maternal animals until weaning. However, the authors did not take into account that none of the effects was consistent over time or sex, there was not a consistent dose–response relationship and/or the effects were not observed in the other study (Tanaka & Inomata, 2017; Tanaka, Suzuki & Inomata, 2018).

(c) Mechanistic studies

In a 28-day study in mice, the effect of ethiprole on liver proliferation and liver P450 enzymes was investigated (Langrand-Lerche, 2002). This study did not provide additional information relevant to the evaluation of ethiprole.

The mechanism of action of ethiprole on thyroid function in the rat was studied in 2-week studies (Webber, 2001a,b,c). These studies did not provide additional information relevant to the evaluation of ethiprole.

A single-dose toxicity study in non-pregnant rabbits was provided (Totis, 2011). The study was evaluated for the sake of completeness; however, as this study was performed in non-pregnant rabbits,

it cannot overrule the effects seen in the does in the developmental toxicity study (see section 2.5(b) above).

This study was designed specifically to investigate whether or not the findings seen in does in the rabbit developmental toxicity study (see section 2.5(b) above) were relevant to the establishment of an acute reference dose (ARfD). Ethiprole (batch no. 101016E021; purity 96.4%) was administered by oral gavage to groups of non-pregnant female New Zealand white rabbits (15 per group and subgroup) at a single dose of 0.85, 1.5 or 3.0 mg/kg bw. A similarly constituted group received the vehicle (0.5% aqueous methylcellulose 400) and acted as a control. Animals were terminated 24 hours (subgroup 1) or 14 days (subgroup 2) after dosing. Clinical observations were performed once for subgroup 1 and daily for subgroup 2. Body weight was measured during the acclimatization period and just before the oral administration in order to calculate the required dosing volume. Body weight and feed consumption were measured every 2 days for subgroup 2. On day 2 (subgroup 1) or day 14 (subgroup 2), blood samples were collected from the central ear artery of each animal. Serum was then separated from whole blood and used for thyroid hormone analysis (T_3 and T_4). Ten out of 15 animals randomly selected from each termination time group were subjected to necropsy, and liver and thyroid were weighed, fixed and examined microscopically. In addition, hepatic cytochrome P450 activities (total, pentoxyresorufin *O*-dealkylation, ethoxyresorufin *O*-deethylation, benzyloxyresorufin *O*-dealkylation) and uridine diphosphate glucuronosyltransferase isoenzymes were assessed in these selected animals.

Based on the absence of any clearly adverse effects in this study, the NOAEL was 3.0 mg/kg bw per day, the highest dose tested (Totis, 2011). It should be noted that comparison with the maternal effects in the rabbit developmental toxicity study (see section 2.5(b) above) is hampered by the fact that the animals in the current study were non-pregnant and the fact that the body weights were expressed to one significant figure only. Therefore, the effects seen at 2.0 mg/kg bw per day in the developmental toxicity study (abortions, body weight changes of <100 g) cannot be overruled by the results of the current study.

(d) Immunotoxic effects

No studies were submitted.

(e) Studies on metabolites

Acute oral toxicity

Acute oral toxicity studies with ethiprole metabolites are summarized in Table 18.

Table 18. Acute oral toxicity studies with ethiprole metabolites

Metabolite	**Species**	**Strain**	**Sex**	**Route**	**Purity (%)**	**LD_{50} (mg/kg bw)**	**Reference**
Amide metabolite of ethiprole (RPA 112916)	Rat	Wistar	M + F	Oral	94.5	>5 000	Dange (2001b)
Sulfone metabolite of ethiprole (RPA 097973)	Rat	Wistar	M + F	Oral	99.1	>2 000	Bigot (1999a)
Sulfide metabolite of ethiprole (RPA 107566)	Rat	Wistar	M + F	Oral	98.1	>2 000	Bigot (1999b)

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

Short-term oral toxicity studies in rats

Groups of 10 male and 10 female Wistar (AF) rats received RPA 112916 (batch no. PAN01/99; purity 94.5%) in the diet at a concentration of 0, 50, 500, 5000 or 10 000 ppm (equal to 0, 5.2, 51.4, 515

and 983 mg/kg bw per day for males and 0, 5.2, 53.5, 512 and 993 mg/kg bw per day for females, respectively) for at least 28 days. Mortality and clinical signs were checked daily. Body weight and feed consumption were measured weekly throughout the study. A neurotoxicity assessment was performed on all animals during the acclimatization period and during week 4 of dosing; ophthalmic examinations were performed on all animals in the acclimatization period and on control and high-dose animals during week 4 of dosing. Clinical chemistry, haematology and urine analysis were performed towards the end of the dosing phase. In addition, a blood sample was collected during week 4 for T_3, T_4 and TSH analysis. All animals were subjected to necropsy, selected organs were weighed and designated tissues were sampled and examined microscopically.

RPA 112916 induced no treatment-related mortalities or clinical signs. Lower body weights (93% of control value) and body weight gains were noted in males at 10 000 ppm. Statistically significant decreases in mean feed consumption were observed in males at 10 000 ppm for the first 3 weeks and in females for the first week (82–88% of control values for males and 86% for females). Increases in prothrombin times were reported in males at 5000 and 10 000 ppm (107% and 115% of control values, respectively). At 5000 and 10 000 ppm, increases in mean total cholesterol (135% and 123% of control values, respectively) and triglyceride concentrations (150% and 110% of control values, respectively) were seen in females.

RPA 112916 induced an imbalance of thyroid hormones in animals treated at 5000 or 10 000 ppm, resulting in higher levels of TSH and lower levels of T_4. In females at 500 ppm, a statistically significant increase in TSH was also observed.

Significant increases in absolute and relative weights of liver, thyroid (males only) and adrenals were observed in males and females at 5000 and 10 000 ppm; at 500 ppm, increases in absolute and relative liver weights (males and females, <115% of controls) and absolute and relative adrenal weights (females) were observed. At histopathological examination, slight to mild centrilobular to panlobular hepatocellular hypertrophy was observed in all animals at 5000 and 10 000 ppm and in one male at 500 ppm. Thyroid follicular cell hypertrophy was found in six males and one female at 5000 ppm and in one male at 10 000 ppm. Slight adrenal microvacuolation of the zona fasciculata was observed at 500 (males only), 5000 and 10 000 ppm.

The NOAEL was 50 ppm (equal to 5.2 mg/kg bw per day), based on increased TSH and increased liver weight with associated histopathological changes at 500 ppm (equal to 51.4 mg/kg bw per day) (Bigot, 2000).

Genotoxicity

Genotoxicity data available for the amide metabolite (RPA 112916) and the sulfone metabolite (RPA 097973) of ethiprole are negative (Table 19).

Table 19. Overview of genotoxicity tests with ethiprole metabolites

Ethiprole metabolite	End-point	Test object	Concentration	Purity (%)	Results	Reference
Amide metabolite (RPA 112916)	Bacterial gene mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537 *Escherichia coli* WP2 pKM101 and WP2 *uvr*A pKM101	Experiment 1: 0, 1.6, 8, 40, 200, 1 000 and 5 000 µg/plate in DMSO (± S9) Experiment 2: 0, 156.25, 312.5, 625, 1 250, 2 500 and 5 000 µg/plate in DMSO (± S9)	99.7	Negative	Ballantyne (2001)

Ethiprole metabolite	End-point	Test object	Concentration	Purity (%)	Results	Reference
Sulfone metabolite (RPA 097973)	Bacterial gene mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537 *E. coli* WP2 *uvr*A	Experiment 1: 0, 8, 40, 200, 1 000 and 5 000 μg/plate in DMSO (± S9) Experiment 2 (except TA100): 0, 20.48, 51.2, 128, 320, 800, 2 000 and 5 000 μg/plate in DMSO (± S9) Experiment 2 (TA100): 4.096, 10.24, 25.6, 64, 160, 400 and 1 000 μg/plate in DMSO (± S9)	99.1	Negative	Dawkes (1999)
Sulfide metabolite (RPA 107566)	Bacterial gene mutation	*S. typhimurium* TA98 and TA100	± S9 Non-GLP screening test, no further details provided	Un-known	Negative	Esdaile (2002)

DMSO: dimethyl sulfoxide; GLP: good laboratory practice; S9: 9000 × *g* supernatant fraction from rat liver homogenate

3. Observations in humans

No medical problems related to the handling of ethiprole at the Bayer AG (CropScience Division) plant in Hangzhou, China, were reported to plant or health and safety management since the start of production in 2010 until the end of 2017.

All employees at the Bayer AG (CropScience Division) plant in Dormagen, Germany, undergo annual examinations, not directly related to ethiprole handling.

No reports are available on any symptoms for researchers handling the product in field trials.

No published human poisoning cases or epidemiological studies have been identified.

Comments

Biochemical aspects

In metabolism studies conducted in rats, ethiprole was rapidly excreted (up to 86% at 48 hours) after administration of a single or repeated low dose of 5 mg/kg bw; at a single high dose of 1000 mg/kg bw, excretion (up to 77%) was slightly lower. After a single low dose, the majority of administered radioactivity was excreted in the faeces, with urinary excretion being 24–36% after 168 hours. Similar results were obtained after repeated low doses. At the high dose, faecal excretion was again the major route of excretion, and urine accounted for only 3–5% of excretion after 168 hours. After bile duct cannulation, the urinary excretion and biliary excretion were, respectively, approximately 11% and 67% in males and 30% and 52% in females at the low dose, and approximately 1% and 9% in males and 2% and 7% in females at the high dose. T_{max} values in whole blood and tissues were 8 and 48 hours at the low and high doses (5 and 1000 mg/kg bw, respectively). Tissue distribution was independent of sex, dose and duration of dosing. The highest radioactive residues were present in the liver and kidney (up to 12% and 1%, respectively, at the low dose, and up to 1% for both at the high dose) and glandular tissues, including thyroid.

Ethiprole is extensively metabolized. The pattern of metabolites in urine was independent of sex, dose and duration of dosing. The major components were the polar glucuronide conjugate of hydroxy-MB 45897, the sulfinic acid RPA 104615, as well as the less polar (non-conjugated) MB 45897 and, in female urine, the carboxylic acid RPA 112705. Three primary parallel metabolic pathways could be derived from the metabolites observed: 1) hydrolysis of the nitrile group to form the amide RPA

112916; 2) reduction of the sulfoxide group to form the sulfide RPA 107566, with subsequent alkyl oxidation; and 3) oxidation of the sulfoxide group to form the major metabolite (i.e. the sulfone RPA 097973), followed by further metabolic reactions, including conjugate formation (McCorquodale & Anderson, 1999).

Toxicological data

The oral median lethal dose (LD_{50}) for ethiprole in rats was greater than 7080 mg/kg bw (Steiblen, 1997).

In a 28-day toxicity study, mice were fed diets containing ethiprole at a concentration of 0, 50, 250, 1000 or 2500 ppm (equal to 0, 9.3, 47.4, 186.2 and 458.0 mg/kg bw per day for males and 0, 11.8, 57.9, 234.4 and 513.0 mg/kg bw per day for females, respectively). The NOAEL was 50 ppm (equal to 9.3 mg/kg bw per day), based on increased liver weights and histopathological changes in the liver at 250 ppm (equal to 47.4 mg/kg bw per day) (Dange, 1999).

In a 28-day toxicity study, rats were fed diets containing ethiprole at a concentration of 0, 20, 100, 500 or 2500 ppm (equal to 0, 1.8, 9.2, 46.1 and 219.3 mg/kg bw per day for males and 0, 2.0, 9.6, 46.3 and 220.2 mg/kg bw per day for females, respectively). The NOAEL was 20 ppm (equal to 1.8 mg/kg bw per day), based on adrenal effects (increased weight with slight increase in vacuolation) and slight effects on thyroid hormones at 100 ppm (equal to 9.2 mg/kg bw per day) (Dange, 2001a).

In a 90-day toxicity study, rats were fed diets containing ethiprole at a concentration of 0, 5, 20, 500 or 2500 ppm (equal to 0, 0.296, 1.17, 30.5 and 155 mg/kg bw per day for males and 0, 0.373, 1.50, 37.6 and 188 mg/kg bw per day for females, respectively). The NOAEL was 20 ppm (equal to 1.17 mg/kg bw per day), based on mortality, changes in thyroid hormone levels, changes in clinical chemistry parameters and increased liver and thyroid weights associated with microscopic changes in these target organs at 500 ppm (equal to 30.5 mg/kg bw per day) (Dange & Foulon, 2002a).

In a 90-day toxicity study, dogs received ethiprole at a dietary concentration of 0, 30, 90 or 200 ppm (equal to 0, 1.0, 3.2 and 7.6 mg/kg bw per day for males and 0, 1.1, 3.6 and 8.5 mg/kg bw per day for females, respectively). The NOAEL was 30 ppm (equal to 1.0 mg/kg bw per day), based on reduced body weight gain and decreased thymus weight with atrophy at 90 ppm (equal to 3.2 mg/kg bw per day) (Bigot, 2002).

In a 1-year toxicity study, dogs received ethiprole at a dietary concentration of 0, 9, 30 or 90 ppm (equal to 0, 0.27, 0.70 and 2.73 mg/kg bw per day for males and 0, 0.22, 0.76 and 2.51 mg/kg bw per day for females, respectively). The NOAEL was 30 ppm (equal to 0.70 mg/kg bw per day), based on overall reduced body weight gain at 90 ppm (equal to 2.51 mg/kg bw per day) (Chevalier, 2001).

The overall NOAEL for oral toxicity in dogs was 30 ppm (equal to 1.0 mg/kg bw per day), based on reduced body weight gain at 90 ppm (equal to 2.51 mg/kg bw per day).

In a 78-week chronic toxicity and carcinogenicity study in mice, ethiprole was administered in the diet at a concentration of 0, 10, 50, 150 or 300 ppm (equal to 0, 1.7, 8.6, 25.6 and 50.8 mg/kg bw per day for males and 0, 1.7, 12.5, 36.3 and 73.5 mg/kg bw per day for females, respectively). The NOAEL for toxicity was 150 ppm (equal to 36.3 mg/kg bw per day), based on a decrease in survival rate in females at 300 ppm (equal to 73.5 mg/kg bw per day). The NOAEL for carcinogenicity was 150 ppm (equal to 36.3 mg/kg bw per day), based on an increase in the incidence of hepatocellular adenomas in females at 300 ppm (equal to 73.5 mg/kg bw per day) (Richard, 2002).

In a 104-week combined chronic toxicity and carcinogenicity study in rats, ethiprole was administered in the diet at 0, 5, 20, 75 or 250 ppm (equal to 0, 0.22, 0.85, 3.21 and 10.8 mg/kg bw per day for males and 0, 0.29, 1.17, 4.40 and 14.7 mg/kg bw per day for females, respectively). The NOAEL for toxicity was 20 ppm (equal to 0.85 mg/kg bw per day), based on effects in the thyroid and/or liver (histopathological changes, increased organ weights and/or altered thyroid hormone or bilirubin levels) at 75 ppm (equal to 3.21 mg/kg bw per day). At 250 ppm (equal to 10.8 mg/kg bw per day), increased incidences of tumours (subcutaneous lipoma in males, hepatocellular adenoma in males and thyroid

adenoma in males and females) were noted, and the slight increase in ovary sex cord tumours was considered equivocal. The NOAEL for carcinogenicity was 75 ppm (equal to 3.21 mg/kg bw per day) (Dange & Foulon, 2002b).

The Meeting concluded that ethiprole is carcinogenic in mice and rats.

Ethiprole was tested for genotoxicity in an adequate range of in vitro and in vivo assays. No evidence of genotoxicity was found (Ballantyne, 1998; Marshall, 1998; Burman, 1999; Fellows, 1999; Howe, 2001).

The Meeting concluded that ethiprole is unlikely to be genotoxic.

In view of the lack of genotoxicity and the fact that tumours were observed only at doses unlikely to occur in humans, the Meeting concluded that ethiprole is unlikely to pose a carcinogenic risk to humans via exposure from the diet.

In a two-generation reproductive toxicity study, ethiprole was administered to rats in the diet at a concentration of 0, 10, 75 or 500 ppm (equal to 0, 0.66, 4.8 and 32 mg/kg bw per day for males and 0, 0.78, 5.8 and 37 mg/kg bw per day for females, respectively, based on the 10-week premating feed intake in F_0 generation animals). The NOAEL for parental toxicity was 75 ppm (equal to 4.8 mg/kg bw per day), based on decreased body weight and body weight gain and effects on liver and thyroid (histopathological effects and increased organ weights) at 500 ppm (equal to 32 mg/kg bw per day). The NOAEL for offspring toxicity was 75 ppm (equal to 4.8 mg/kg bw per day), based on reduced body weight with associated delays in acquisition of puberty at 500 ppm (equal to 32 mg/kg bw per day). The NOAEL for reproductive toxicity was 500 ppm (equal to 32 mg/kg bw per day), the highest dose tested (Tyl, Myers & Marr, 2001).

In a developmental toxicity study in rats, ethiprole was administered by oral gavage at a dose of 0, 3, 10 or 30 mg/kg bw per day on days 6–20 of gestation. The NOAEL for maternal toxicity was 10 mg/kg bw per day, based on the slight decrease in body weight at GDs 6–8, decreased feed consumption and liver effects at 30 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 10 mg/kg bw per day, based on the increased incidence of enlarged thymus and ossification delays in several bones at 30 mg/kg bw per day (Foulon, 2000).

In a developmental toxicity study in rabbits, ethiprole was administered orally by gavage from GDs 6 through 28 at a dose of 0, 0.25, 0.5, 2.0 or 4.0 mg/kg bw per day. The NOAEL for maternal toxicity was 0.5 mg/kg bw per day, based on excessive maternal toxicity (abortion, decreased body weight and reduced feed consumption) at 2.0 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 0.5 mg/kg bw per day, based on increased incidences of ossification delays in several bones (metacarpal, phalanges, pubis), enlarged fontanelles and the presence of 27 presacral vertebrae (variation) at 2.0 mg/kg bw per day (Foulon, 2002).

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

In an acute neurotoxicity study in rats, ethiprole was administered orally by gavage at a dose of 0, 100, 500 or 2000 mg/kg bw. No NOAEL was identified. The LOAEL was 100 mg/kg bw, the lowest dose tested, based on decreased landing foot splay (both sexes) and a lower level of activity compared with controls (females only) on the day of dosing only. There was no evidence of neuropathology after 14 days (Palmer, 2001a).

In a second acute neurotoxicity study in rats, ethiprole was administered orally by gavage at a dose of 0, 10, 25, 35 or 250 mg/kg bw. The NOAEL for acute neurotoxicity was 25 mg/kg bw, based on the lower level of arousal and a higher incidence of closure of the eyes at 35 mg/kg bw on the day of dosing only. There was no evidence of neuropathology after 14 days (Palmer, 2001b).

In a 90-day dietary neurotoxicity study, rats were fed ethiprole at a concentration of 0, 20, 100 or 400 ppm (equal to 0, 1.4, 7.2 and 28.7 mg/kg bw per day for males and 0, 1.7, 8.4 and 33.0 mg/kg bw per day for females, respectively). The NOAEL for systemic toxicity was 20 ppm (equal to 1.4 mg/kg bw per day), based on an increase in thyroid weight in males at 100 ppm (equal to 7.2 mg/kg bw

per day). No signs of neurotoxicity were observed at 400 ppm (equal to 28.7 mg/kg bw per day), the highest dose tested. There was no evidence of neuropathology at any dose (Palmer, 2001c).

The Meeting concluded that ethiprole induces transient neurobehavioural effects.

No studies on immunotoxic effects were submitted.

Toxicological data on metabolites and/or degradates

RPA 112916 (plant and rat metabolite)

The acute oral LD_{50} for the amide metabolite of ethiprole (RPA 112916) in rats was greater than 5000 mg/kg bw (Dange, 2001b).

In a 28-day dietary toxicity study, rats were fed RPA 112916 at a concentration of 0, 50, 500, 5000 or 10 000 ppm (equal to 0, 5.2, 51.4, 515 and 983 mg/kg bw per day for males and 0, 5.2, 53.5, 512 and 993 mg/kg bw per day for females, respectively). The NOAEL was 50 ppm (equal to 5.2 mg/kg bw per day), based on increased TSH and increased liver weight with associated histopathological changes at 500 ppm (equal to 51.4 mg/kg bw per day) (Bigot, 2000).

RPA 112916 was not mutagenic in an Ames test (Ballantyne, 2001).

RPA 112916 is found in bile of rats in low amounts (2–3% of the total radioactive residues) and is structurally very similar to its parent compound.

These findings indicate that the toxicological profile of RPA 112916 is very similar to that of its parent, ethiprole. It is concluded that RPA 112916 is not more potent than ethiprole.

RPA 097973 (plant metabolite)

The oral LD_{50} for the sulfone metabolite of ethiprole (RPA 097973) in rats was greater than 5000 mg/kg bw (Bigot, 1999a).

RPA 097973 was not mutagenic in an Ames test (Dawkes, 1999).

RPA 097973 is found in urine at over 10% of the dose, and its toxicity is considered to be covered by the parent compound.

N-Glucuronide of RPA 107566 (goat metabolite)

The *N*-glucuronide of RPA 107566 is not found in the rat. RPA 107566 is a minor metabolite in rat, found in faeces at less than 5%. The acute oral LD_{50} for RPA 107566 is greater than 2000 mg/kg bw (Bigot, 1999b), and RPA 107566 is negative in a screening Ames test (Esdaile, 2002). The *N*-glucuronide of RPA 107566 is expected to be less toxic than RPA 107566.

For chronic toxicity, the threshold of toxicological concern approach (Cramer class III) could be applied to the *N*-glucuronide of RPA 107566.

Human data

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

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

Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) of 0–0.005 mg/kg bw, based on the NOAEL of 0.5 mg/kg bw per day for maternal (abortion, decreased body weight and reduced feed consumption) and embryo/fetal toxicity (ossification delays in several bones, enlarged fontanelles and the presence of 27 presacral vertebrae) in the developmental toxicity study in rabbits and using a safety factor of 100. The margin between the upper bound of the ADI and the LOAEL for liver, thyroid and skin tumours in rats is approximately 2000.

The Meeting established an ARfD of 0.005 mg/kg bw, based on the NOAEL of 0.5 mg/kg bw per day for maternal toxicity (decreased body weight and reduced feed consumption) in the developmental toxicity study in rabbits and using a safety factor of 100.

The Meeting concluded that the metabolites ethiprole-amide (RPA 112916) and ethiprole-sulfone (RPA 097973) would be covered under the ADI and the ARfD for ethiprole.

Levels relevant to risk assessment of ethiprole

Species	Study	Effect	NOAEL	LOAEL
Mouse	Seventy-eight-week study of toxicity and carcinogenicity[a]	Toxicity	150 ppm, equal to 36.3 mg/kg bw per day	300 ppm, equal to 73.5 mg/kg bw per day
		Carcinogenicity	150 ppm, equal to 36.3 mg/kg bw per day	300 ppm, equal to 73.5 mg/kg bw per day
Rat	Acute neurotoxicity study[b]	Neurotoxicity	25 mg/kg bw	35 mg/kg bw
	Two-year study of toxicity and carcinogenicity[a]	Toxicity	20 ppm, equal to 0.85 mg/kg bw per day	75 ppm, equal to 3.21 mg/kg bw per day
		Carcinogenicity	75 ppm, equal to 3.21 mg/kg bw per day	250 ppm, equal to 10.8 mg/kg bw per day
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	500 ppm, equal to 32 mg/kg bw per day[c]	–
		Parental toxicity	75 ppm, equal to 4.8 mg/kg bw per day	500 ppm, equal to 32 mg/kg bw per day
		Offspring toxicity	75 ppm, equal to 4.8 mg/kg bw per day	500 ppm, equal to 32 mg/kg bw per day
	Developmental toxicity study[b]	Maternal toxicity	10 mg/kg bw per day	30 mg/kg bw per day
		Embryo and fetal toxicity	10 mg/kg bw per day	30 mg/kg bw per day
Rabbit	Developmental toxicity study[b]	Maternal toxicity	0.5 mg/kg bw per day	2.0 mg/kg bw per day
		Embryo and fetal toxicity	0.5 mg/kg bw per day	2.0 mg/kg bw per day

Species	Study	Effect	NOAEL	LOAEL
Dog	Thirteen-week and 1-year studies of toxicity[a,d]	Toxicity	30 ppm, equal to 1.0 mg/kg bw per day	90 ppm, equal to 2.51 mg/kg bw per day

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

Acceptable daily intake (ADI) (applies to ethiprole, ethiprole-amide and ethiprole-sulfone, expressed as ethiprole)

0–0.005 mg/kg bw

Acute reference dose (ARfD) (applies to ethiprole, ethiprole-amide and ethiprole-sulfone, expressed as ethiprole)

0.005 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 ethiprole

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid; extensive (~80%) at 5 mg/kg bw; low (~10%) at 1 000 mg/kg bw
Dermal absorption	No data
Distribution	Highest residues in liver, kidney and glandular tissues, including thyroid
Potential for accumulation	No evidence of accumulation
Rate and extent of excretion	Largely complete within 48 hours, primarily in bile and faeces
Metabolism in animals	Extensive, independent of sex, dose and duration of dosing; hydrolysis of nitrile group, reduction of sulfoxide group, oxidation of sulfoxide group
Toxicologically significant compounds in animals and plants	Ethiprole
Acute toxicity	
Rat, LD_{50}, oral	>7 080 mg/kg bw
Rat, LD_{50}, dermal	No data
Rat, LC_{50}, inhalation	No data
Rabbit, dermal irritation	No data
Rabbit, ocular irritation	No data
Guinea-pig, dermal sensitization	No data

Short-term studies of toxicity	
Target/critical effect	Body weight gain
Lowest relevant oral NOAEL	1.0 mg/kg bw per day (dog)
Lowest relevant dermal NOAEL	No data
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Thyroid and liver
Lowest relevant NOAEL	0.85 mg/kg bw per day (rat)
Carcinogenicity	Carcinogenic in mice and rats[a]
Genotoxicity	
	No evidence of genotoxicity in vitro or in vivo[a]
Reproductive toxicity	
Target/critical effect	Body weight, liver, thyroid
Lowest relevant parental NOAEL	4.8 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	4.8 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	32 mg/kg bw per day, highest dose tested (rat)
Developmental toxicity	
Target/critical effect	Abortion, body weight, feed consumption, ossification delays, several skeletal variations
Lowest relevant maternal NOAEL	0.5 mg/kg bw per day (rabbit)
Lowest relevant embryo/fetal NOAEL	0.5 mg/kg bw per day (rabbit)
Neurotoxicity	
Acute neurotoxicity NOAEL	25 mg/kg bw
Subchronic neurotoxicity NOAEL	28.7 mg/kg bw per day, highest dose tested
Developmental neurotoxicity NOAEL	No data
Other toxicological studies	
Immunotoxicity	No data
Studies on toxicologically relevant metabolites	
RPA 112916	Acute oral LD_{50}: >5 000 mg/kg bw (rat)
	28-day dietary toxicity NOAEL: 5.2 mg/kg bw per day (rat)
	Ames: Not mutagenic
RPA 097973	Acute oral LD_{50}: >5 000 mg/kg bw (rat)
	Ames: Not mutagenic
Human data	
	No adverse effects in manufacturing personnel

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

Summary

	Value	Study	Safety factor
ADI	0–0.005 mg/kg bw[a]	Developmental toxicity study in rabbits	100
ARfD	0.005 mg/kg bw[a]	Developmental toxicity study in rabbits	100

[a] Applies to ethiprole, ethiprole-amide and ethiprole-sulfone, expressed as ethiprole.

References

Ballantyne M (1998). RPA107382: reverse mutation in four histidine-requiring strains of *Salmonella typhimurium* and one tryptophan-requiring strain of *Escherichia coli*. Unpublished report no. 198/115-D5140/R016832. Covance Laboratories Ltd, Harrogate, United Kingdom. Bayer File No. M-192448-01-1. Submitted to WHO by Bayer.

Ballantyne M (2001). RPA112916 (metabolite of ethiprole): reverse mutation in four histidine-requiring strains of *Salmonella typhimurium* and two tryptophan-requiring strains of *Escherichia coli*. Unpublished report no. 1905/64-D6171/C016325. Covance Laboratories Ltd, Harrogate, United Kingdom. Bayer File No. M-202186-01-1. Submitted to WHO by Bayer.

Bigot D (1999a). Acute oral toxicity in the rat: RPA097973. Unpublished report no. SA 98546/R016844. Rhone-Poulenc Agro, Sophia Antipolis, France. Bayer File No. M-192471-01-1. Submitted to WHO by Bayer.

Bigot D (1999b). Acute oral toxicity in the rat: RPA107566. Unpublished report no. SA 98545/R016845. Rhone-Poulenc Agro, Sophia Antipolis, France. Bayer File No. M-192473-01-1. Submitted to WHO by Bayer.

Bigot D (2000). RPA112916 metabolite of ethiprole (RPA107382): 28-day toxicity study in the rat by dietary administration. Unpublished report no. SA 99387/R016688. Aventis CropScience S.A., Sophia Antipolis, France. Bayer File No. M-192137-01-1. Submitted to WHO by Bayer.

Bigot D (2002). RPA 107382: 90-day toxicity study in the dog by dietary administration. Amended report. Unpublished report no. SA 98340, C023975/C014168. Aventis CropScience S.A., Sophia Antipolis, France. Bayer File No. M-206098-02-1. Submitted to WHO by Bayer.

Burman M (1999). RPA 107382: induction of micronuclei in the bone marrow of treated mice. Unpublished report no. 198/119-D5140/R000181. Covance Laboratories Ltd, Harrogate, United Kingdom. Bayer File No. M-158055-01-1. Submitted to WHO by Bayer.

Chevalier G (2001). Ethiprole (RPA107382): 52-week dietary toxicity in beagle dogs. Unpublished report no. 17445 TCC, C013286. CIT Centre Internationale de Toxicologie, Evreux, France. Bayer File No. M-204326-01-1. Submitted to WHO by Bayer.

Dange M (1999). RPA 107382: preliminary 28 day toxicity study in the C57 B1/6N mice by dietary administration. Unpublished report no. SA 98331, R005957. Rhone-Poulenc Agro, Sophia Antipolis, France. Bayer File No. M-171883-01-1. Submitted to WHO by Bayer.

Dange M (2001a). Ethiprole RPA 107382: 28-day toxicity study in the rat by dietary administration. Unpublished report no. SA 99383, C011658. Aventis CropScience S.A., Sophia Antipolis, France. Bayer File No. M-201261-01-1. Submitted to WHO by Bayer.

Dange M (2001b). Acute oral toxicity in the rat: RPA 112916. Unpublished report no. SA 01040/C012671. Aventis CropScience S.A., Sophia Antipolis, France. Bayer File No. M-203164-01-1. Submitted to WHO by Bayer.

Dange M, Foulon O (2002a). RPA 107382: 90-day toxicity study in the rat by dietary administration. Unpublished report no. SA 97233, C021079/C016682. Aventis CropScience S.A., Sophia Antipolis, France. Bayer File No. M-192116-02-1. Submitted to WHO by Bayer.

Dange M, Foulon O (2002b). RPA 107382: chronic toxicity and carcinogenicity study of ethiprole (RPA 107382) in the Wistar rat by dietary administration. Unpublished report no. SA 97593, C021077/C012607. Aventis CropScience S.A., Lyon, France. Bayer File No. M-203024-02-1. Submitted to WHO by Bayer.

Dawkes N (1999). RPA097973: reverse mutation in four histidine-requiring strains of *Salmonella typhimurium* and one tryptophan-requiring strain of *Escherichia coli*. Amended final report. Unpublished report no. 198/126-D5140/R016656. Covance Laboratories Ltd, Harrogate, United Kingdom. Bayer File No. M-192043-01-1. Submitted to WHO by Bayer.

Esdaile DJ (2002). RPA 107566: intermediate of RPA 107382. Industrial hygiene hazard identification screen. Unpublished report no. SA 98065. Bayer CropScience, Sophia Antipolis, France. Bayer File No. M-217373-01-1. Submitted to WHO by Bayer.

Fellows M (1999). RPA 107382: Mutation at the thymidine kinase (*tk*) locus of mouse lymphoma L5178Y cells (MLA) using the microtitre fluctuation technique. Unpublished report no. 198/114-D5140/R000204. Covance Laboratories Ltd, Harrogate, United Kingdom. Bayer File No. M-158105-01-1. Submitted to WHO by Bayer.

Foulon O (1999a). Ethiprole (RPA107382). Range-finding study for developmental toxicity in the rat by gavage. Unpublished report no. SA 99036, R016879. Aventis CropScience S.A., Sophia Antipolis, France. Bayer File No. M-192545-01-1. Submitted to WHO by Bayer.

Foulon O (1999b). Ethiprole (RPA107382). Range-finding study for developmental toxicity in the rabbit by gavage. Unpublished report no. SA 99078, R016881. Aventis CropScience S.A., Sophia Antipolis, France. Bayer File No. M-192549-01-1. Submitted to WHO by Bayer.

Foulon O (2000). Ethiprole (RPA107382). Developmental toxicity study in the rat by gavage. Unpublished report no. SA 99146, R018797. Aventis CropScience S.A., Sophia Antipolis, France. Bayer File No. M-196111-01-1. Submitted to WHO by Bayer.

Foulon O (2002). Ethiprole (RPA107382). Developmental toxicity study in the rabbit by gavage. Unpublished report no. SA 99088, R016684. Aventis CropScience S.A., Sophia Antipolis, France. Bayer File No. M-192124-02-1. Submitted to WHO by Bayer.

Howe J (2001). Ethiprole (RPA 107382): measurement of unscheduled DNA synthesis in rat liver using an in vivo/in vitro procedure. Unpublished report no. 2014/31-D6173/C021030. Covance Laboratories Ltd, Harrogate, United Kingdom. Bayer File No. M-210455-01-1. Submitted to WHO by Bayer.

Langrand-Lerche C (2002). 28-day explanatory toxicity study in the C57BL/6 mouse. Ethiprole. Unpublished report no. SA 01416/C026077. Bayer CropScience S.A., Sophia Antipolis, France. Bayer File No. M-210729-01-1. Submitted to WHO by Bayer.

Marshall R (1998). RPA107382: induction of chromosome aberrations in cultured human peripheral blood lymphocytes. Unpublished report no. 198/116-D5140/R016593. Covance Laboratories Ltd, Harrogate, United Kingdom. Bayer File No. M-191907-01-1. Submitted to WHO by Bayer.

McCorquodale GY, Anderson AR (1999). [^{14}C]-RPA 107382: absorption, distribution, metabolism and excretion in the rat. Unpublished report no. C025330. Inveresk Research International Ltd, Tranent, United Kingdom. Bayer File No. M-192087-02-1. Submitted to WHO by Bayer.

Palmer HA (2001a). RPA107382: neurotoxicity to rats by acute oral administration. Unpublished report no. RNP/558, C016157. Huntingdon Life Sciences Ltd, Huntingdon, United Kingdom. Bayer File No. M-201848-01-1. Submitted to WHO by Bayer.

Palmer HA (2001b). RPA107382: neurotoxicity study by a single oral gavage administration to CD rats followed by a 14-day observation period. Unpublished report no. RNP/608, C016526. Aventis CropScience S.A., Sophia Antipolis, France. Bayer File No. M-202573-01-1. Submitted to WHO by Bayer.

Palmer HA (2001c). RPA107382: 13 week neurotoxicity study in rats by dietary administration. Unpublished report no. RNP/610/992922, C016595. Huntingdon Life Sciences Ltd, Huntingdon, United Kingdom. Bayer File No. M-202708-01-1. Submitted to WHO by Bayer.

Richard J (2002). Ethiprole (RPA107382): carcinogenicity study by oral route (dietary admixture) in C57BL/6 mice. Amended report. Unpublished report no. 17351 TCS, C023452/C014046. CIT Centre International de Toxicologie, Evreux, France. Bayer File No. M-205769-02-1. Submitted to WHO by Bayer.

Steiblen G (1997). RPA107382: acute oral toxicity in the rat. Unpublished report no. SA 97421, R016801. Rhone-Poulenc Agro, Sophia Antipolis, France. Bayer File No. M-192389-01-1. Submitted to WHO by Bayer.

Tanaka T, Inomata A (2017). Reproductive and neurobehavioral effects of ethiprole administered to mice in the diet. Birth Defects Res. 109:1568–85.

Tanaka T, Suzuki T, Inomata A (2018). Reproductive and neurobehavioral effects of maternal exposure to ethiprole in F_1-generation mice. Birth Defects Res. 110(3):259–75.

Totis M (2011). Ethiprole – Acute toxicity study in the rabbit by gavage. Unpublished report no. SA 10376. Bayer S.A.S., Bayer CropScience, Sophia Antipolis, France. Bayer File No. M-416191-01-1. Submitted to WHO by Bayer.

Tyl RW, Myers CB, Marr MC (2001). Ethiprole 2-generation reproductive toxicity evaluation of ethiprole (RP107382) administered in the feed to CD (Sprague-Dawley) rats. Unpublished report no. 65C.07407.300, B003509. Research Triangle Institute, Research Triangle Park, NC, USA. Bayer File No. M-240522-01-1. Submitted to WHO by Bayer.

Webber C (2001a). Ethiprole investigation into potential effects on thyroid function after two weeks of treatment in male rats using the perchlorate discharge test. Unpublished report no. RNP/613 / C017842. Huntingdon Life Sciences Ltd, Huntingdon, United Kingdom. Bayer File No. M-205038-01-1. Submitted to WHO by Bayer.

Webber C (2001b). The effect of repeated oral doses of ethiprole on the biliary excretion of intravenously administered ^{125}I-thyroxine (T_4) in male rats. Unpublished report no. C017804. Huntingdon Life Sciences Ltd, Huntingdon, United Kingdom. Bayer File No. M-204974-01-1. Submitted to WHO by Bayer.

Webber C (2001c). The effect of repeated oral doses of ethiprole on the pharmacokinetics of intravenously administered ^{125}I-thyroxine (T_4) in male rats. Unpublished report no. C016605. Huntingdon Life Sciences Ltd, Huntingdon, United Kingdom. Bayer File No. M-202728-01-1. Submitted to WHO by Bayer.

FENPICOXAMID

First draft prepared by
Kimberley Low[1] and Angelo Moretto[2]

[1] Health Evaluation Directorate, Pest Management Regulatory Agency, Health Canada, Ottawa, Ontario, Canada
[2] Department of Biomedical and Clinical Sciences, University of Milan, and International Centre for Pesticides and Health Risk Prevention, ASST Fatebenefratelli Sacco, Milan, Italy

Explanation 57
Evaluation for acceptable intake 58
1. Biochemical aspects 58
1.1 Absorption, distribution and excretion 62
(a) Oral and intravenous routes 62
(b) Dermal route 66
1.2 Biotransformation 69
(a) In vivo studies 69
(b) In vitro studies 71
1.3 Toxicokinetics 72
2. Toxicological studies 74
2.1 Acute toxicity 74
(a) Lethal doses 74
(b) Dermal irritation 75
(c) Ocular irritation 75
(d) Dermal sensitization 75
(e) Phototoxicity 75
2.2 Short-term studies of toxicity 75
(a) Oral administration 75
(b) Dermal application 81
(c) Exposure by inhalation 81
2.3 Long-term studies of toxicity and carcinogenicity 81
2.4 Genotoxicity 91
(a) In vitro studies 91
(b) In vivo studies 91
2.5 Reproductive and developmental toxicity 92
(a) Multigeneration studies 92
(b) Developmental toxicity 93
2.6 Special studies 95
(a) Neurotoxicity 95
(b) Immunotoxicity 95
(c) Studies on metabolites 95
(d) Investigation of impurities 95
(e) Microbiological effects 96
3. Observations in humans 97
Comments 97
Toxicological evaluation 100
References 102

Explanation

Fenpicoxamid is the International Organization for Standardization–approved common name for [2-[[(3*R*,7*R*,8*R*,9*S*)-7-benzyl-9-methyl-8-(2-methylpropanoyloxy)-2,6-dioxo-1,5-dioxonan-3-yl]-carbamoyl]-4-methoxypyridin-3-yl]oxymethyl 2-methylpropanoate, with the Chemical Abstracts Service (CAS) number 517875-34-2.

Fenpicoxamid is a foliar fungicide whose mode of action is by the inhibition of mitochondrial complex III to disrupt spore germination and germ tube elongation.

Fenpicoxamid 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) and were conducted in accordance with relevant national or international test guidelines, unless otherwise specified. No additional information from a literature search was identified that complemented the toxicological information submitted for the current assessment.

Evaluation for acceptable intake

1. Biochemical aspects

The absorption, distribution, metabolism and excretion of fenpicoxamid, as well as its toxicokinetics, have been investigated in F344 and Sprague Dawley rats, Crl:CD1 mice, New Zealand white rabbits and beagle dogs. Summaries of the relevant data are presented below.

The absorption, distribution, metabolism and elimination (ADME) of fenpicoxamid were investigated using fenpicoxamid uniformly labelled (UL) on the phenyl ring (Fig. 1) or labelled at the pyridine-2 position (Fig. 2). The test item in the main studies was a mixture of ^{14}C-labelled and unlabelled fenpicoxamid, with a probe study performed with a mixture of ^{12}C-, ^{13}C- and ^{14}C-labelled fenpicoxamid. The study design is summarized in Table 1.

Fig. 1. [^{14}C-phenyl-UL]Fenpicoxamid

*XDE-777 phenyl-UL-^{14}C

Fig. 2. [^{14}C-2-pyridine]Fenpicoxamid

*XDE-777 pyridine-2-^{14}C

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

Test group	Dose (mg/kg bw) (route)	Number of animals of each sex	Remarks	Reference
Probe study – F344/NTac rats – time course rat blood (group 1)	65 (oral)	1 M	Single-dose mixture of [^{14}C-phenyl-UL]fenpicoxamid, [^{13}C-phenyl-UL]fenpicoxamid and unlabelled fenpicoxamid with target radioactivity of 3.0 MBq/kg bw in 0.5% methylcellulose Excreta, blood (plasma and RBCs), expired air, volatiles and time course plasma collected	Hansen et al. (2012)
Probe study – F344/NTac rats – rat excreta (group 2)	65 (oral)	1 M, 1 F	Single dose of [^{13}C-phenyl-UL]fenpicoxamid in 0.5% methylcellulose Excreta and blood collected	Hansen et al. (2012)
Probe study – F344/NTac rats – C_{max} rat blood (group 3)	65 (oral)	1 M	Single-dose mixture of [^{14}C-phenyl-UL]fenpicoxamid, [^{13}C-phenyl-UL]fenpicoxamid and unlabelled fenpicoxamid with target radioactivity of 3.0 MBq/kg bw in 0.5% methylcellulose Killed at C_{max}, and blood collected	Hansen et al. (2012)
Probe study – F344/NTac rats – C_{max} rat blood (group 4)	65 (oral)	1 F	Single dose of [^{13}C-phenyl-UL]fenpicoxamid in 0.5% methylcellulose Killed at C_{max}, and blood collected	Hansen et al. (2012)
Probe study – Crl:CD1 mice – time course mouse blood (group 5)	65 (oral)	2 M, 2 F	Single-dose mixture of [^{14}C-phenyl-UL]fenpicoxamid, [^{13}C-phenyl-UL]fenpicoxamid and fenpicoxamid with target radioactivity of 3.0 MBq/kg bw in 0.5% methylcellulose Excreta, blood, expired air and volatiles collected	Hansen et al. (2012)
Probe study – Crl:CD1 mice – C_{max} mouse blood (group 6)	65 (oral)	2 M, 2 F	Single-dose mixture of [^{14}C-phenyl-UL]fenpicoxamid, [^{13}C-phenyl-UL]fenpicoxamid and unlabelled fenpicoxamid with target radioactivity of 3.0 MBq/kg bw in 0.5% methylcellulose Killed at C_{max}, and blood collected	Hansen et al. (2012)
Probe study – F344/NTac rats – C_{max} rat blood (group 7)	65 (oral)	1 M, 1 F	Single-dose mixture of [^{14}C-phenyl-UL]fenpicoxamid and unlabelled fenpicoxamid with target radioactivity of 26	Hansen et al. (2012)

Test group	Dose (mg/kg bw) (route)	Number of animals of each sex	Remarks	Reference
			MBq/kg bw in 0.5% methylcellulose Killed 6 hours post-dosing, and excreta and blood collected; whole body autoradiography performed	
Probe study – F344/NTac rats – ½ C_{max} rat blood (group 8)	65 (oral)	1 M, 1 F	Single-dose mixture of [^{14}C-phenyl-UL]fenpicoxamid and unlabelled fenpicoxamid with target radioactivity of 26 MBq/kg bw in 0.5% methylcellulose Killed 12 hours post-dosing, and excreta and blood collected	Hansen et al. (2012)
Probe study – New Zealand white rabbits – time course rabbit blood (group 9)	30 (oral)	2 F	Single-dose mixture of [^{14}C-phenyl-UL]fenpicoxamid and unlabelled fenpicoxamid in 0.5% methylcellulose Excreta and blood collected Time course blood collected	Hansen et al. (2012)
Probe study – New Zealand white rabbits – time course rabbit blood (group 10)	10 (iv)	2 F	Single-dose mixture of [^{14}C-phenyl-UL]fenpicoxamid and unlabelled fenpicoxamid in 10% intralipid emulsion Excreta and blood collected Time course blood collected	Hansen et al. (2012)
Probe study – F344/NTac rats – time course rat blood (group 11)	10 (oral)	2 M	Single-dose mixture of [^{14}C-phenyl-UL]fenpicoxamid and unlabelled fenpicoxamid in 0.5% methylcellulose Time course plasma, excreta and blood collected	Hansen et al. (2012)
Probe study – F344/NTac rats – time course rat blood (group 12)	1 (iv)	2 M	Single-dose mixture of [^{14}C-phenyl-UL]fenpicoxamid and unlabelled fenpicoxamid in 10% intralipid emulsion Time course plasma, excreta and blood collected	Hansen et al. (2012)
Probe study – beagle dogs – time course dog blood and excreta	30 (oral)	1 M, 1 F	Single-dose mixture of [^{14}C-pyridine]fenpicoxamid and unlabelled fenpicoxamid in 0.5% (w/v) METHOCEL Time course blood, urine and faeces collected	Thomas (2012)
Tissue distribution – F344/DuCrl rats – C_{max}	10 (oral)	4 M, 4 F	Single-dose mixture of [^{14}C-phenyl-UL]fenpicoxamid and unlabelled fenpicoxamid Killed at C_{max} (2 hours post-dosing)	Hansen, Clark & Staley (2012)

Test group	Dose (mg/kg bw) (route)	Number of animals of each sex	Remarks	Reference
Tissue distribution – F344/DuCrl rats – ½ C_{max}	10 (oral)	4 M, 4 F	Single-dose mixture of [^{14}C-phenyl-UL]fenpicoxamid and unlabelled fenpicoxamid Killed at ½ C_{max} (6 hours post-dosing)	Hansen, Clark & Staley (2012)
Tissue distribution – F344/DuCrl rats – C_{max}	300 (oral)	4 M, 4 F	Single-dose mixture of [^{14}C-phenyl-UL]fenpicoxamid and unlabelled fenpicoxamid Killed at C_{max} (2 hours post-dosing)	Hansen, Clark & Staley (2012)
Tissue distribution – F344/DuCrl rats – ½ C_{max}	300 (oral)	4 M, 4 F	Single-dose mixture of [^{14}C-phenyl-UL]fenpicoxamid and unlabelled fenpicoxamid Killed at ½ C_{max} (12 hours post-dosing)	Hansen, Clark & Staley (2012)
Absorption and excretion – Sprague Dawley rats – bile duct cannulation – time course rat bile and excreta	10 (oral)	4 M, 4 F	Single-dose mixture of [^{14}C-2-pyridine]fenpicoxamid and unlabelled fenpicoxamid in 0.5% (w/v) METHOCEL Time course bile, urine and faeces collected Killed at 72 hours post-dosing	Press & Reynolds (2013)
Absorption and excretion – Sprague Dawley rats – bile duct cannulation – time course rat bile and excreta	300 (oral)	4 M, 4 F	Single-dose mixture of [^{14}C-2-pyridine]fenpicoxamid and unlabelled fenpicoxamid in 0.5% (w/v) METHOCEL Time course bile, urine and faeces collected	Press & Reynolds (2013)
Pharmacokinetics and metabolism – F344/DuCrl rats	10 (oral)	4 M, 4 F	Single-dose mixture of [^{14}C-phenyl-UL]fenpicoxamid and unlabelled fenpicoxamid	Hansen et al. (2013)
Pharmacokinetics and metabolism – F344/DuCrl rats	300 (oral)	4 M, 4 F	Single-dose mixture of [^{14}C-phenyl-UL]fenpicoxamid and unlabelled fenpicoxamid	Hansen et al. (2013)
Pharmacokinetics and metabolism – F344/DuCrl rats	10 (oral)	4 M, 4 F	Single-dose mixture of [^{14}C-2-pyridine]fenpicoxamid and unlabelled fenpicoxamid	Hansen et al. (2013)
Pharmacokinetics and metabolism – F344/DuCrl rats	10 (oral)	4 M, 4 F	Repeated oral doses of unlabelled fenpicoxamid (14 doses) and single-dose mixture of [^{14}C-phenyl-UL]fenpicoxamid and unlabelled fenpicoxamid	Hansen et al. (2013)
In vitro comparative metabolism study – mouse liver microsome	0.065 μmol/L		Single exposure to mixture of [^{14}C-2-pyridine]fenpicoxamid and unlabelled fenpicoxamid	Zhang et al. (2014)
In vitro comparative metabolism study – rat liver microsome	0.065 μmol/L		Single exposure to mixture of [^{14}C-2-pyridine]fenpicoxamid and unlabelled fenpicoxamid	Zhang et al. (2014)

Test group	Dose (mg/kg bw) (route)	Number of animals of each sex	Remarks	Reference
In vitro comparative metabolism study – human liver microsome	0.065 µmol/L		Single exposure to mixture of [14C-2-pyridine]fenpicoxamid and unlabelled fenpicoxamid	Zhang et al. (2014)

bw: body weight; C_{max}: maximum concentration; F: female; iv: intravenous; M: male; RBCs: red blood cells; UL: uniformly labelled; w/v: weight per volume

1.1 Absorption, distribution and excretion

(a) Oral and intravenous routes

Preliminary ADME assays were performed on rats, mice and rabbits. Orally administered fenpicoxamid was rapidly absorbed in the rat and rabbit. Time to reach maximum concentration values (T_{max}) were 6 and 2 hours, respectively, and area under the plasma concentration–time curve from time 0 to infinity values ($AUC_{0–\infty}$) were 38.4 and 24.1 µg·h/g, respectively, in rats and rabbits given an oral dose of 65 mg/kg body weight (bw). Of the animals given 65 mg/kg bw orally, male rats and male mice excreted 12% of the administered dose in the urine, whereas female mice excreted 25% and female rabbits excreted 42% of the administered dose in the urine. In rats given 10 mg/kg bw, 15% was excreted in the urine of rats dosed orally, whereas 26% was excreted in the urine of rats dosed intravenously. Rabbits given 10 mg/kg bw intravenously died immediately upon dosing (Hansen et al., 2012).

A preliminary ADME assay was performed in beagle dogs. The T_{max} was 2 hours post-dosing, with 1% of the administered dose excreted in the urine and 90% excreted in the faeces. Biliary excretion was not investigated, nor was the recovered radioactivity characterized to determine whether the parent was metabolized. Excretion was rapid, occurring mostly in 24 hours (Thomas, 2012).

Tissue distribution was investigated in F344/DuCrl rats (four of each sex per dose) following administration of a single radiolabelled dose of 0, 10 or 300 mg [14C-phenyl-UL]fenpicoxamid/kg bw. Tissues were collected at the maximum concentration (C_{max}) and ½ C_{max}. Tissue distribution was extensive, but low at the C_{max}, with most tissues accounting for less than 0.01% of the administered dose. The majority of radioactivity (92–99%) was recovered in the gastrointestinal tract, regardless of dose or sex, followed by the urinary bladder, liver and kidneys (Table 2).

Table 2. Distribution of radioactivity in rat tissues/organs at C_{max} and ½ C_{max} after administration of 14C-labelled fenpicoxamid[a]

Tissue/ organ	Concentration of radioactivity in tissues/organs							
	10 mg/kg bw				300 mg/kg bw			
	C_{max}		½ C_{max}		C_{max}		½ C_{max}	
	Males	Females	Males	Females	Males	Females	Males	Females
Bladder								
µg eq/g tissue	2.97 ± 0.67	5.08 ± 4.82	3.22 ± 1.60	2.19 ± 1.41	17.70 ± 7.77	2.21 ± 1.17	5.33 ± 1.28	9.45 ± 6.30
% AD	0.01	0.02	0.01	0.01	<0.01	<0.01	<0.01	<0.01
Blood								
µg eq/g tissue	0.62 ± 0.07	0.76 ± 0.10	0.15 ± 0.10	0.58 ± 0.11	1.98 ± 0.30	2.07 ± 0.32	0.87 ± 0.23	1.18 ± 0.21

Tissue/ organ	Concentration of radioactivity in tissues/organs							
	10 mg/kg bw				300 mg/kg bw			
	C_{max}		½ C_{max}		C_{max}		½ C_{max}	
	Males	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
% AD	0.19	0.22	0.12	0.17	0.02	0.02	0.01	0.01
Carcass								
µg eq/g tissue	0.17 ± 0.03	0.20 ± 0.05	0.14 ± 0.02	0.15 ± 0.06	0.51 ± 0.13	0.86 ± 0.39	0.32 ± 0.13	0.37 ± 0.07
% AD	0.89	1.05	0.72	0.81	0.10	0.18	0.07	0.07
GI tract								
µg eq/g tissue	120.56 ± 7.15	106.93 ± 5.73	98.79 ± 6.31	79.63 ± 6.31	3 809 ± 201	3 098 ± 232	1 274 ± 257	1 414 ± 346
% AD	92.23	92.87	86.89	87.42	98.68	97.82	52.36	58.96
Kidney								
µg eq/g tissue	1.14 ± 0.18	1.05 ± 0.12	0.91 ± 0.13	0.88 ± 0.15	3.93 ± 0.67	3.34 ± 0.38	3.38 ± 1.86	1.77 ± 0.30
% AD	0.08	0.07	0.06	0.06	0.01	0.01	0.01	<0.01
Liver								
µg eq/g tissue	2.60 ± 0.42	2.32 ± 0.20	1.22 ± 0.46	1.32 ± 0.21	8.18 ± 1.56	6.64 ± 0.73	3.82 ± 1.02	2.83 ± 0.27
% AD	0.72	0.61	0.35	0.40	0.08	0.07	0.05	0.03
Lungs								
µg eq/g tissue	0.56 ± 0.05	0.67 ± 0.10	0.44 ± 0.10	0.52 ± 0.07	1.76 ± 0.73	2.10 ± 0.75	1.06 ± 0.34	1.05 ± 0.19
% AD	0.03	0.04	0.02	0.03	<0.01	<0.01	<0.01	<0.01
Skin								
µg eq/g tissue	0.23 ± 0.04	0.25 ± 0.07	0.15 ± 0.02	0.22 ± 0.04	0.66 ± 0.10	0.62 ± 0.12	0.38 ± 0.12	0.34 ± 0.04
% AD	0.43	0.44	0.28	0.37	0.05	0.04	0.03	0.02
Testes								
µg eq/g tissue	0.27 ± 0.05	NA	0.21 ± 0.04	NA	0.69 ± 0.13	NA	0.83 ± 0.19	NA
% AD	0.03	NA	0.02	NA	<0.01	NA	<0.01	NA
Final cage wash (% AD)	1.73	1.30	3.73	5.88	0.17	0.25	42.19	34.63
Total recovered radioactivity (% AD)	96.37 ± 1.42	96.69 ± 0.45	92.23 ± 1.89	95.19 ± 2.60	99.13 ± 1.29	98.41 ± 0.36	94.72 ± 1.80	93.74 ± 2.99

AD: administered dose; bw: body weight; eq: equivalents; C_{max}: maximum concentration; GI: gastrointestinal; NA: not applicable

[a] Tissues/organs lacking quantifiable radioactivity or radioactivity counts greater than 0.01% of the administered dose in at least one dose group were excluded from the table.

Source: Data obtained from pages 30–41 in Hansen, Clark & Staley (2012)

At the high dose, tissue/organ concentrations were 2- to 3-fold higher than at the low dose, and tissue/organ concentrations did not scale proportionately to the administered dose. However, there were no major changes to the pattern of tissue/organ distribution between doses or between sexes. Rapid elimination occurred in all tissues (Hansen, Clark & Staley, 2012).

In an excretion study in bile duct–cannulated Sprague Dawley rats, animals were given [^{14}C-2-pyridine]fenpicoxamid at a single oral dose of 10 or 300 mg/kg bw. Concentrations in the blood, cellular fraction and plasma confirmed that absorption was rapid, peaking at 2 hours, but concentrations did not scale proportionately between the low and high doses (Table 3). At the high dose, blood plasma concentrations were higher in males than in females, and plasma concentrations were higher than concentrations in the cellular fraction. At the low dose, sex differences were less marked, although plasma concentrations were higher than concentrations in the cellular fraction.

Table 3. Concentrations of radioactivity in blood, cellular fraction and plasma of bile duct–cannulated rats following a single oral dose of [^{14}C-2-pyridine]fenpicoxamid at 10 or 300 mg/kg bw

	Mean (± SD) concentrations of radioactivity (ng fenpicoxamid eq/g)					
	10 mg/kg bw			**300 mg/kg bw**		
Time (h)	**Blood**	**Cellular fraction**	**Plasma**	**Blood**	**Cellular fraction**	**Plasma**
Males						
2	224 ± 51.3	125 ± 24.1	418 ± 123	1 230 ± 630	620 ± 296	2 230 ± 1 150
9	103 ± 36.5	55.2 ± 8.1	190 ± 61	1 170 ± 878	610 ± 319	2 110 ± 1 690
Females						
2	237 ± 50.5	118 ± 18.7	403 ± 89.7	809 ± 93.3	436 ± 58.8	1 230 ± 201
9	165 ± 57.9	90.1 ± 44.1	261 ± 103	680 ± 135	353 ± 81.4	1 080 ± 255

bw: body weight; eq: equivalents; SD: standard deviation
Source: Data obtained from pages 26–27 in Press & Reynolds (2013)

Recovery of administered radioactivity in the excreta and carcass was 89–99%. Biliary excretion was higher than excretion in the urine, indicating that absorption was higher than estimated in the other studies; however, the highest levels of recovered radioactivity were in the faeces (Table 4). Regardless of dose, elimination was rapid, peaking at 8 hours in the urine and in the first 24 hours in the faeces and bile.

Table 4. Recovery of radioactivity in excreta and carcass of bile duct–cannulated rats after administration of a single dose of [^{14}C-2-pyridine]fenpicoxamid

	% of administered dose recovered (± SD)			
	10 mg/kg bw		**300 mg/kg bw**	
Excreta with collection intervals (h)	**Males**	**Females**	**Males**	**Females**
Urine				
0–4	0.10 ± 0.18	0.13 ± 0.27	0.02 ± 0.03	0.01 ± 0.02
4–8	1.24 ± 0.58	1.09 ± 0.26	0.19 ± 0.05	0.09 ± 0.05
8–12	0.31 ± 0.10	0.18 ± 0.19	0.06 ± 0.05	0.04 ± 0.04
12–24	0.34 ± 0.16	0.99 ± 0.26	0.45 ± 0.63	0.11 ± 0.03
24–48	0.10 ± 0.03	0.31 ± 0.13	0.23 ± 0.39	0.05 ± 0.02

Excreta with collection intervals (h)	% of administered dose recovered (± SD)			
	10 mg/kg bw		300 mg/kg bw	
	Males	**Females**	**Males**	**Females**
48–72	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.02	0.01 ± 0.01
Total	2.10 ± 0.61	2.75 ± 0.35	0.96 ± 1.10	0.31 ± 0.06
Faeces				
0–12	35.7 ± 11.7	7.97 ± 14.2	45.0 ± 15.7	8.46 ± 14.6
12–24	32.47 ± 9.98	58.9 ± 17.1	35.3 ± 5.21	76.8 ± 20.0
24–48	3.10 ± 0.69	12.4 ± 7.9	5.60 ± 3.40	11.1 ± 5.02
48–72	0.16 ± 0.15	1.81 ± 2.65	0.22 ± 0.15	0.21 ± 0.22
Total	71.7 ± 6.44	81.0 ± 3.17	86.1 ± 13.3	96.5 ± 3.01
Cage rinse				
0–4	0.67 ± 0.80	0.77 ± 0.56	0.23 ± 0.16	0.05 ± 0.03
4–8	0.57 ± 0.25	0.65 ± 0.28	0.10 ± 0.09	0.09 ± 0.02
8–12	0.26 ± 0.12	0.31 ± 0.19	0.11 ± 0.09	0.04 ± 0.02
12–24	0.13 ± 0.06	0.36 ± 0.08	0.07 ± 0.05	0.04 ± 0.01
24–48	0.04 ± 0.02	0.08 ± 0.03	0.07 ± 0.10	0.02 ± 0.01
Total	1.66 ± 0.47	2.17 ± 0.32	0.57 ± 0.39	0.23 ± 0.04
Final cage wash	0.04 ± 0.03	0.06 ± 0.02	0.07 ± 0.10	0.02 ± 0.01
Final cage wipe	0.50 ± 0.37	0.84 ± 0.30	0.21 ± 0.18	0.07 ± 0.07
Bile				
0–4	4.75 ± 1.45	3.12 ± 1.40	0.09 ± 0.18	0.39 ± 0.03
4–8	5.18 ± 0.70	3.49 ± 0.79	0.31 ± 0.13	0.45 ± 0.03
8–12	2.20 ± 0.57	2.50 ± 0.46	0.27 ± 0.18	0.36 ± 0.04
12–24	1.18 ± 0.40	2.51 ± 0.39	0.28 ± 0.22	0.48 ± 0.13
24–48	0.10 ± 0.06	0.33 ± 0.25	0.10 ± 0.03	0.17 ± 0.07
48–72	0.003 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.04
Total	13.4 ± 0.89	12.0 ± 2.31	1.05 ± 0.65	1.86 ± 0.13
Bile cannula	0.003 ± 0.002	0 ± 0	0.002 ± 0.003	0 ± 0
Jacket rinse[a]	0 ± 0	0 ± 0	0.001 ± 0.001	0 ± 0
Residual carcass	0.035 ± 0.004	0.04 ± 0.02	0.03 ± 0.12	0.009 ± 0.017
Total recovery	89.4 ± 7.17	98.9 ± 1.14	89.0 ± 11.8	99.0 ± 2.85

bw: body weight; SD: standard deviation

[a] Jacket-and-tether system used to hold bile cannula in place was rinsed to collect residual radioactivity.

Source: Data obtained from pages 28–31 in Press & Reynolds (2013)

Pharmacokinetics was examined with the phenyl and pyridine labels in single-dose and repeated-dose oral studies with rats (Table 5). Absorption was rapid (T_{max} 2–4 hours), regardless of dose, dose regimen or label, but low, at 5–20% of the administered dose. The pyridine label had a generally lower AUC value than the phenyl label, with higher AUC values in females than in males. The exception was in the high-dose group, where there were no sex differences in AUC.

Table 5. Plasma and blood pharmacokinetic parameters for rats given a single dose of ^{14}C-labelled fenpicoxamid

	Phenyl label				Pyridine label	
	10 mg/kg bw		300 mg/kg bw		10 mg/kg bw	
Parameter	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
Plasma						
T_{max} (h)	2 ± 0	2.25 ± 0.5	4.67 ± 2.31	2 ± 0	2 ± 0	1.75 ± 0.5
C_{max} (µg/g)	1.86 ± 0.64	2.13 ± 0.39	3.54 ± 0.67	4.50 ± 0.48	1.29 ± 0.16	1.50 ± 0.23
Absorption $t_{½}$ (h)	0.58 ± 0.21	0.59 ± 0.43	0.56 ± 0.10	0.58 ± 0.09	0.59 ± 0.17	0.45 ± 0.17
Elimination $t_{½α}$ (h)	4.37 ± 1.03	5.92 ± 1.80	12.11 ± 1.47	9.72 ± 1.35	4.97 ± 0.88	6.37 ± 1.15
Elimination $t_{½β}$ (h)	35.38 ± 25.48	23.73 ± 5.75	NA	NA	53.39 ± 34.53	61.75 ± 14.48
$AUC_{0–168 h}$ (µg·h/g)	18.95 ± 2.23	23.73 ± 5.75	NA	NA	13.17 ± 0.97	18.89 ± 3.45
$AUC_{0–48 h}$ (µg·h/g)	15.52 ± 3.07	22.25 ± 5.31	74.41 ± 13.24	73.32 ± 12.43	NA	NA
Blood cells[a]						
T_{max} (h)	1.75 ± 0.5	1.75 ± 0.96	–	–	2 ± 0	1.5 ± 0.58
C_{max} (µg/g)	0.19 ± 0.03	0.29 ± 0.11	–	–	0.12 ± 0.03	0.18 ± 0.07
Absorption $t_{½}$ (h)	0.27 ± 0.08	0.28 ± 0.15	–	–	0.45 ± 0.19	0.43 ± 0.12
Elimination $t_{½α}$ (h)	57.64 ± 5.18	50.80 ± 2.44	–	–	0.66 ± 0.44	1.26 ± 1.24
Elimination $t_{½β}$ (h)	NA	NA	–	–	9.39 ± 2.59	11.21 ± 1.38
$AUC_{0–168 h}$ (µg·h/g)	10.40 ± 1.99	11.25 ± 1.53	–	–	NA	NA
$AUC_{0–24 h}$ (µg·h/g)	2.23 ± 0.88	3.53 ± 0.44	–	–	0.84 ± 0.27	1.29 ± 0.39

AUC: area under the concentration–time curve; bw: body weight; C_{max}: maximum concentration; NA: parameter not calculated for this group; $t_{½}$: half-life; T_{max}: time to reach maximum concentration

[a] Not enough data points to model red blood cell pharmacokinetic parameters for 300 mg/kg bw.

Source: Data obtained from Tables 7 and 8, pages 65–66, in Hansen et al. (2013)

Comparison of tissue distribution showed low retention in the tissues and no major differences between labels (Table 6). There was no evidence of bioaccumulation.

Excretion was rapid (58–90% of the administered dose within 24 hours) and was predominantly via the faeces. Almost all radioactivity had been excreted by 48 hours. Comparatively more radioactivity was excreted via the urine at low doses with the phenyl label than at high doses, and generally less was excreted in the urine with the pyridine label (Table 7).

(b) Dermal route

No studies were submitted.

Table 6. Distribution of radioactivity in rat tissues/organs 168 hours after administration of ^{14}C-labelled fenpicoxamid[a]

	Concentration of radioactivity in tissues/organs							
	Phenyl label						Pyridine label	
	1 × 10 mg/kg bw		1 × 300 mg/kg bw		15 × 10 mg/kg bw[b]		1 × 10 mg/kg bw	
Tissue/organ	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
Blood								
µg eq/g tissue	0.013 ± 0.002	0.006 ± 0.007	NQ	NQ	0.012 ± 0.003	0.017 ± 0.004	NQ	0.004 ± 0.005
% AD	<0.01	<0.01	NQ	NQ	<0.01	0.01	NQ	<0.01
Heart								
µg eq/g tissue	0.003 ± 0.001	0.003 ± 0.001	NQ	NQ	0.002 ± 0.001	0.003 ± 0.001	0.001 ± 0.0	0.002 ± 0.0
% AD	<0.01	<0.01	NQ	NQ	<0.01	<0.01	<0.01	<0.01
Kidneys								
µg eq/g tissue	0.010 ± 0.002	0.015 ± 0.005	NQ	NQ	0.009 ± 0.002	0.014 ± 0.003	NQ	0.008 ± 0.001
% AD	<0.01	<0.01	NQ	NQ	<0.01	<0.01	NQ	<0.01
Liver								
µg eq/g tissue	0.013 ± 0.004	0.006 ± 0.006	NQ	NQ	0.013 ± 0.003	0.006 ± 0.005	NQ	NQ
% AD	0.01	<0.01	NQ	NQ	0.01	<0.01	NQ	NQ
Lungs								
µg eq/g tissue	0.005 ± 0.001	0.005 ± .001	0.054 ± 0.012	0.050 ± 0.007	0.005 ± 0.001	0.006 ± 0.001	0.002 ± 0.0	0.003 ± 0.0
% AD	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Pancreas								
µg eq/g tissue	0.002 ± 0.0	0.003 ± 0.001	0.035 ± 0.008	NQ	0.003 ± 0.003	NQ	NQ	NQ
% AD	<0.01	<0.01	<0.01	NQ	<0.01	NQ	NQ	NQ
Plasma								
µg eq/g tissue	–	–	–	–	0.005 ± 0.001	0.006 ± 0.002	–	–
% AD					<0.01	<0.01		
RBCs								
µg eq/g tissue	–	–	–	–	0.019 ± 0.004	0.023 ± 0.005	–	–
% AD					<0.01	<0.01		
Skin								
µg eq/g tissue	0.002 ± 0.002	0.018 ± 0.005	0.116 ± 0.086	0.081 ± 0.094	0.004 ± 0.003	0.024 ± 0.007	NQ	0.006 ± 0.003
% AD	0.01	0.04	0.01	0.01	0.01	0.05	NQ	0.01

Tissue/organ	Concentration of radioactivity in tissues/organs							
	Phenyl label						Pyridine label	
	1 × 10 mg/kg bw		1 × 300 mg/kg bw		15 × 10 mg/kg bw[b]		1 × 10 mg/kg bw	
	Males	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
Spleen								
µg eq/g tissue	0.003 ± 0.001	0.003 ± 0.001	0.028 ± 0.004	NQ	0.004 ± 0.001	0.005 ± 0.001	0.001 ± 0.0	0.002 ± 0.0
% AD	<0.01	<0.01	<0.01	NQ	<0.01	<0.01	<0.01	<0.01
Thymus								
µg eq/g tissue	0.003 ± 0.001	0.002 ± 0.001	NQ	NQ	NQ	NQ	NQ	NQ
% AD	<0.01	<0.01	NQ	NQ	NQ	NQ	NQ	NQ
Uterus								
µg eq/g tissue	NA	0.003 ± 0.001	NA	NQ	NA	NQ	NA	NQ
% AD	NA	<0.01	NA	NQ	NA	NQ	NA	NQ

AD: administered dose; bw: body weight; eq: equivalents; NA: not applicable; NQ: non-quantifiable (i.e. samples with radioactivity measurements less than twice the concurrently run background); RBCs: red blood cells; UL: uniformly labelled

[a] Tissues/organs without quantifiable radioactivity in any dose group were excluded from the table.

[b] Fourteen daily doses of unlabelled fenpicoxamid followed by a single oral dose of a mixture of unlabelled fenpicoxamid and [^{14}C-phenyl-UL]fenpicoxamid.

Source: Data obtained from Table 3, pages 53–56, in Hansen et al. (2013)

Table 7. Recovery of radioactivity in tissues and excreta of rats after administration of ^{14}C-labelled fenpicoxamid

Excreta/tissues with collection intervals (h)	% of administered dose recovered (± SD)							
	Phenyl label						Pyridine label	
	1 × 10 mg/kg bw		1 × 300 mg/kg bw		15 × 10 mg/kg bw[a]		1 × 10 mg/kg bw	
	Males	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
Expired air	–	–	–	–	–	–	–	–
Cage wash	0.27 ± 0.33	0.15 ± 0.09	7.03 ± 10.12	2.37 ± 3.16	1.33 ± 1.89	NQ	0.06 ± 0.03	0.04 ± 0.03
Urine/rinse								
0–12	7.95 ± 2.84	10.76 ± 0.37	1.16 ± 0.37	2.75 ± 2.69	7.10 ± 1.62	7.57 ± 0.76	4.68 ± 0.44	5.75 ± 0.44
12–24	6.40 ± 2.13	6.49 ± 2.00	2.37 ± 1.39	0.94 ± 0.29	4.25 ± 0.72	5.93 ± 1.86	1.28 ± 0.22	1.62 ± 1.03
24–48	2.78 ± 1.03	2.66 ± 1.42	1.82 ± 1.23	0.75 ± 0.40	1.33 ± 0.36	2.62 ± 1.67	0.33 ± 0.11	1.51 ± 1.36
48–72	0.33 ± 0.14	0.21 ± 0.11	0.87 ± 0.78	0.10 ± 0.08	0.22 ± 0.04	0.26 ± 0.14	0.07 ± 0.03	0.09 ± 0.06
72–96	0.10 ± 0.06	0.06 ± 0.03	0.92 ± 1.11	0.25 ± 0.33	0.08 ± 0.03	0.06 ± 0.04	0.02 ± 0.01	0.02 ± 0.01
96–120	0.03 ± 0.0	0.04 ± 0.03	0.73 ± 1.22	0.07 ± 0.08	0.04 ± 0.01	0.03 ± 0.02	0.02 ± 0.01	0.01 ± 0

Excreta/tissues with collection intervals (h)	% of administered dose recovered (± SD)							
	Phenyl label						Pyridine label	
	1 × 10 mg/kg bw		1 × 300 mg/kg bw		15 × 10 mg/kg bw[a]		1 × 10 mg/kg bw	
	Males	Females	Males	Females	Males	Females	Males	Females
120–144	0.02 ± 0.01	0.03 ± 0.01	0.31 ± 0.35	0.02 ± 0.03	0.03 ± 0.02	0.03 ± 0.04	0.01 ± 0.01	0.01 ± 0
144–168	0.02 ± 0.01	0.02 ± 0.01	0.24 ± 0.27	0.04 ± 0.05	0.04 ± 0.03	0.04 ± 0.06	0.01 ± 0.01	0.01 ± 0
Total	17.63 ± 2.58	20.28 ± 3.63	8.43 ± 5.26	4.91 ± 3.83	13.09 ± 2.48	16.54 ± 3.03	6.43 ± 0.51	9.01 ± 1.95
Faeces								
0–24	74.89 ± 8.94	76.55 ± 12.68	72.68 ± 14.05	85.76 ± 8.45	79.70 ± 4.59	66.92 ± 13.11	73.53 ± 10.97	50.09 ± 25.17
24–48	10.20 ± 4.08	13.52 ± 9.96	8.99 ± 4.59	8.11 ± 3.72	5.75 ± 2.44	12.24 ± 5.27	12.76 ± 12.76	26.03 ± 15.08
48–72	1.64 ± 1.25	0.73 ± 0.49	0.92 ± 0.50	0.5 ± 0.67	0.55 ± 0.17	1.39 ± 1.19	0.57 ± 0.30	1.50 ± 1.0
72–96	0.17 ± 0.10	0.1 ± 0.09	0.15 ± 0.09	0.06 ± 0.09	0.08 ± 0.04	0.15 ± 0.13	0.08 ± 0.04	0.13 ± 0.13
96–120	0.17 ± 0.28	NQ	0.10 ± 0.11	0.15 ± 0.29	0.03 ± 0.01	0.04 ± 0.03	0.03 ± 0.03	0.02 ± 0.01
120–144	NQ	NQ	0.10 ± 0.12	NQ	0.01 ± 0.01	0.01 ± 0.02	NQ	0.01 ± 0.01
144–168	0.01 ± 0.01	NQ	0.07 ± 0.11	NQ	0.01 ± 0.0	NQ	0.17 ± 0.33	NQ
Total	87.08 ± 5.07	91.04 ± 5.69	83.0 ± 15.92	94.62 ± 6.12	86.14 ± 3.06	80.76 ± 7.86	87.14 ± 1.41	77.77 ± 11.01
Tissues	0.02 ± 0.01	0.05 ± 0.03	0.02 ± 0.02	0.10 ± 0.19	0.01 ± 0.01	0.06 ± 0.02	NQ	0.01 ± 0.0
Total recovery	105 ± 4.25	111 ± 5.67	98.5 ± 2.09	102 ± 1.00	101 ± 1.26	97.5 ± 5.89	93.6 ± 1.06	86.8 ± 12.07

bw: body weight; NQ: non-quantifiable (i.e. samples with radioactivity measurements less than twice the concurrently run background); SD: standard deviation; UL: uniformly labelled

[a] Fourteen daily doses of unlabelled fenpicoxamid followed by a single oral dose of a mixture of unlabelled fenpicoxamid and [^{14}C-phenyl-UL]fenpicoxamid.

Source: Data obtained from Table 3, pages 45–52, in Hansen et al. (2013)

1.2 Biotransformation

(a) In vivo studies

In rats administered radiolabelled fenpicoxamid, unchanged parent was not excreted in the urine, but was a major component of the radioactivity recovered in the faeces. In the urine of groups dosed with the phenyl label, the major metabolites were UK-2A diol with an open ring (X1236349), UK-2A diol (X696872) and unidentified metabolite 4. Another metabolite, UK-2A open ring (X737057), was seen only in the high-dose groups. In animals given the pyridine label, the two major urinary metabolites were UK-2A diol (X696872) and unidentified metabolite 2 (Table 8).

Table 8. Metabolite profile in excreta of rats dosed with ^{14}C-labelled fenpicoxamid

	% of administered dose							
	Phenyl label						Pyridine label	
	1 × 10 mg/kg bw		1 × 300 mg/kg bw		15 × 10 mg/kg bw[a]		1 × 10 mg/kg bw	
Compound	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
Urine								
Parent	–	–	–	–	–	–	–	–
UK-2A diol open ring	7.96	9.24	3.74	2.08	4.89	7.48	–	–
UK-2A diol	6.90	7.20	2.02	1.34	5.66	5.27	2.61	3.90
UK-2A open ring	–	–	0.72	0.43	–	–	–	–
Unidentified metabolite 2	–	–	–	–	–	–	3.81	5.11
Unidentified metabolite 3	–	–	0.55	0.26	–	–	–	–
Unidentified metabolite 4	2.77	3.89	1.41	0.79	2.53	3.78	–	–
Faeces[b]								
Parent	18.3	ND	49.1	52.6	51.1	34.1	38.0	29.0
UK-2A open ring	6.68	ND	–	–	9.76	7.70	9.73	9.13
UK-2A	6.13	ND	–	–	6.20	6.28	6.79	6.47
Unidentified metabolite 1	–	ND	–	–	4.87	3.43	11.6	19.5
Total unextracted radioactivity	56.0	ND	33.9	42.0	14.2	29.3	21.0	13.6
Total								
Total identified	45.97	20.3	55.58	56.45	77.61	60.83	57.13	48.5
Total unidentified[c]	58.77	91.04[d]	34.96	43.05	21.6	36.51	36.41	38.21
Total accounted for[e]	105	111	90.5	99.5	99.2	97.3	93.5	86.7
Lost/unaccounted for[f]	0	0	9	0.5	0.8	2.7	6.5	13.3

–: non-quantifiable (i.e. samples with radioactivity measurements less than twice the concurrently run background); bw: body weight; ND: no data; UL: uniformly labelled

[a] Fourteen daily doses of unlabelled fenpicoxamid followed by a single oral dose of a mixture of unlabelled fenpicoxamid and [^{14}C-phenyl-UL]fenpicoxamid.

[b] No faeces data for low-dose single-dose [^{14}C-phenyl-UL]fenpicoxamid females due to low recoveries of radioactivity from faecal homogenate extracts.

[c] Total unidentified = (unidentified metabolites) + (total unextracted radioactivity).

[d] Total unidentified for low-dose single-dose [^{14}C-phenyl-UL]fenpicoxamid females was assumed to be equivalent to the percentage of administered dose in faeces, as presented in Table 7 above.

[e] Total accounted for = (total identified) + (total unidentified).

[f] 100 − (total accounted for).

Source: Data obtained from Table 2, page 145, in Hansen et al. (2013)

Although the largest percentage of recovered radioactivity in the faeces was identified as unchanged parent, a large portion, regardless of dose or label, was unextracted radioactivity (Table 8). The unextracted radioactivity represents the fraction of the administered dose that became irreversibly

bound within the faeces and may contribute to the low apparent absorption of fenpicoxamid. This bound fraction is lowest in the repeated-dose groups, and there are sex differences that may account for durational effects and sex differences seen in the database. This apparent overwhelming of the faecal binding seen in the repeated-dose metabolism study may relate to durational effects in the remainder of the database. Relative availability of fenpicoxamid is described in the toxicokinetics from individual studies compiled in section 1.3 below.

The proposed metabolic pathway for fenpicoxamid in rats is shown in Fig. 3. The principal routes of metabolism of fenpicoxamid involved hydrolysis of various ester groups, as well as *O*-dealkylation of the hydroxymethoxy side-chain of the pyridine ring.

Fig. 3. Proposed metabolic pathway for fenpicoxamid in rats

XDE-777
Parent
MW = 614
(Peak-9, Feces)

X642188
UK-2A
MW = 514
(Peak-8, Feces)

MW = 548
(Peak-5, Urine)

AND Enantiomer

X696872
UK-2A diol
MW = 444
(Peak-6, Urine)

X737057
UK-2A open ring
MW = 532
(Peak-7, Urine & Feces)

X12326349
UK-2A diol open ring
MW = 462
(Peak-4, Urine)

Source: Hansen et al. (2013)

(b) In vitro studies

In an in vitro comparative metabolism study, rat, mouse and human liver microsomes were exposed to a 0.065 µmol/L concentration of fenpicoxamid (purity 79.4%) and [^{14}C-2-pyridine]fenpicoxamid (radiopurity 99.1%). Owing to the low concentrations and an excess of enzyme activity in all three species, the study did not inform on species-specific total capacity to metabolize the parent compound. Qualitative differences between the species were identified.

The parent was identified only in the microsome-free incubations, indicating that all three species were capable of metabolism. Eight metabolites were detected, although only X1236349 and X737057 were identified. Three metabolites, including X1236349, were detected in the absence of microsomes, indicating that they are impurities of the test substance when detected at those levels.

The human microsome incubations formed only three substances as metabolites, “d”, “e” and X737057. All three species formed “e” at comparable levels of approximately 25% of the applied dose. Metabolite “d” was found in the human microsome incubations at approximately 3% of the applied dose, but in the mouse microsome incubations at approximately 15% of the applied dose. X737057 was found in the human microsome incubations at higher levels than in the rat microsome incubations (~21% in humans versus ~11% in rats). Both the rat and mouse microsome incubations formed metabolite “a”, a very polar metabolite (~8–12% of the applied dose over background), whereas metabolite “b” was found only in the mouse microsome incubations, and metabolites “c” and “g” (both ~1% of the applied dose) were found only in the rat microsome incubations. The presence of metabolite “g” at background levels in the rat microsome incubations suggests that it is an impurity; however, its absence in the mouse and human microsome incubations suggests that it can be metabolized into another product. Microsomal protein precipitate was noted and accounted for approximately 10% of the unextracted label, suggesting label binding (Zhang et al., 2014).

1.3 Toxicokinetics

Urine and blood samples were taken from a representative sample of the toxicity studies to confirm the steady-state systemic concentrations of fenpicoxamid as described in section 1.1 above and to confirm levels of internal exposure to the test substance.

Toxicokinetic analysis was performed in three mouse studies. In a 28-day dietary study, blood concentrations of the parent compound were too low to allow an analysis to be performed. Concentrations of metabolite X737057 in the blood were sublinear at the high dose (6000 parts per million [ppm]) in females and at the middle dose (3000 ppm) in males. Urinary concentrations of fenpicoxamid were above the lower level of quantification (LLQ) in all dose groups in males and above the LLQ in females at the middle and high doses. In male mice, concentrations of the parent compound were sublinear at the middle dose, whereas concentrations were linear in females. Urinary concentrations of metabolite X737057 were equivalent to those of the parent compound in males and 4.5 times higher than those of the parent compound in females. Both sexes exhibited non-linear concentrations at the middle dose for the metabolite (Thomas, Murray & McCoy, 2012).

In a 90-day dietary study, fenpicoxamid was present in the blood above the LLQ in females but not in males, whereas it was present in urine above the LLQ in males but not in females, indicating separate and more efficient pathways in males. Metabolite X737057 was found in both blood and urine of males and females. Blood fenpicoxamid concentrations were statistically linear but visually non-linear at the low–middle dose (1500 ppm) in females. Urinary fenpicoxamid concentrations were sublinear at the high dose (6000/9000 ppm) in males. Blood concentrations of metabolite X737057 were 17% and 65% of the blood concentrations of fenpicoxamid in females given the low (300 ppm) and high doses (6000 ppm), respectively. Blood concentrations were less than proportional at the higher doses, with the inflection point at the middle–high dose (3000 ppm) in males and females. Urinary concentrations of metabolite X737057 were non-linear at the high dose in males and statistically linear, but visually non-linear, at the middle–high dose in females (Thomas, Murray & McCoy, 2014).

Concentration analyses in an 18-month study revealed that fenpicoxamid was rapidly metabolized and/or excreted and was not present at quantifiable levels in the majority of blood and urine samples collected after 3, 6 and 12 months of treatment. However, the major metabolite, X12326349, and a lesser metabolite, X737057, were both present at quantifiable levels in most samples obtained from treated animals. Dose proportionality in the blood and urine was inconsistent for metabolite X12326349 at 3 and 6 months. At 12 months, levels of metabolite X12326349 in the blood were dose proportionate; however, test substance intake was decreased. In the urine, excretion was higher at 12 months than at 3 and 6 months; however, dose proportionality was regained (Thomas, Murray & McCoy, 2013).

Overall, the data from the three mouse studies suggest that there are changes in the kinetics of fenpicoxamid and its metabolites over time and that this change in kinetics may be complicated by

multiple, possibly competing, mechanisms of non-linearity. Levels of the minor metabolite, X737057, were more likely to be less than dose proportionate in the blood, but linear in urinary excretion (Thomas, Murray & McCoy, 2012, 2013, 2014).

In the 28-day, 90-day and 1-year dog studies, blood concentrations of fenpicoxamid and its two metabolites, X737057 and X12326349, were generally low. Where analysis of the AUC was possible, blood concentrations were statistically linear, but visually non-linear, with plateaus at the highest doses administered. Urinary concentrations accounted for less than 0.1% of the administered dose in all studies, including the 1-year study, where urinary elimination of parent compound, metabolite X737057 and metabolite X12326349 accounted for less than 0.01%, less than 0.05% and less than 0.07% of the administered dose, respectively, regardless of the dose. Analysis of urinary elimination was possible only for metabolite X737057 in the 28-day study, where it was sublinear. In the 90-day study, urinary elimination was linear for the parent compound, statistically but visually non-linear for metabolite X12326349 and, for metabolite X737057, non-linear at the top dose for males and statistically linear, but visually non-linear, in females (Heward, 2013a,b, 2014).

Toxicokinetics were evaluated in rats in the 29-day, 90-day (diurnal variation assessment) and 2-year dietary toxicity studies as well as in the reproductive toxicity study and developmental toxicity study. In the short-term and long-term toxicity studies, blood concentrations of fenpicoxamid were low to undetectable. Urinary concentrations were generally higher, non-linear over a shorter duration of dosing and linear in the 90-day and 2-year studies. Samples were analysed for metabolite X737057 in all studies. Blood concentrations after 29 days were dose dependent in males but not in females. After 90 days, blood concentrations were non-linear in males at the middle (6000 ppm) and high doses (11 500/14 000 ppm) and statistically linear, but visually non-linear, in females at the same doses. Neither of the metabolites in the 2-year study, X737057 and X12326349, showed linearity of blood concentrations; however, the point of departure for blood linearity was unclear in the absence of dosing below 100 mg/kg bw per day. Urinary concentrations of metabolite X737057 were higher than blood concentrations, but did not follow a linear increase pattern after 29 or 90 days of exposure. In the 2-year study, concentrations of metabolites X12326349 and X737057 in the urine were visually non-linear at the middle dose (10 000 ppm) and high doses (30 000 ppm) and statistically non-linear at the high dose. It was noted that the low blood concentrations of the parent compound and two metabolites and shallow dose–response curves indicate that there was little internal exposure (Stebbins, Murray & McCoy, 2012; Stebbins et al., 2012, 2014).

In the preliminary reproductive toxicity study in rats, the fenpicoxamid concentration in blood was below the LLQ in all doses in males and sublinear at the middle dose (9000 ppm) in females. Concentrations of the parent compound were sublinear in milk at the low dose (4500 ppm). Fenpicoxamid was isolated in the blood of pups, but there was evidence of cross-contamination, and the results were considered unreliable. Metabolite X737057 was found in males, females, offspring and milk. Concentrations were sublinear in adults and milk, but linear in pups. Although the levels of the metabolite were higher than the levels of the parent compound in the blood of adults, they were lower than fenpicoxamid levels in the milk (Rasoulpour et al., 2012a).

In the preliminary developmental toxicity study in rats, only plasma was investigated. Fenpicoxamid was undetected in dams and fetuses, whereas metabolite X737057 was found in dams and fetuses, and at comparable levels in both. In dams, the concentrations were sublinear between the low (4500 ppm) and middle doses (9000 ppm), although they were effectively linear in fetuses. Concentrations of the metabolite were 6- to 10-fold higher than in the main study, in which whole blood was analysed. In the main study, fenpicoxamid was not found above the LLQ in the dams, but was found in two of the 12 fetal samples from treated groups. The samples were analysed for both metabolites X12326349 and X737057, which were found above the LLQ in both dams and fetuses. Metabolite X12326349 was found at higher concentrations than metabolite X737057, and fetal concentrations were approximately 76% and 32% of the maternal blood levels, respectively. Blood levels of metabolite X737057 were sublinear at the middle dose, whereas levels of metabolite X12326349 were statistically linear, but visually non-linear, at the high dose (13 500 ppm) (Rasoulpour et al., 2012b).

In the preliminary developmental toxicity study in rabbits, only the low-dose (12 250 ppm) animals were sampled. Concentrations of metabolite X12326349 were found to be above the LLQ; however, the parent compound was found at trace levels in two of the five samples, and metabolite X737057 was not found in any of the samples. In the main study, neither fenpicoxamid nor metabolite X737057 was found above the LLQ in does or fetuses. Metabolite X12326349 was found in the blood samples of both. Metabolite concentrations in the fetal and maternal samples were statistically linear and visually non-linear. Whereas metabolite concentrations in maternal and fetal samples were roughly equal at 5000 and 15 000 ppm, fetal concentrations at the low dose were approximately 28% of those of the maternal animals (Rasoulpour et al., 2012c; Ellis-Hutchings, Bell & McCoy, 2013).

Overall, the low internal exposure, as evidenced by low levels of fenpicoxamid and the major metabolites in the samples, and lack of linearity in many of the studies mean that less weight can be placed on the dose–response relationship.

2. Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

In an acute oral toxicity study, rats were given a limit dose of fenpicoxamid (purity 78.0%) in 0.5% methylcellulose in distilled water at 2000 mg/kg bw. There were no mortalities and no clinical signs of toxicity. Body weights decreased in two animals between day 7 and day 14, although all other animals gained weight throughout the study. There were no gross changes at necropsy (Durando, 2011a).

In an acute dermal toxicity study, rats were given a limit dose of fenpicoxamid (purity 78.0%) moistened with 0.5% methylcellulose in distilled water at 5000 mg/kg bw for 24 hours. There were no mortalities and no signs of systemic toxicity. Yellow staining was noted at the test site in both males and females. One male and one female each either lost weight or did not gain weight in the first week of observation; however, all animals gained weight by the end of the observation period. There were no gross changes at necropsy (Durando, 2011b).

In an acute inhalation study, rats were given a maximum achievable concentration of fenpicoxamid (purity 78.0%) at 0.53 mg/L for 4 hours. There were no mortalities. Clinical signs of toxicity were limited to periocular staining in one female on day 14 and day 15. Body weights were decreased following exposure; however, all animals gained weight from day 4 until the end of the observation period. There were no gross changes at necropsy (Krieger & Garlinghouse, 2012).

Fenpicoxamid is therefore of low acute oral and dermal toxicity and slight inhalation toxicity in rats (Table 9).

Table 9. Acute toxicity of fenpicoxamid

Species	Strain	Sex	Route	Purity (%)	Result	Reference
Rat	F344	Female	Oral	78.0	LD_{50} > 2 000 mg/kg bw	Durando (2011a)
Rat	F344	Male and female	Dermal	78.0	LD_{50} > 5 000 mg/kg bw	Durando (2011b)
Rat	F344	Male and female	Inhalation	78.0	LC_{50} > 0.53 mg/L	Krieger & Garlinghouse (2012)

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

(b) Dermal irritation

In a dermal irritation study, 0.5 mg of fenpicoxamid (purity 78.0%) was moistened with 0.5% methylcellulose in water and applied on a 2.5 cm × 2.5 cm gauze patch to the skin of three male New Zealand white rabbits for 4 hours. Very slight erythema was noted at the test site in one of the three animals, and no oedema was noted. All scores at the 24-, 48- and 72-hour marks were zero. Fenpicoxamid is considered to be non-irritating to the skin of rabbits (Durando, 2011c).

(c) Ocular irritation

In an eye irritation study, 0.04–0.05 g of fenpicoxamid (purity 78.0%) was instilled into the conjunctival sac of one eye of three female New Zealand white rabbits. The eyes were left unwashed following instillation, and the opposite eyes were left as controls. Slight to marked redness, slight chemosis and slight to marked discharge were noted in all animals. All signs of irritation had abated by 72 hours. Fenpicoxamid is considered to be minimally irritating to the eyes of rabbits based on persistence of irritation past 24 hours (Durando, 2011d).

(d) Dermal sensitization

In a dermal sensitization study using a local lymph node assay, five female CBA/J mice per dose were exposed to three daily applications of 0%, 5%, 25% or 75% fenpicoxamid (purity 78.0%) in dimethyl sulfoxide for 3 consecutive days. Following intravenous injection of 740 kBq of ^{3}H-labelled thymidine, individual animal disintegrations per minute counts were obtained, and the stimulation index was calculated for each dose group. The stimulation indices for the 5%, 25% and 75% groups were 0.9, 1.2 and 1.3, respectively. A concurrent positive control assay was performed with α-hexylcinnamaldehyde, which validated the study. Fenpicoxamid is not considered to be a dermal sensitizer in mice (Boverhof & Sosinski, 2012).

(e) Phototoxicity

In an in vitro 3T3 NRU phototoxicity test, mouse BALB/c 3T3 cells cultured in vitro were exposed to fenpicoxamid (purity 84.2%) dissolved in dimethyl sulfoxide. Concentrations of 0.244, 0.488, 0.977, 1.953, 3.906, 7.813, 15.63 and 31.25 µg/mL were investigated in the presence and absence of artificial sunlight irradiance. A mean phototoxic effect (MPE) of 0.061 was recorded, which corresponded with no phototoxic potential predicted (i.e. MPE < 0.1). Similarly, photo-irritancy factor data did not fall in the phototoxic range. No cytotoxic effects were observed after treatment of cells in either the presence or the absence of artificial sunlight. The positive controls induced the appropriate response. There was no evidence that fenpicoxamid had any phototoxic effects on BALB/c 3T3 cells (Roth, 2015).

2.2 Short-term studies of toxicity

(a) Oral administration

Mice

In a 7-day palatability study, groups of three female Crl:CD1 (ICR) mice received fenpicoxamid (purity 78.0%) in the diet at a concentration of 0, 1432, 2864 or 5727 ppm (equal to 0, 286, 584 and 1164 mg/kg bw per day, respectively). Animals were monitored daily for general health, significant clinical abnormalities, body weight, body weight gain and feed consumption. Animals were killed on day 7, with the exception of control animals, which were returned to the vivarium, and a gross necropsy was performed.

There were no mortalities. There were no effects on body weight or feed consumption, and body weight gains in the high-dose group were slightly higher than those of controls. Mesenteric lymph nodes were increased in size in one mid-dose and two high-dose animals, and renal lymph nodes were increased in size in one high-dose animal. Peyer's patches were of increased prominence throughout the gastrointestinal tract of one high-dose animal (Thomas, Murray & Sura, 2010).

In a 28-day toxicity study, groups of five Crl:CD1 (ICR) mice received fenpicoxamid (purity 78.0%) in the diet at a concentration of 0, 1923, 3846 or 7692 ppm (0, 1500, 3000 and 6000 ppm, corrected for test substance purity; equal to 0, 216, 444 and 901 mg/kg bw per day for males and 0, 295, 652 and 1177 mg/kg bw per day for females, respectively). Animals were monitored twice daily for mortality and moribundity and daily for clinical signs of toxicity. Cage-side observations were performed daily, and detailed clinical observations were performed weekly. Body weight, body weight gain and feed consumption were measured twice in the first week and weekly thereafter. Ophthalmological examinations were performed pre-exposure and prior to scheduled necropsy. Haematology and clinical chemistry parameters were analysed from samples taken prior to terminal kill, and toxicokinetics was analysed in urine and whole blood. Gross examinations were performed, organs were weighed and histopathological examination was performed on animals from the control and high-dose groups, including the one high-dose male that died during the study.

There was one mortality attributed to the stress of handling. There were no treatment-related effects on clinical signs, body weight, feed consumption, ophthalmology, haematology parameters or gross pathological observations.

Changes to the adrenal glands consisted of increased weights at and above 3000 ppm in males and in all treated groups in females. There was an increase in the incidence of very slight hypertrophy of the zona fasciculata at and above 3000 ppm in females and at 6000 ppm in males. As there were no histopathological adrenal changes in the 90-day study (see below) at similar doses and in larger dose groups, these changes were considered equivocal.

Changes in the liver consisted of increased liver weights in males at 3000 and 6000 ppm. In females, liver weights were increased at 1500 ppm; however, as the dose increased, the extent of the liver weight increase was less compared with controls. The incidence of slight hepatocellular hypertrophy was increased at 3000 and 6000 ppm in males and females. Blood albumin concentrations were decreased in females in all treated groups. Liver changes were consistent with those seen in the 90-day toxicity study (see below) (Thomas, Murray & McCoy, 2012).

In a 90-day toxicity study, groups of 10 Crl:CD1 (ICR) mice received fenpicoxamid (purity 78.0%) in the diet at a concentration (corrected for purity) of 0, 300, 1500, 3000 or 6000/9000 ppm (increased at day 57) (equal to 0, 40, 192, 399 and 921 mg/kg bw per day, respectively) for males and 0, 300, 1500, 3000 or 6000 ppm (equal to 0, 49, 303, 566 and 1107 mg/kg bw per day, respectively) for females. Additional groups of 10 animals received 0 and 3000/6000 ppm for males and 0 and 6000 ppm for females for 90 days and were given untreated diet for an additional 28-day recovery period. Animals were monitored twice daily for mortality and moribundity and daily for clinical signs of toxicity. Cage-side observations were performed daily, and detailed clinical observations were performed weekly. Body weight, body weight gain and feed consumption were measured twice in the first week and weekly thereafter. Ophthalmological examinations were performed pre-exposure and prior to scheduled necropsy. Haematology and clinical chemistry parameters were analysed from samples taken prior to terminal kill, and toxicokinetics was analysed in urine and whole blood. Gross examinations were performed, organs were weighed and histopathological examination was performed on animals from the control and high-dose groups.

There were no effects on mortality, clinical signs of toxicity, body weight, body weight gain, feed consumption, ophthalmology or haematology. Changes were limited to the liver at and above 1500 ppm, with increased organ weight and hepatocellular hypertrophy with altered tinctorial properties in males and females, decreased serum triglycerides, increased hepatocyte fatty change and

multifocal single-cell necrosis in males and decreased serum albumin in females. At 3000 ppm, serum albumin was decreased in males. At 6000 ppm, serum total protein and cholesterol were decreased in females. There were no treatment-related changes following the 28-day recovery period.

The no-observed-adverse-effect level (NOAEL) was 300 ppm (equal to 40 mg/kg bw per day), based on increased liver weights and hepatocellular hypertrophy in males and females, decreased serum triglycerides, hepatocyte fatty change and multifocal single-cell necrosis in males and decreased serum albumin in females at 1500 ppm (equal to 192 mg/kg bw per day) (Thomas, Murray & McCoy, 2014).

Rats

In a 7-day palatability study, groups of three female F344/DuCrl rats received fenpicoxamid (purity 78.0%) at a concentration of 0, 2269, 4537 or 9074 ppm (equal to 0, 246, 473 and 1000 mg/kg bw per day, respectively, corrected for test material purity). Animals were monitored daily for general health, significant clinical abnormalities, body weight, body weight gain and feed consumption. Animals were killed on day 7, with the exception of control animals, which were returned to the vivarium, and a gross necropsy was performed.

There were no effects on mortality, clinical signs of toxicity, body weight, body weight gain, feed consumption or gross necropsy (Sura & Murray, 2010).

In a 29-day toxicity study, groups of five F344/DuCrl rats of each sex received fenpicoxamid (purity 78.0%) at a concentration of 0, 2949, 5769 or 11 538 ppm (0, 2300, 4500 and 9000 ppm, corrected for test material purity; equal to 0, 196, 395 and 788 mg/kg bw per day for males and 0, 197, 377 and 764 mg/kg bw per day for females, respectively). Animals were monitored twice daily for mortality and moribundity and daily for clinical signs of toxicity. Cage-side observations were performed daily, and detailed clinical observations were performed weekly. Body weight, body weight gain and feed consumption were measured twice in the first week and weekly thereafter. Ophthalmological examinations were performed pre-exposure and prior to scheduled necropsy. Haematology and clinical chemistry parameters were analysed from fasted samples taken prior to terminal kill, and urine analysis was performed on samples taken from the metabolism cages the week prior to terminal kill. Urinary sediment was characterized from pooled samples. Toxicokinetics was analysed in urine and whole blood. Gross examinations were performed, organs were weighed and histopathological examination was performed on animals from the control and high-dose groups and on gross lesions in all dose groups.

There were no effects on mortality, clinical signs of toxicity, body weight, body weight gain, feed consumption, haematology, organ weights, gross pathology or histopathology. At 9000 ppm, cholesterol was increased in males and females, urine volume was increased in females and specific gravity was decreased in females; however, owing to the lack of a dose–response relationship, the adversity and relationship to treatment were unknown.

The NOAEL was 9000 ppm (equal to 764 mg/kg bw per day), the highest dose tested (Stebbins, Murray & McCoy, 2012).

In a 90-day toxicity study, groups of 15 F344/DuCrl rats of each sex received fenpicoxamid (purity 78.0%) at a dietary concentration of 0, 3000, 6000 or 11 500/14 000 ppm (dose increased at day 71) (corrected for test material purity; equal to 0, 180, 365 and 732 mg/kg bw per day for males and 0, 205, 413 and 834 mg/kg bw per day for females, respectively). Animals were monitored twice daily for mortality and moribundity and daily for clinical signs of toxicity. Cage-side observations were performed daily, and detailed clinical observations were performed weekly. Body weight, body weight gain and feed consumption were measured twice in the first week and weekly thereafter.

Ophthalmological examinations were performed pre-exposure and prior to scheduled necropsy. Haematology and clinical chemistry parameters were analysed from fasted samples taken prior to terminal kill, and urine analysis was performed on samples taken from the metabolism cages the week prior to terminal kill. Urinary sediment was characterized from pooled samples. Toxicokinetics was analysed in urine and whole blood. Gross examinations were performed, organs were weighed and histopathological examination was performed on animals from the control and high-dose groups and on gross lesions in all dose groups. Five animals of each sex per group were subjected to neurobehavioural examination in all dose groups and to neurohistopathology in the control and high-dose groups.

There were no effects on mortality, clinical signs of toxicity, body weight, body weight gain, feed consumption, haematology, clinical chemistry, organ weights, gross pathology or histopathology. There were no effects in the neurological subgroups.

The NOAEL was 11 500/14 000 ppm (equal to 732 mg/kg bw per day), the highest dose tested (Stebbins et al., 2012).

Dogs

In a 5-day palatability study, groups of two female beagle dogs received fenpicoxamid (purity 79.4%) at a dietary concentration of 0, 15 000 or 30 000 ppm (equivalent to 0, 375 and 750 mg/kg bw per day), and a group of two male beagle dogs received fenpicoxamid (purity 78.0%) at a dietary concentration of 30 000 ppm (equivalent to 750 mg/kg bw per day). Animals were observed for morbidity, mortality, injury, body weight and feed consumption twice daily and for clinical signs daily. At the end of the palatability study, animals were subjected to a 3-week washout period and transferred into the 28-day oral toxicity study.

Feed consumption and body weights were slightly decreased in the first day or two of exposure in all treated animals. Both had returned to levels comparable to control values by the second day (Heward, 2012).

In a 28-day toxicity study, groups of two female beagle dogs received fenpicoxamid (purity 79.4%) at a dietary concentration of 0, 15 000 or 30 000 ppm (equal to 0, 446.1 and 1110.1 mg/kg bw per day, respectively; 0, 354 and 881 mg/kg bw per day, respectively, corrected for test substance purity), and a group of two male beagle dogs received fenpicoxamid (purity 78.0%) at a concentration of 30 000 ppm (equal to 915.9 mg/kg bw per day; 727 mg/kg bw per day, corrected for purity). Animals were observed for morbidity, mortality and injury twice daily and for clinical signs daily. Body weight and feed consumption were measured weekly. Ophthalmoscopic examinations were conducted and haematology and clinical chemistry parameters were measured pretest and prior to terminal necropsy. Urine analysis was performed in week 4. Gross pathological and histopathological examinations were carried out in all groups, and organ weights were measured in all groups.

There were no effects on mortality or clinical signs of toxicity. Males and females in the 30 000 ppm group lost body weight during the study, and one of two females in the 15 000 ppm group lost body weight. Feed consumption was decreased in the first week of treatment in one male and one female given 30 000 ppm. There were no effects on ophthalmology, haematology, clinical chemistry or urine analysis parameters. There were no effects on gross pathology, histopathology or organ weights (Heward, 2013a).

In a 90-day toxicity study, groups of four beagle dogs of each sex received fenpicoxamid (purity 79.4%) at a dietary concentration of 0, 3000, 10 000 or 30 000 ppm (corrected for test material purity; equal to 0, 100, 408 and 939 mg/kg bw per day for males and 0, 122, 353 and 1115 mg/kg bw per day for females, respectively). Animals were observed for morbidity, mortality and injury twice daily and for clinical signs weekly. Body weight and feed consumption were measured daily for the

first 2 weeks of the study and weekly thereafter. Ophthalmoscopic examinations were performed in the pretest period and prior to scheduled necropsy. Haematology, clinical chemistry and urine analysis parameters were measured pretest and at weeks 7 and 13. Gross pathological and histopathological examinations were performed in all groups, and organ weights were measured in all groups.

There were no effects on mortality, body weight, body weight gain, feed consumption, haematology, clinical chemistry or urine analysis parameters, gross pathological or histopathological examinations or organ weights.

The NOAEL was 30 000 ppm (equal to 939 mg/kg bw per day), the highest dose tested (Heward, 2013b).

In a 1-year toxicity study, groups of four beagle dogs of each sex received fenpicoxamid (purity 79.4% in weeks 1–28 and 83.7% in weeks 29–52) at a dietary concentration of 0, 3000, 10 000 or 30 000 ppm (equal to 0, 84, 300 and 981 mg/kg bw per day for males and 0, 80, 273 and 1011 mg/kg bw per day for females, respectively, corrected for test substance purity). Animals were observed for morbidity, mortality and injury twice daily and for clinical signs weekly. Body weight and feed consumption were measured weekly, with the exception of twice during week 2 during a period of low feed consumption. Ophthalmoscopic examinations were performed in the pretest period and prior to scheduled necropsy. Haematology, clinical chemistry and urine analysis parameters were measured pretest and at 3, 6, 9 and 12 months. Faecal samples were examined for ova and parasites 3 months into the study period. Gross pathological and histopathological examinations were performed in all groups, and organ weights were measured in all groups.

There were no effects on mortality, ophthalmology, haematology or urine analysis parameters or gross pathology. Clinical signs of toxicity consisted of an increased incidence of thin appearance at 10 000 ppm and above in both males and females. Overall body weight was decreased compared with controls in males starting at 10 000 ppm, and males exhibited a week of body weight loss at and above 10 000 ppm and reduced body weight gain thereafter. Body weights were decreased in females at 30 000 ppm. Total bilirubin was increased in males and females at 10 000 ppm, and relative liver weights were increased in males in all treated groups. Hepatocellular hypertrophy was increased in males at 10 000 ppm (Table 10). The combined effect on liver parameters was considered adverse at 10 000 ppm in males.

Table 10. Effects in the 1-year oral toxicity study in dogs

	Males				**Females**			
	0 ppm	**3 000 ppm**	**10 000 ppm**	**30 000 ppm**	**0 ppm**	**3 000 ppm**	**10 000 ppm**	**30 000 ppm**
Clinical signs								
Excretion								
Faeces discoloured, red	0/0[a]	0/0	0/0	1/1	1/1	0/0	0/0	0/0
Faeces, mucoid	2/1	0/0	0/0	1/1	5/3	3/2	1/1	2/1
Faeces, soft	181/4	600/4	583/4	889/4	72/4	139/4	231/4	491/4
Faeces, watery	14/4	257/4	279/4	447/4	1/1	0/0	78/2	63/4
External appearance								

	Males				Females			
	0 ppm	**3 000 ppm**	**10 000 ppm**	**30 000 ppm**	**0 ppm**	**3 000 ppm**	**10 000 ppm**	**30 000 ppm**
Thin	0/0	0/0	87/3	248/2	5/1	0/0	20/1	235/2
Body weight (kg)								
Day −1	8.21 ± 1.17	8.14 ± 0.77	8.60 ± 0.81	8.68 ± 1.00	8.05 ± 0.72	7.79 ± 0.89	7.99 ± 1.04	7.86 ± 0.46
Day 7	8.30 ± 1.19	8.14 ± 0.73	8.34 ± 0.94	8.28 ± 1.08	7.89 ± 0.61	7.85 ± 0.87	7.65 ± 1.12	7.36 ± 0.63
Day 14	8.29 ± 1.33	8.22 ± 0.85	8.36 ± 0.99	8.19 ± 1.03	7.80 ± 0.66	7.96 ± 0.89	7.54 ± 1.16	7.46 ± 0.52
Day 28	8.50 ± 1.17	8.23 ± 0.65	8.21 ± 1.14	7.96 ± 0.93 (−6.4%)	7.85 ± 0.76	8.21 ± 0.82	7.55 ± 1.23	7.54 ± 0.24
Day 42	8.71 ± 1.23	8.33 ± 0.75	8.31 ± 1.10	8.04 ± 0.74 (−7.7%)	7.86 ± 0.96	8.35 ± 0.62	7.74 ± 1.17	7.71 ± 0.35
Day 56	8.93 ± 1.19	8.43 ± 0.69	8.29 ± 1.07 (−7.2%)	8.00 ± 0.83 (−10.4%)	7.60 ± 1.18	8.46 ± 0.63	7.73 ± 1.20	7.65 ± 0.44
Day 70	9.00 ± 1.09	8.56 ± 0.64	8.28 ± 1.17 (−8.0%)	8.05 ± 0.74 (−10.6%)	7.65 ± 1.51	8.51 ± 0.60	7.69 ± 1.31	7.44 ± 0.42
Day 84	9.09 ± 0.97	8.46 ± 0.58 (−6.9%)	8.21 ± 1.15 (−9.7%)	8.14 ± 0.70 (−10.5%)	7.58 ± 1.38	8.35 ± 0.64	7.76 ± 1.29	7.40 ± 0.32
Day 98	9.18 ± 1.03	8.49 ± 0.50 (−7.5%)	8.26 ± 1.00 (−10.0%)	8.26 ± 0.66 (−10.0%)	7.69 ± 1.22	8.38 ± 0.49	7.76 ± 1.30	7.35 ± 0.32
Day 196	9.80 ± 0.87	9.03 ± 0.70 (−7.9%)	8.71 ± 1.11 (−11.1%)	8.83 ± 0.42 (−9.9%)	8.43 ± 1.68	8.74 ± 0.42	8.38 ± 1.60	7.85 ± 0.72 (−6.9%)
Day 210	9.78 ± 0.82	9.10 ± 0.67 (−7.0%)	8.79 ± 1.11 (−10.1%)	8.90 ± 0.54 (−9.0%)	8.60 ± 1.74	8.80 ± 0.35	8.45 ± 1.67	7.89 ± 0.59 (−8.3%)
Day 364	10.01 ± 0.54	9.36 ± 0.94 (−6.5%)	8.86 ± 0.94 (−11.5%)	9.35 ± 0.47 (−6.6%)	9.20 ± 2.28	8.91 ± 0.45	8.75 ± 1.60	8.16 ± 0.90 (−11.3%)
Body weight gain (kg)	1.8	1.22	0.26	0.67	1.15	1.12	0.76	0.30
Clinical chemistry								
Total bilirubin (mg/dL)								
Pretest	0.18 ± 0.050	0.18 ± 0.050	0.15 ± 0.058	0.15 ± 0.058	0.15 ± 0.058	0.18 ± 0.050	0.18 ± 0.050	0.18 ± 0.050
3 months	0.15 ± 0.058	0. 20 ± 0.000	0.30 ± 0.000	0.38 ± 0.050	0.20 ± 0.000	0.30 ± 0.000	0.33 ± 0.050	0.40 ± 0.082
6 months	0.20 ± 0.000	0.28 ± 0.050	0.35 ± 0.058	0.45 ± 0.058	0.23 ± 0.050	0.30 ± 0.000	0.38 ± 0.050	0.50 ± 0.000
9 months	0.20 ± 0.000	0.25 ± 0.058	0.35 ± 0.058	0.50 ± 0.000	0.20 ± 0.000	0.28 ± 0.050	0.35 ± 0.058	0.55 ± 0.100

	Males				Females			
	0 ppm	**3 000 ppm**	**10 000 ppm**	**30 000 ppm**	**0 ppm**	**3 000 ppm**	**10 000 ppm**	**30 000 ppm**
12 months	0.18 ± 0.050	0.25 ± 0.058	0.38 ± 0.050	0.50 ± 0.000	0.20 ± 0.000	0.25 ± 0.058	0.38 ± 0.050	0.50 ± 0.000
Organ weights								
Liver								
Absolute (g)	243 ± 25	261 ± 41 (7%)	251 ± 44 (3%)	278 ± 20 (14%)	217 ± 15	216 ± 16	209 ± 37	226 ± 27
Relative (%)	2.47 ± 0.11	2.85 ± 0.19** (15%)	2.90 ± 0.17** (17%)	3.13 ± 0.12** (27%)	2.58 ± 0.64	2.48 ± 0.17	2.48 ± 0.10	2.94 ± 0.36
Histopathology								
Hypertrophy, with altered tinctorial properties, hepatocyte, panlobular – very slight	0	1	4	3	0	0	0	0

ppm: parts per million; **: $P < 0.01$ (statistical analysis performed only on organ weights)
[a] Number of times observed/total number of animals affected.
Source: Heward (2014)

The NOAEL was 3000 ppm (equal to 80 mg/kg bw per day), based on an increased incidence of thin appearance and increased total bilirubin in males and females and decreased body weight and increased liver changes in males at 10 000 ppm (equal to 273 mg/kg bw per day) (Heward, 2014).

(b) Dermal application

No studies were submitted.

(c) Exposure by inhalation

No studies were submitted.

2.3 Long-term studies of toxicity and carcinogenicity

Mice

In an 18-month carcinogenicity study, groups of 50 Crl:CD1 (ICR) mice of each sex received fenpicoxamid (purity 79.4%) in the diet at a concentration of 0, 50, 300 or 1500 ppm (males) or 0, 50, 300 or 3000 ppm (females) (corrected for test substance purity; equal to 0, 5.3, 32 and 156 mg/kg bw per day for males and 0, 6.8, 40 and 388 mg/kg bw per day for females, respectively). Animals were monitored daily for mortality and moribundity and biweekly for clinical signs of toxicity. Detailed clinical observations were performed on 10 animals of each sex per group monthly for the first 13 months of treatment and at months 15 and 18. Body weight, body weight gain and feed consumption were measured weekly for the first 13 months of treatment and monthly thereafter. Ophthalmological examinations were performed pre-exposure and prior to scheduled necropsy. White blood cell counts were analysed from samples taken from the pedal vein at 12 months and orbital sinus at 18 months,

and toxicokinetics was analysed in urine and whole blood. Gross examinations were performed and organs were weighed in all animals, and histopathological examination was performed on animals from the control and high-dose groups and all animals killed in extremis or found dead.

There were no effects on mortality, clinical signs of toxicity, body weight, feed consumption or white blood cell counts. There were no gross changes to the liver; however, liver weights were increased in males in all treated groups and in females at 3000 ppm. Based on the lack of histopathological changes and dose–response relationship, the increased liver weights were considered adverse only at 1500/3000 ppm in both sexes. There was an increase in calculi in the lumen of gall bladders in males and females at 300 ppm and above, indicating a possible alteration of liver metabolism. Hypertrophy with altered tinctorial properties was increased in males at 300 ppm and above and in females at 3000 ppm. Multifocal single-cell necrosis was increased in males in all treated groups and in females at 3000 ppm; however, the changes were considered treatment related and adverse only at the high dose in both sexes. Hepatocyte vacuolization consistent with fatty change was increased at 1500 ppm in males in the midzonal and centrilobular regions of the liver, whereas in females the vacuolization was multifocal and involved individual cells at 3000 ppm. There was an increase in intrahepatocellular erythrocytes in males at 1500 ppm. In males, hepatocellular adenomas were increased, although within the historical control range, at 300 ppm, and adenomas and carcinomas were increased at 1500 ppm (Table 11).

Table 11. Effects in the 18-month oral toxicity study in mice

	Males				**Females**			
	0 ppm	**50 ppm**	**300 ppm**	**1 500 ppm**	**0 ppm**	**50 ppm**	**300 ppm**	**3 000 ppm**
Organ weights								
Liver								
Absolute (g)	2.44 ± 0.43	3.16 ± 3.12	2.58 ± 0.56	2.86 ± 0.63*	2.22 ± 0.58	1.94 ± 0.29	2.30 ± 1.50	2.51 ± 0.62*
Relative (g/100 g)	5.03 ± 0.97	6.51 ± 4.99	5.56 ± 1.28	5.95 ± 1.23	5.25 ± 1.40	4.85 ± 0.61	5.36 ± 3.08	6.034 ± 1.78*
Histopathology								
Adrenal (no. examined)	50	50	50	50	50	50	50	50
Hypertrophy; zona fasciculata; unilateral; focal; slight	3	1	0	0	0	0	0	0
Hypertrophy; zona fasciculata; unilateral; diffuse; slight	1	0	0	0	0	0	0	0
Gall bladder (no. examined)	46	45	38	47	49	50	48	49
Calculi, lumen	4	2	10	14*	2	2	6	10*
Liver (no. examined)	50	50	50	50	50	50	50	50
Hypertrophy, with altered tinctorial properties, hepatocyte, centrilobular/midzonal	5	4	21*	37*	2	5	5	32*
- very slight	4	4	20*	15*	2	3	5	11*
- slight	1	0	1	20*	0	2	0	21*

	Males				Females			
	0 ppm	50 ppm	300 ppm	1 500 ppm	0 ppm	50 ppm	300 ppm	3 000 ppm
- moderate	0	0	0	2	0	0	0	0
Necrosis; hepatocyte; individual cells; multifocal	1	4	3	12*	0	1	0	2
- very slight	1	4	3	11*	0	1	0	2
- slight	0	0	0	1	0	0	0	0
Vacuolization; consistent with fatty change; hepatocyte; centrilobular/midzonal	5	3	4	15*	0	0	1	2
- very slight	1	3	2	9*	0	0	1	2
- slight	3	0	2	5	0	0	0	0
- moderate	1	0	0	1	0	0	0	0
Vacuolization; consistent with fatty change; hepatocyte; individual cells; multifocal; very slight	0	0	0	1	1	0	0	10*
Intrahepatocellular erythrocytes; multifocal	1	2	1	11*	0	0	0	0
- very slight	1	2	1	8*	0	0	0	0
- slight	0	0	0	3	0	0	0	0
Tumour incidences								
Adrenal glands								
Adenoma; unilateral; subcapsular; benign; primary	0	1	1	3	0	0	0	0
Liver								
Adenoma; hepatocyte; benign; primary	4	2	7	8	1	0	1	0
Adenoma; two; hepatocyte; benign; primary	0	0	3	2	0	0	0	0
Adenoma; three; hepatocyte; benign; primary	0	1	1	0	0	0	0	0
Adenoma; five; hepatocyte; benign; primary	0	0	0	1	0	0	0	0
Total no. of animals with one or more adenomas; hepatocyte; benign;	4	3	11	11	1	0	1	1

	Males				Females			
	0 ppm	50 ppm	300 ppm	1 500 ppm	0 ppm	50 ppm	300 ppm	3 000 ppm
primary								
Historical controls		*4–12*						
Carcinoma; hepatocyte; malignant without metastasis; primary	1	3	0	4	0	0	0	0
Historical controls		*0–2*						
Total no. of animals with one or more adenomas and/or carcinomas; hepatocyte; benign; primary	5	6	11	13	1	0	1	0
Historical controls		*4–13*						

ppm: parts per million
Source: Thomas, Murray & McCoy (2013)

The NOAEL for toxicity was 50 ppm (equal to 5.3 mg/kg bw per day), based on altered liver metabolism and increased hepatocellular hypertrophy in males at 300 ppm (equal to 32 mg/kg bw per day). The NOAEL for carcinogenicity was 50 ppm (equal to 5.3 mg/kg bw per day), based on an equivocal increase in the incidence of liver adenomas in males at 300 ppm (equal to 32 mg/kg bw per day) (Thomas, Murray & McCoy, 2013).

Rats

In an oral 2-year toxicity and carcinogenicity study, groups of 50 F344/DuCrl rats of each sex received fenpicoxamid (purity 79.4%) in the diet at a targeted dose of 0, 100, 300 or 1000 mg/kg bw per day (equivalent to concentrations of 0, 3000, 10 000 and 30 000 ppm, respectively, corrected for test substance purity; achieved doses of 0, 101, 303 and 1009 mg/kg bw per day for males and 0, 101, 302 and 1009 mg/kg bw per day for females, respectively). An additional 10 rats of each sex per group served as a 12-month satellite group at the same doses. Animals were monitored daily for mortality and moribundity and biweekly for clinical signs of toxicity. Detailed clinical observations were performed on 10 animals of each sex per group monthly for the first 12 months of treatment and at months 15, 18, 21 and 24. Body weight, body weight gain and feed consumption were measured weekly for the first 13 months of treatment and monthly thereafter. Ophthalmological examinations were performed pre-exposure and prior to scheduled necropsy. Haematology, clinical chemistry and urine analysis parameters were examined in samples taken from 10 animals of each sex per dose at 3, 6, 12, 18 and 24 months, and toxicokinetics was analysed in urine and whole blood. Thyroid hormone and blood iodine analyses were performed on retained sera samples from the 3- and 12-month samples and the 24-month sample, respectively. Gross examinations were performed and organs were weighed in all animals, and histopathological examination was performed on animals from the control and high-dose groups and on all animals killed in extremis or found dead.

There were no effects on mortality, clinical signs of toxicity, feed consumption, haematological or coagulation parameters or gross pathology.

Final body weights were decreased in 30 000 ppm females only (Table 12).

Table 12. Effects in the 2-year oral toxicity and carcinogenicity study in rats

	Males				**Females**			
	0 ppm	**3 000 ppm**	**10 000 ppm**	**30 000 ppm**	**0 ppm**	**3 000 ppm**	**10 000 ppm**	**30 000 ppm**
Body weight								
Initial (g)	149.4 ± 12.7	149.2 ± 13.0	148.1 ± 13.1	148.1 ± 11.9	101.8 ± 5.8	100.5 ± 5.2	99.8 ± 5.2	99.2* ± 5.1
Final (g)	419.7 ± 31.1	424.3 ± 25.1	412.7 ± 29.9	411.7 ± 23.1	280.6 ± 21.0	270.7 ± 18.8	271.5 ± 20.2	263.2* ± 15.9 (↓ 6%)
Body weight gain (g) (% of controls)								
Week 1	32.5 (21.8%)	31.1 (20.8%)	31.1 (21%)	30.8 (20.8%)	17.8 (17.4%)	17.6 (17.5%)	17.5 (17.5%)	17.8 (17.9%)
Weeks 1–13	178.8 (119.7%)	176.5 (118.3%)	179.3 (121%)	179 (120.8%)	88.2 (86.6%)	83.6 (83.2%)	83.3 (84.1%)	80.7 (81.4%)
Weeks 13–26	231.1 (154.7%)	227.5 (152.5%)	232.3 (156.8%)	228.7 (154.4%)	103.5 (101.7%)	98.2 (97.7%)	100.8 (101%)	95.3 (96.1%)
Weeks 26–52	282.6 (189.2%)	279.7 (187.5%)	285.1 (192.5%)	277.1 (187.1%)	116.8 (114.7%)	110.9 (110.3%)	112.6 (112.8%)	110.9 (111.8%)
Weeks 52–75	300.6 (201.2%)	298.3 (200%)	302.3 (204.1%)	290.1 (195.8%)	152.6 (150%)	144.2 (143.5%)	146.3 (146.6%)	140.9 (142%)
Overall weeks −1 to 104	270	275.1	264.6	263.6	178.8	170.2	171.7	164
Blood thyroid hormone levels (% change)								
3 months (no. examined)	9	10	7	10	9	10	9	10
T_3 (ng/dL)	110 ± 9.6	109 ± 10.7 (↓ 1.2%)	105 ± 18.4 (↓ 5.0%)	101 ± 17.4 (↓ 8.0%)	116 ± 16.9	105 ± 23.2 (↓ 9.5%)	103 ± 15.3 (↓ 11.1%)	95 ± 16.9 (↓ 17.8%)
T_4 (µg/dL)	7.3 ± 1.0	6.3 ± 1.3 (↓ 13.3%)	6.0 ± 1.4 (↓ 17.9%)	6.1 ± 1.4 (↓ 16.6%)	3.6 ± 1.2	4.0 ± 1.2 (↑ 10.0%)	3.2 ± 1.1 (↓ 12.2%)	3.3 ± 0.9 (↓ 9.7%)

	Males				Females			
	0 ppm	3 000 ppm	10 000 ppm	30 000 ppm	0 ppm	3 000 ppm	10 000 ppm	30 000 ppm
12 months (no. examined)	10	10	10	10	10	10	10	10
T_3 (ng/dL)	102 ± 10.7	90 ± 14.9 (↓ 12.4%)	99 ± 10.7 (↓ 3.5%)	88 ± 10.1* (↓ 13.8%)	128 ± 34.7[a]	115 ± 17.1 (↓ 10.2%)	113 ± 16.0 (↓ 12.2%)	109 ± 11.3 (↓ 15.4%)
					118 ± 14.4[b]	(↓ 2.5%)	(↓ 4.2%)	(↓ 7.6%)
T_4 (µg/dL)	5.5 ± 1.4	5.4 ± 1.3 (↓ 2.5%)	4.5 ± 1.2 (↓ 17.8%)	4.5 ± 0.5 (↓ 18.3%)	2.8 ± 0.7	3.2 ± 1.4 (↑ 11.6%)	3 ± 0.7 (↑ 6.0%)	2.5 ± 0.7 (↓ 11.6%)
TSH (ng/mL)	2.3 ± 0.4	2.2 ± 0.9 (↓ 5.6%)	2.6 ± 0.9 (↑ 10.7%)	2.4 ± 0.6 (↑ 3.4%)	3.1 ± 1.1	2.1 ± 0.6 (↓ 31.2%)	2.2 ± 0.6 (↓ 29.6%)	2.1 ± 0.3 (↓ 31.8%)
24 months (no. examined)	20	20	20	20	20	20	20	20
T_3 (ng/dL)	83 ± 9.5	86 ± 14.7 (↑ 3.1%)	83 ± 16.5 (↓ 0.4%)	81 ± 7.5 (↓ 3.5%)	97 ± 11	105 ± 12.9 (↑ 8.7%)	99 ± 15.0 (↑ 2.6%)	105 ± 12.8 (↑ 8.2%)
T_4 (µg/dL)	3.6 ± 0.8	3.3 ± 0.8 (↓ 7.3%)	3.8 ± 0.8 (↑ 10.3%)	3.3 ± 0.5 (↓ 7.8%)	2.7 ± 0.4	2.7 ± 0.4 (↑ 0.4%)	2.4 ± 0.5 (↓ 10.7%)	2.2 ± 0.5* (↓ 18.8%)
TSH (ng/mL)	1.7 ± 0.5	1.9 ± 1.2 (↑ 10.3%)	1.8 ± 0.5 (↑ 5.7%)	2.1 ± 0.8 (↑ 19.5%)	1.8 ± 0.5	1.6 ± 0.3 (↓ 7.9%)	1.8 ± 0.3 (↑ 4.0%)	1.9 ± 0.4 (↑ 5.6%)
Whole blood iodide (ng/g)								
Organic iodide, calculated from T_3 and T_4[c]								
3 months	46 ± 7	42 ± 8	40 ± 9	40 ± 9	24 ± 8	27 ± 8	21 ± 7	22 ± 6
12 months	37 ± 9	36 ± 8	30 ± 8	30 ± 3	19 ± 4	21 ± 9	20 ± 5	17 ± 4
24 months	24 ± 6	22 ± 5	25 ± 5	22 ± 4	18 ± 3	19 ± 3	17 ± 3	15 ± 3
Measured total iodide (% change)								
24 months	107 ± 40	115 ± 52 (↑ 7%)	134 ± 41 (↑ 25%)	215 ± 80* (↑ 100%)	84 ± 55	77 ± 40 (↓ 8.3%)	104 ± 51 (↑ 23.8%)	161 ± 91* (↑ 91.6%)

	Males				Females			
	0 ppm	3 000 ppm	10 000 ppm	30 000 ppm	0 ppm	3 000 ppm	10 000 ppm	30 000 ppm
95% CI	± 25	± 32	± 25	± 50	± 34	± 25	± 31	± 57
Range	82–132	83–147	109–159	165–265	50–118	52–102	73–135	104–218
Adjusted mean[d]	3	11	30	111	7	0	27	84
Maximum calculated iodide intake due to fenpicoxamid in µg/day (µg/kg bw per day)								
24 months[e]	0	2.4 (5.8)	7.3 (17.4)	24.2 (58)	0	1.6 (5.8)	4.7 (17.4)	15.7 (58)
Urine analysis: pH (% of animals with urinary pH of 7.5 and higher)								
3 months	7.4 (20%)	7.35 (0%)	7.9 (60%)	8.1 (90%)	7.4 (20%)	7.55 (30%)	7.65 (50%)	8.15 (80%)
6 months	7.7 (40%)	7.9 (60%)	8.25 (100%)	8.4 (100%)	7.5 (30%)	8.25 (90%)	8.1 (70%)	8.35 (90%)
12 months	8.05 (70%)	7.75 (40%)	8.5 (90%)	8.5 (100%)	8.1 (80%)	8.2 (60%)	8.0 (60%)	8.55 (100%)
18 months	8.05 (80%)	7.8 (50%)	7.8 (60%)	8.25 (90%)	7.8 (50%)	7.4 (20%)	7.8 (30%)	8.1 (80%)
24 months	7.3 (20%)	7.15 (0%)	7.45 (10%)	7.8 (50%)	7.2 (0%)	7.2 (0%)	7.25 (0%)	7.45 (20%)
Organ weights (% change)								
Absolute kidney (g)								
12 months	2.357 ± 0.176	2.361 ± 0.136	2.390 ± 0.072 (↑ 1%)	2.525 ± 0.122* (↑ 7%)	1.384 ± 0.067	1.359 ± 0.084	1.414 ± 0.076 (↑ 2%)	1.443 ± 0.094 (↑ 4%)
24 months	2.594 ± 0.21	2.705 ± 0.20 (↑ 4%)	2.712 ± 0.24 (↑ 5%)	2.787 ± 0.21* (↑ 7%)	1.735 ± 0.11	1.757 ± 0.16 (↑ 1%)	1.793 ± 0.14 (↑ 3%)	1.830 ± 0.11* (↑ 5%)
Relative kidney (g/100 g)								
12 months	0.571 ± 0.029	0.578 ± 0.020	0.584 ± 0.025	0.605 ± 0.021*	0.689 ± 0.035	0.692 ± 0.026	0.716 ± 0.021	0.740 ± 0.036*

	Males				Females			
	0 ppm	3 000 ppm	10 000 ppm	30 000 ppm	0 ppm	3 000 ppm	10 000 ppm	30 000 ppm
			(↑ 2%)	(↑ 6%)			(↑ 4%)	(↑ 7%)
24 months	0.657 ± 0.09	0.681 ± 0.06 (↑ 4%)	0.705 ± 0.07* (↑ 7%)	0.722 ± 0.06* (↑ 10%)	0.663 ± 0.056	0.690 ± 0.062 (↑ 4%)	0.700 ± 0.053* (↑ 6%)	0.740 ± 0.049* (↑ 12%)
Absolute thyroid (mg)								
12 months	17.3 ± 2.9	17.6 ± 2.2	17.4 ± 1.8	19.4 ± 0.8* (↑ 12%)	11.3 ± 0.7	11.2 ± 1.1	11.7 ± 1.2 (↑ 4%)	13.5 ± 0.7* (↑ 16%)
24 months	22.0 ± 4.4	25.4 ± 10.6 (↑ 15%)	24.1 ± 3.6$ (↑ 10%)	26.9 ± 4.6$ (↑ 22%)	17.8 ± 5.9	18.2 ± 6.8 (↑ 2%)	19.4 ± 3.7$ (↑ 11%)	20.9 ± 3.4$ (↑ 17%)
Relative thyroid (mg/100 g)								
12 months	4.2 ± 0.4	4.3 ± 0.4	4.2 ± 0.3	4.7 ± 0.2* (↑ 12%)	5.6 ± 0.4	5.7 ± 0.6	5.9 ± 0.6 (↑ 5%)	6.9 ± 0.4* (↑ 23%)
24 months	5.6 ± 1.2	6.2 ± 2.7 (↑ 11%)	6.3 ± 0.9$ (↑ 13%)	6.9 ± 1.1$ (↑ 23%)	6.8 ± 2.0	7.1 ± 2.5 (↑ 4%)	7.6 ± 1.9 (↑ 12%)	8.4 ± 1.5* (↑ 23%)
Histopathology								
12 months								
Thyroid (no. examined)	10	10	10	10	10	10	10	10
Dilatation, increased, follicle, multifocal	1	5	6	2	3	5	2	1
- very slight	0	2	1	0	0	2	0	1
- slight	1	3	5	2	3	3	2	0
Dilatation, increased, follicle, diffuse	0	4	4	9	0	5	8	9
Aggregates of macrophages/histiocytes, multifocal	3	5	5	9	1	7	6	9

	Males				Females			
	0 ppm	3 000 ppm	10 000 ppm	30 000 ppm	0 ppm	3 000 ppm	10 000 ppm	30 000 ppm
Kidney (no. examined)	10	0	0	10	10	0	0	10
Chronic progressive glomerulonephropathy	10	0	0	10	4	0	0	2
- very slight	10	0	0	9	4	0	0	2
- slight	0	0	0	1	0	0	0	0
24 months								
Thyroid (no. examined; % change)	49	49	50	50	50	50	50	50
Dilatation, increased, follicle, multifocal – slight	9	35* (↑ 74%)	25* (↑ 64%)	2 (↓ 77.8%)	21	32* (↑ 34%)	40* (↑ 47.5%)	17 (↓ 19%)
Dilatation, increased, follicle, diffuse – slight	5	5	24* (↑ 79%)	48* (↑ 90%)	3	5 (↑ 40%)	6 (↑ 50%)	32* (↑ 91%)
Dilatation, increased, follicle, multifocal or diffuse – slight	14	40* (↑ 65%)	49* (↑ 71%)	50* (↑ 72%)	24	37* (↑ 35%)	46* (↑ 48%)	49* (↑ 51%)
Aggregates of macrophages/ histiocytes, focal – very slight	2	6	4	0	12	8	6	5
Aggregates of macrophages/ histiocytes, multifocal – very slight	25	31 (↑ 19%)	37* (↑ 32%)	46* (↑ 46%)	11	24* (↑ 54%)	25* (↑ 56%)	37* (↑ 70%)
Aggregates of macrophages/ histiocytes, multifocal – slight	0	0	0	0	1	0	0	0
Aggregates of macrophages/ histiocytes, focal or multifocal – very slight or slight	27	37 (↑ 27%)	41* (↑ 34%)	46* (↑ 41%)	24	32 (↑ 25%)	31 (↑ 23%)	42* (↑ 43%)
Kidneys (no. examined)	50	50	50	50	50	50	50	50
Chronic progressive glomerulonephropathy								

	Males				Females			
	0 ppm	3 000 ppm	10 000 ppm	30 000 ppm	0 ppm	3 000 ppm	10 000 ppm	30 000 ppm
- very slight	37	36	29	22*	42	13	7	37
- slight	8	9	17 (↑ 53%)	19* (↑ 58%)	2	0	2	2
- moderate	4	3	3	8 (↑ 50%)	0	0	0	0
- severe	1	2	1	1	0	0	0	0
Ovaries (no. examined)	–	–	–	–	50	14	11	49
Adenocarcinoma; malignant with metastasis; primary	–	–	–	–	0	0	0	2
Uterus (no. examined)	–	–	–	–	50	26	20	49
Adenocarcinoma; malignant with metastasis; primary	–	–	–	–	1	0	0	1
Adenocarcinoma; malignant without metastasis; primary	–	–	–	–	0	0	0	3

bw: body weight; CI: confidence interval; ppm: parts per million; T_3: triiodothyronine; T_4: thyroxine; TSH: thyroid stimulating hormone; *: $P < 0.05$ (Dunnett's test); [$]: $P < 0.05$ (Wilcoxon's test)

[a] Statistical outlier = 219.26 ng/dL.

[b] With statistical outlier removed.

[c] Calculated by the reviewer from the sum of T_3 and T_4 levels assuming the density of blood is 1 g/mL.

[d] Based on the intercept value in ng/g for the male (iodine = 0.1096 × dose + 104.39, $R^2 = 0.9975$) and female (iodine = 0.0833 × dose + 77.35, $R^2 = 0.9719$) linear regressions against treatment dose.

[e] Calculations were based on an iodide impurity level of 58 ppm in the test substance, 100, 300 and 1000 mg/kg bw per day of test material intake, and body weights of 0.417 kg for males and 0.272 kg for females.

Source: Stebbins et al. (2014)

There was an increase in urine alkalinization at 6 months in males and females at 3000 ppm, at 3 and 12 months in males at 10 000 ppm and at 18 and 24 months in males and females at 30 000 ppm. The changes were considered adverse at 10 000 ppm in the presence of an increase in relative kidney weights at 24 months and an increased incidence of chronic progressive glomerulonephropathy; however, the treatment-related nature of the changes is unknown, as this is a common lesion of the ageing rat.

Triiodothyronine (T_3) was decreased at increasing time points in males and females starting at 3000 ppm, and thyroxine (T_4) was decreased in males at 3000 ppm and above and in females at 10 000 ppm and above. Thyroid stimulating hormone (TSH), however, was increased only at 10 000 ppm and above in males at 12 and 24 months and in females at 24 months (Table 12). The effects on T_3 and T_4 were considered adverse only in the presence of effects on TSH. Thyroid weights were increased at 3000 ppm and above. Histopathologically, there is an increase in multifocal to diffuse dilatation of follicles and aggregates of macrophages/histiocytes at 3000 ppm and above in both sexes. According to the study authors, the thyroid toxicity is a result of iodide impurities in the active ingredient, as evidenced by the increased blood iodide level in males at 3000 ppm and in females at 10 000 ppm (Table 12). However, there was insufficient evidence to support this proposed mode of action. The effects on the thyroid hormones were limited, and the additional 6 µg/kg bw per day iodide over the approximately 15 µg/kg bw per day already present in the diet of the low-dose animals would not be expected to have as large an effect as is seen in this study. In addition, the finding of aggregates of macrophages/histiocytes at the lowest dose tested is sufficiently rare as to cause concern.

Although not statistically significant, ovarian tumours in the rat are a very rare tumour type, and the increase at 30 000 ppm would be considered treatment related.

A NOAEL for toxicity was not identified, based on increased thyroid weight and thyroid histopathology changes and increased blood iodide at 3000 ppm (equal to 101 mg/kg bw per day), the lowest dose tested.

The NOAEL for carcinogenicity was 10 000 ppm (equal to 302 mg/kg bw per day), based on an increased incidence of ovarian adenocarcinomas in females at 30 000 ppm (equal to 1009 mg/kg bw per day) (Stebbins et al., 2014).

2.4 Genotoxicity

(a) In vitro studies

A range of in vitro studies of the genotoxicity of fenpicoxamid was conducted to assess its potential for inducing chromosomal aberration, gene mutation and reverse mutation (summarized in Table 13). There was one positive result, the chromosomal aberration assay in rat lymphocytes. There was a significant increase in aberrations (primarily chromatid breaks and exchanges) in the absence of S9 (the 9000 × *g* supernatant fraction from rat liver homogenate) after 4 hours at 100 µg/mL compared with both current and historical controls in both experiments, and there was a positive linear trend in aberrations in the presence of S9 fraction in the second experiment. There were no effects on polyploidy after 24 hours of exposure.

(b) In vivo studies

Unscheduled DNA synthesis and micronucleus assays were conducted to assess the potential of fenpicoxamid to damage DNA and impede repair in vivo (summarized in Table 13). There was no evidence of genotoxicity.

As there was no evidence of genotoxic effects in the in vivo studies, the concern from the in vitro chromosomal aberration study is lessened. Overall, there was no evidence for genotoxicity or mutagenicity in the presence or absence of metabolic activation.

Table 13. Genotoxicity studies with fenpicoxamid

End-point	Test object	Concentration	Purity (%)	Results	Reference
In vitro					
Reverse mutation	*Salmonella typhimurium* and *Escherichia coli*	1.5, 5.0, 15, 50, 150, 500, 1 500 or 5 000 µg/plate ± S9	78.0	Negative	Dakoulas & Divi (2010)
Chromosomal aberration	Rat lymphocytes	25, 50 or 100 µg/mL ± S9	78.0	Positive	Schisler (2011a)
Mammalian forward mutation	Chinese hamster ovary cells (CHO/HPRT)	6.25–100 µg/mL ± S9	78.0	Negative	Schisler (2011b)
In vivo					
Mouse micronucleus	CD-1 mouse bone marrow, males	0, 500, 1 000 or 2 000 mg/kg bw	78.0	Negative	Schisler (2011c)
Unscheduled DNA synthesis	Hsd:ICR (CD-1) mouse liver cells, males	2 000 mg/kg bw	79.4	Negative	Pant (2014)

bw: body weight; CHO/HPRT: Chinese hamster ovary hypoxanthine–guanine phosphoribosyl transferase; S9: 9000 × *g* supernatant fraction from liver homogenate from phenobarbital- and 5,6-benzoflavone-treated rats

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

In a range-finding one-generation reproductive toxicity study, groups of four Crl:CD(SD) rats of each sex received fenpicoxamid (purity 78.0%) at a dietary concentration of 0, 5769, 11 538 or 17 308 ppm (0, 4500, 9000 and 13 500 ppm, respectively, corrected for test substance purity; equal to 0, 370, 748 and 1113 mg/kg bw per day for males and 0, 350, 697 and 1049 mg/kg bw per day for females, respectively). Animals were monitored daily for mortality and moribundity and weekly for clinical signs of toxicity. Body weight and body weight gain were measured weekly in males and weekly during the prebreeding phase and on gestation days (GDs) 0, 7, 14 and 20 and lactation days (LDs) 0, 1, 4, 7, 14 and 21 in females. Feed consumption was measured weekly during prebreeding but was not measured during breeding due to co-housing. Breeding commenced 2 weeks after the start of treatment, litters were recorded as soon as possible following delivery and litters were culled on LD 4. Toxicokinetics was analysed in whole blood of males and females, milk of lactating females and whole blood of culled F_1 pups. Gross examinations were performed and organs were weighed in all adults, and the uteri of all females were stained for examination. Pups were subjected to a gross external examination. Histopathological examination was performed on all adults from the control and high-dose groups.

In the parental animals, there were no effects on mortality, clinical signs of toxicity, body weight, body weight gain or feed consumption. At 4500 ppm, liver weights were slightly increased in males. At 13 500 ppm, there were histopathological changes in the liver of both males and females, consisting of multifocal necrosis with accompanying inflammation and vacuolization consistent with fatty change in males and periportal vacuolization consistent with fatty change in females.

There were no effects on reproductive parameters.

In the offspring, there were decreases in pup weight compared with controls starting at 4500 ppm (Rasoulpour et al., 2012a).

In a two-generation reproductive toxicity study, groups of 25 Crl:CD(SD) rats of each sex received fenpicoxamid (purity 79.4%) at a dietary concentration of 0, 1729, 5052 or 16 614 ppm

(equal to 0, 107, 322 and 1066 mg/kg bw per day, respectively, corrected for purity of test substance) for males and 0, 1362, 4005 or 13 383 ppm (equal to 0, 105, 315 and 1052 mg/kg bw per day, respectively, corrected for purity of test substance) for females. Animals were monitored twice daily for mortality and moribundity and weekly for clinical signs of toxicity. Body weight and body weight gain were measured weekly in males and weekly during the prebreeding phase and on GDs 0, 7, 14 and 20 and LDs 0, 1, 4, 7, 14 and 21 in females. Feed consumption was measured weekly during prebreeding but was not measured during breeding due to co-housing. Breeding commenced 2 weeks after the start of treatment, litters were recorded as soon as possible following delivery and litters were culled on LD 4. F_1 litters were weaned on postnatal day (PND) 21, and one male and one female from each litter were randomly selected as F_1 parental animals to produce the second (F_2) generation. Selected F_1 animals were monitored daily for onset of puberty. Gross examinations were performed and organs were weighed in all adults, and the uteri of all females were stained for examination. Histopathological examination was performed on all adults from the control and high-dose groups. Three pups of each sex per litter were subjected to a gross necropsy after random selection on PND 22, and one of the three pups of each sex per litter was examined grossly for select organ weights.

In parental animals, there were no effects on mortality, body weight, feed consumption, clinical signs of toxicity, gross pathology, histopathology or organ weights.

There were no effects on reproductive parameters.

In offspring, there were no effects on litter parameters, pup body weights, gross necropsy or organ weights.

The NOAEL for parental toxicity was 13 383 ppm (equal to 1052 mg/kg bw per day), the highest dose tested.

The NOAEL for reproductive toxicity was 13 383 ppm (equal to 1052 mg/kg bw per day), the highest dose tested.

The NOAEL for offspring toxicity was 13 383 ppm (equal to 1052 mg/kg bw per day), the highest dose tested (Ellis-Hutchings et al., 2013).

(b) Developmental toxicity

Rats

In a preliminary developmental toxicity study, groups of six premated female Crl:CD(SD) rats were given fenpicoxamid (purity 78.0%) at a concentration of 0, 4500, 9000 or 13 500 ppm (corrected for test substance purity; equal to 0, 322, 639 and 977 mg/kg bw per day, respectively) in the diet from GD 6 to GD 21. Animals were monitored twice daily for mortality and moribundity and daily for clinical signs of toxicity. Body weight and body weight gain were measured daily during treatment (GDs 6–21), and feed consumption was measured every 3 days from GD 6 to GD 21. A blood sample was taken for toxicokinetics at necropsy, and all surviving animals were subjected to gross necropsy and uterine staining. Fetuses were examined for external malformations.

In dams, there were no effects on mortality, clinical signs of toxicity, body weight, body weight gain, feed consumption, caesarean section parameters or gross necropsy.

In fetuses, there were no developmental effects noted (Rasoulpour et al., 2012b).

In a developmental toxicity study, groups of 24 premated female Crl:CD(SD) rats were given fenpicoxamid (purity 79.4%) at a concentration of 0, 1350, 4050 or 13 500 ppm (corrected for purity of the test substance; equal to 0, 103, 311 and 1036 mg/kg bw per day, respectively) in the diet from GD 6 to GD 21. Animals were monitored twice daily for mortality and moribundity and daily for clinical signs of toxicity. Body weight and body weight gain were measured daily during treatment (GDs 6–21), and feed consumption was measured every 3 days from GD 6 to GD 21. A blood sample

was taken for toxicokinetics at necropsy, and all surviving animals were subjected to gross necropsy and uterine staining. All fetuses were examined for body weight, sex and external malformations. Half of each group was examined for visceral malformations, and the remaining half was stained and examined for skeletal malformations.

In dams, there were no effects on mortality, body weight or feed consumption, clinical signs of toxicity, caesarean section parameters or gross necropsy findings.

In fetuses, there were no effects on viability, sex ratios or body weights or treatment-related effects on malformations or variations.

The NOAEL for maternal toxicity was 13 500 ppm (equal to 1036 mg/kg bw per day), the highest dose tested.

The NOAEL for embryo and fetal toxicity was 13 500 ppm (equal to 1036 mg/kg bw per day), the highest dose tested (Rasoulpour, Marshall & McCoy, 2012).

Rabbits

In a preliminary developmental toxicity study, groups of five female premated New Zealand white rabbits were given fenpicoxamid (purity 79.4%) at a concentration of 0, 12 250 or 24 500 ppm (corrected for test substance purity; equal to 0, 460 and 630 mg/kg bw per day, respectively) in the diet from GD 7 to GD 28. Animals were monitored twice daily for mortality and moribundity and daily for clinical signs of toxicity. Body weight and feed consumption were measured daily during treatment (GDs 7–28), and statistical analyses were conducted on GDs 0, 7, 10, 13, 16, 20, 24 and 28. A blood sample was taken for toxicokinetics at GD 27, and all surviving animals were subjected to gross necropsy and uterine staining. Fetuses were weighed; however, no further examinations were performed.

The 24 500 ppm group was killed in extremis due to body weight loss, decreased feed consumption and decreased faecal output on GD 14. There were no treatment-related effects in the 12 250 ppm group (Rasoulpour et al., 2012c).

In a developmental toxicity study, groups of 25 female premated New Zealand white rabbits were given fenpicoxamid (purity 79.4%) at a dietary concentration of 0, 1889, 6297 or 18 891 ppm (0, 1500, 5000 and 15 000 ppm, respectively, corrected for test substance purity; equal to 0, 52.8, 177 and 495 mg/kg bw per day, respectively) from GD 7 to GD 28. Animals were monitored twice daily for mortality and moribundity and daily for clinical signs of toxicity. Body weight and feed consumption were measured daily during treatment (GDs 7–8), and statistical analyses were conducted on GDs 0, 7, 10, 13, 16, 20, 24 and 28. A blood sample was taken for toxicokinetics on GD 27, and all surviving animals were subjected to gross necropsy and uterine staining. All fetuses were examined for body weight, sex, and external and visceral malformations. Half of each group was stained and examined for craniofacial malformations, and all fetuses were stained and examined for skeletal malformations.

In the dams at 1500 ppm, there were decreases in body weight gain and feed consumption that were not considered adverse, as there were no effects on body weight. At 5000 ppm and above, animals exhibited decreased feed consumption, decreased body weight gain and decreased faecal output. At 15 000 ppm, there was body weight loss and decreased feed consumption.

In fetuses, there were no treatment-related effects on developmental parameters.

The NOAEL for maternal toxicity was 1500 ppm (equal to 52.8 mg/kg bw per day), based on decreased body weight gain, feed consumption and faecal output at 5000 ppm (equal to 177 mg/kg bw per day).

The NOAEL for embryo and fetal toxicity was 15 000 ppm (equal to 495 mg/kg bw per day), the highest dose tested (Ellis-Hutchings, Bell & McCoy, 2013).

2.6 Special studies

(a) Neurotoxicity

No studies were submitted.

(b) Immunotoxicity

No studies were submitted.

(c) Studies on metabolites

The acute toxicity and genotoxicity of metabolite X642188 are summarized in Tables 14 and 15, respectively.

Table 14. Acute toxicity study on a metabolite of fenpicoxamid

Test substance	Species	Strain	Sex	Route	Purity (%)	Result	Reference
X642188	Rat	Wistar	Female	Oral	99	LD_{50} > 2 000 mg/kg bw	Dalal (2013)

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

Table 15. Genotoxicity study on a metabolite of fenpicoxamid

Test substance	End-point	Test object	Concentration	Purity (%)	Results	Reference
In vitro						
X642188	Reverse mutation	*Salmonella typhimurium*	51.2, 128, 320, 800, 2 000 and 5 000 µg/plate ± S9	99	Negative	Patel (2012)

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

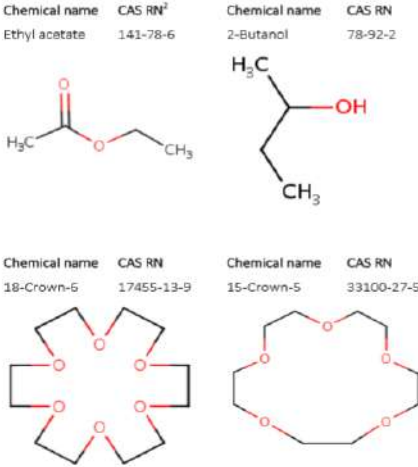
Of the metabolites identified as process hydrolysis degradates, X12335723, X12264475 and X12019520 were flagged for possible genotoxic effects. X12335723 and X12264475 were also embedded in the parent. As such, if weight of evidence suggests that the parent is non-genotoxic, the level of concern is also low for hydrolysis degradates. X12314005 was not seen as a metabolite in the ADME studies, but had no flags for genotoxicity. X12019520 is not a known metabolite in the rat and, due to its small size, is not considered covered by the alerts in the parent. Read-across data from an analogue, RN-3734-22-3, would lessen concern for genotoxicity in humans (Dow, date unknown).

Insufficient information was available to evaluate the toxicological relevance of the metabolites X12326349 (animal metabolite), X696872 (animal metabolite) and X12019520 (simulated processing product).

(d) Investigation of impurities

The purity of the fenpicoxamid used in the toxicity studies ranged between 78.0% and 84.2%. In addition to the iodine cited as an impurity in the 2-year rat oral toxicity study, four impurities were isolated from the test substance: ethyl acetate (CAS no. 141-78-6), 2-butanol (CAS no. 78-92-2), 18-crown-6 (CAS no. 17455-13-9) and 15-crown-5 (CAS no. 33100-27-5) (Fig. 4).

Fig. 4. Structures of impurities found in fenpicoxamid



Source: O'Connell & Hopkins (2017)

The sponsor commissioned a consulting group to perform a search on the names and CAS numbers of the four impurities using the bibra TRACE database, the TOXNET system of databases, e-ChemPortal, the European Chemicals Agency Information on Chemicals database, PubMed and the Registry of Toxic Effects of Chemical Substances.

Ethyl acetate did not induce micronuclei in the bone marrow of mice or hamsters. It caused a weak positive reaction in the induction of aberrations in Chinese hamster lung fibroblast cells without S9 fraction and induced sister chromatid exchanges (SCEs) in Chinese hamster ovary (CHO) cells in the presence of S9. It did not induce unscheduled DNA synthesis in human lymphocytes. Ames tests were negative in *S. typhimurium* strains; however, aneuploidy and other genotoxicity were induced in *Saccharomyces cerevisiae*.

2-Butanol induced clastogenicity in a disputed study, and the evidence of genotoxicity in mammals was weak. Otherwise, 2-butanol was negative in chromosomal aberration assays in CHO cells, Ames tests in *S. typhimurium* or *E. coli* and a gene conversion assay in *S. cerevisiae*.

18-Crown-6 was considered possibly to have anti-mutagenic or co-mutagenic properties due to the ionophoric characteristics of crown ethers and the possibility of altering the structure of membranes to allow for enhanced uptake of other substances. No relevant in vivo mammalian data were identified. 18-Crown-6 did not induce chromosomal aberrations in the rat liver or CHO AA8 cells or SCEs in Chinese hamster (V79) cells. It was negative in *S. typhimurium* and *E. coli* in the Ames assay and in the gene conversion assay in *S. cerevisiae*.

15-Crown-6 was considered possibly to have anti-mutagenic or co-mutagenic properties due to the ionophoric characteristics of crown ethers and the possibility of altering the structure of membranes to allow for enhanced uptake of other substances. No relevant in vivo mammalian data were identified. 15-Crown-6 did not induce chromosomal aberrations in CHO AA8 cells. It was negative in *S. typhimurium* in the Ames assay (O'Connell & Hopkins, 2017).

(e) Microbiological effects

The fungicide fenpicoxamid was evaluated at the 2018 JMPR to determine its impact on microbiota in the gastrointestinal tract. As no data were submitted by the sponsors, a literature search was performed using a number of search engines. These included BioOne (http://www.bioone.org/), Google (https://www.google.com/), Google Scholar (http://scholar.google.com/), PubMed (http://www.ncbi.nlm.nih.gov/pubmed), ScienceDirect (http://www.sciencedirect.com/) and Web of Science (https://apps.webofknowledge.com).

The search strategy used the input keywords of the fungicide chemical name (fenpicoxamid), chemical structure, antimicrobial mode of action, antimicrobial spectrum of activity, antimicrobial resistance, resistance mechanisms and genetics, microbiome, microbiota, gut microbiota, gut microbiome, gastrointestinal microbiota, gastrointestinal microbiome, etc., as well as the Boolean operators AND, OR and NOT.

The extensive search and review of the scientific literature did not find any reports on the effects of fenpicoxamid on the intestinal microbiome to include in the toxicological risk assessment.

3. Observations in humans

In reports on manufacturing plant personnel, no adverse health effects were noted (Dow, 2016).

No information on accidental or intentional poisoning in humans is available.

Comments

Biochemical aspects

In ADME studies conducted in rats using fenpicoxamid labelled with ^{14}C at the phenyl-UL and 2-pyridine positions, the absorption of fenpicoxamid was rapid (T_{max} 2–5 hours) but low (5–20%). Plasma $AUC_{0–168\ h}$ values ranged from 13 to 24 µg·h/g at the low dose (10 mg/kg bw) and from 73 to 74 µg·h/g at the high dose (300 mg/kg bw), indicating a lack of dose proportionality. Elimination was rapid (58–90% of the administered dose within 24 hours) and predominately via the faeces. Biliary excretion of radiolabel was higher than urinary excretion and accounted for only a small fraction of the faecal excretion. Absorbed fenpicoxamid was completely metabolized, with no parent found in the urine. A significant portion of radioactivity was found bound within the faeces (~56% at the single low dose and ~34% at the single high dose), which may account for the lack of absorption. However, following repeated exposure, the portion bound in the faeces was reduced to approximately 14%, suggesting saturation of the binding sites in faecal material (Hansen et al., 2013; Press & Reynolds, 2013).

Tissue distribution was measured at C_{max} and ½ C_{max} (2 and 6–12 hours post-dosing, respectively). At those time points, the highest concentrations of radioactivity were in the gastrointestinal tract, urinary bladder, liver and kidneys. One hundred and sixty-eight hours following dosing, concentrations of radioactivity in all tissues were at or below 0.01% of the administered dose (Hansen, Clark & Staley, 2012; Hansen et al., 2013).

The principal routes of metabolism of fenpicoxamid involved hydrolysis of various ester groups, as well as *O*-dealkylation of the hydroxymethoxy side-chain of the pyridine ring. The primary metabolites were X1236349 and X696872; however, X1236349 also occurs as an impurity (Hansen et al., 2013; Zhang et al., 2014).

Toxicokinetics was analysed in all the short- and long-term studies of toxicity referenced below under "Toxicological data". Overall, there was a low internal exposure to fenpicoxamid, as evidenced by low levels of parent compound and major metabolites in the urine and blood samples; saturation of absorption led to small increases in internal dose, even with large increases in the administered dose. Therefore, a proportionate dose–response relationship would not be expected.

Toxicological data

In rats, fenpicoxamid had an acute oral median lethal dose (LD_{50}) greater than 2000 mg/kg bw, an acute dermal LD_{50} greater than 5000 mg/kg bw and an acute inhalation median lethal concentration (LC_{50}) greater than 0.53 mg/L (Durando, 2011a,b; Krieger & Garlinghouse, 2012).

Fenpicoxamid was non-irritating to the skin and minimally irritating to the eyes of rabbits (Durando, 2011c,d). It was not a dermal sensitizer in mice (Boverhof & Sosinski, 2012). Fenpicoxamid did not cause phototoxicity in vitro (Roth, 2015).

The main toxic effects of fenpicoxamid in short- and long-term studies were liver changes, including adenomas and carcinomas, in the mouse; liver and thyroid changes in the rat; and body weight effects and liver changes in the dog.

In a 90-day study, mice received a dietary concentration of fenpicoxamid of 0, 300, 1500, 3000 or 6000/9000 ppm (males, increased at day 57) or 6000 ppm (females) (equal to 0, 40, 192, 399 and 921 mg/kg bw per day for males and 0, 49, 303, 566 and 1107 mg/kg bw per day for females, respectively), with recovery groups of 0, 6000 (females) and 3000/6000 (males) ppm given untreated diet for 28 days. The NOAEL was 300 ppm (equal to 40 mg/kg bw per day), based on increased liver weights and hepatocellular hypertrophy in males and females, decreased serum triglycerides, hepatocyte fatty change and multifocal single-cell necrosis in males and decreased serum albumin in females at 1500 ppm (equal to 192 mg/kg bw per day) (Thomas, Murray & McCoy, 2014).

In a 29-day study in which rats received a dietary concentration of fenpicoxamid of 0, 2300, 4500 or 9000 ppm (equal to 0, 196, 395 and 788 mg/kg bw per day for males and 0, 197, 377 and 764 mg/kg bw per day for females, respectively), the NOAEL was 9000 ppm (equal to 764 mg/kg bw per day), the highest dose tested (Stebbins, Murray & McCoy, 2012).

In a 90-day study in which rats received a dietary concentration of fenpicoxamid of 0, 3000, 6000 or 11 500/14 000 ppm (dose increased at day 71; equal to 0, 180, 365 and 732 mg/kg bw per day for males and 0, 205, 413 and 834 mg/kg bw per day for females, respectively), the NOAEL was 11 500/14 000 ppm (equal to 732 mg/kg bw per day), the highest dose tested (Stebbins et al., 2012).

In a 90-day study in which dogs received a dietary concentration of fenpicoxamid of 0, 3000, 10 000 or 30 000 ppm (equal to 0, 100, 408 and 939 mg/kg bw per day for males and 0, 122, 353 and 1115 mg/kg bw per day for females, respectively), the NOAEL was 30 000 ppm (equal to 939 mg/kg bw per day), the highest dose tested (Heward, 2013b).

In a 1-year study in which dogs received a dietary concentration of fenpicoxamid of 0, 3000, 10 000 or 30 000 ppm (equal to 0, 84, 300 and 981 mg/kg bw per day for males and 0, 80, 273 and 1011 mg/kg bw per day for females, respectively), the NOAEL was 3000 ppm (equal to 80 mg/kg bw per day), based on an increased incidence of thin appearance and increased total bilirubin in males and females and decreased body weight and increased liver changes in males at 10 000 ppm (equal to 273 mg/kg bw per day) (Heward, 2014).

In an 18-month carcinogenicity study in which mice received a dietary concentration of fenpicoxamid of 0, 50, 300 or 1500 ppm (males) or 0, 50, 300 or 3000 ppm (females) (equal to 0, 5.3, 32 and 156 mg/kg bw per day for males and 0, 6.8, 40 and 388 mg/kg bw per day for females, respectively), the NOAEL for toxicity was 50 ppm (equal to 5.3 mg/kg bw per day), based on altered liver metabolism and increased hepatocellular hypertrophy in males at 300 ppm (equal to 32 mg/kg bw per day). The NOAEL for carcinogenicity was 50 ppm (equal to 5.3 mg/kg bw per day), based on an equivocal increase in the incidence of liver adenomas in males at 300 ppm (equal to 32 mg/kg bw per day) (Thomas, Murray & McCoy, 2013).

In a 2-year toxicity and carcinogenicity study in which rats received a dietary concentration of fenpicoxamid of 0, 3000, 10 000 or 30 000 ppm adjusted to achieve intakes of 0, 101, 302 and 1009 mg/kg bw per day, a NOAEL for toxicity could not be identified, as increased thyroid weight, histopathological changes in the thyroid and increased blood iodide were observed at 101 mg/kg bw per day, the lowest dose tested. The NOAEL for carcinogenicity was 302 mg/kg bw per day, based on an increased incidence of ovarian adenocarcinomas at 1009 mg/kg bw per day (Stebbins et al., 2014).

The Meeting concluded that fenpicoxamid is carcinogenic in male mice and female rats, but not in female mice or male rats.

Fenpicoxamid was tested for genotoxicity in an adequate range of in vitro and in vivo assays. It was not mutagenic in bacterial or mammalian cells in vitro (Dakoulas & Divi, 2010; Schisler,

2011b,c). It caused chromosomal aberrations in rat lymphocytes in vitro (Schisler, 2011a), but it gave negative results in vivo in a mouse micronucleus test and unscheduled DNA synthesis assay (Schisler, 2011c; Pant, 2014).

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

As fenpicoxamid is unlikely to be genotoxic in vivo and there is a clear threshold for liver adenomas in male mice and ovarian adenocarcinomas in female rats, the Meeting concluded that fenpicoxamid is unlikely to pose a carcinogenic risk to humans from the diet.

In a two-generation reproductive toxicity study in which rats received a dietary concentration of fenpicoxamid of 0, 1729, 5052 or 16 614 ppm in males and 0, 1362, 4005 or 13 383 ppm in females adjusted to provide achieved intakes of, respectively, 0, 107, 322 and 1066 mg/kg bw per day for males and 0, 105, 315 and 1052 mg/kg bw per day for females, the NOAEL for parental toxicity, reproductive toxicity and offspring toxicity was 1052 mg/kg bw per day, the highest dose tested (Ellis-Hutchings et al., 2013).

In a developmental toxicity study in which female rats received fenpicoxamid in the diet at a concentration of 0, 1350, 4050 or 13 500 ppm (equal to 0, 103, 311 and 1036 mg/kg bw per day, respectively) from GD 6 to GD 21, the NOAEL for maternal and embryo/fetal toxicity was 13 500 ppm (equal to 1036 mg/kg bw per day), the highest dose tested (Rasoulpour et al., 2012b).

In a developmental toxicity study in which female rabbits received fenpicoxamid in the diet at a concentration of 0, 1500, 5000 or 15 000 ppm (equal to 0, 52.8, 177 and 495 mg/kg bw per day, respectively) from GD 7 to GD 28, the NOAEL for maternal toxicity was 1500 ppm (equal to 52.8 mg/kg bw per day), based on decreased body weight gain, feed consumption and faecal output at 5000 ppm (equal to 177 mg/kg bw per day). The NOAEL for embryo and fetal toxicity was 15 000 ppm (equal to 495 mg/kg bw per day), the highest dose tested (Ellis-Hutchings, Bell & McCoy, 2013).

No neurotoxicity or immunotoxicity studies were available, but there was no indication of neurotoxic or immunotoxic effects in the short- or long-term toxicity studies.

No information on the potential effects of fenpicoxamid on the microbiome of the human gastrointestinal tract is available.

Toxicological data on metabolites and/or degradates

The acute oral LD_{50} of X642188, a rat and crop metabolite, was greater than 2000 mg/kg bw (Dalal, 2013). X642188 was negative in the Ames test (Patel, 2012). For chronic toxicity, the threshold of toxicological concern (TTC) approach (Cramer class III) could be applied.

No toxicity information was available for any other metabolites and/or degradates. However, based on structure–activity considerations, the TTC approach (Cramer class III) could be applied to X12264475 (animal metabolite and processing product), X12314005 (crop metabolite and processing product) and X12335723 (processing product) for the assessment of chronic toxicity.

Insufficient information was available to evaluate the toxicological relevance of the metabolites X12326349 (animal metabolite), X696872 (animal metabolite) and X12019520 (simulated processing product).

Human data

In reports on manufacturing plant personnel, no adverse health effects were noted (Dow, 2016).

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

Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) of 0–0.05 mg/kg bw, on the basis of the NOAEL of 5.3 mg/kg bw per day from the 18-month mouse carcinogenicity study for liver changes and an equivocal increase in the incidence of liver adenomas at 32 mg/kg bw per day in males. A safety factor of 100 was applied. The Meeting noted that there was a margin of 600 between the upper bound of the ADI and the LOAEL for adenomas in mice and a margin of 2000 relative to the LOAEL (the lowest dose tested) for non-neoplastic effects on the thyroid in the 2-year study in rats.

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

Levels relevant to risk assessment of fenpicoxamid

Species	Study	Effect	NOAEL	LOAEL
Mouse	Eighteen-month study of carcinogenicity[a]	Toxicity	50 ppm, equal to 5.3 mg/kg bw per day	300 ppm, equal to 32 mg/kg bw per day
		Carcinogenicity	50 ppm, equal to 5.3 mg/kg bw per day	300 ppm, equal to 32 mg/kg bw per day
Rat	Two-year study of toxicity and carcinogenicity[a]	Toxicity	–	101 mg/kg bw per day[b]
		Carcinogenicity	302 mg/kg bw per day	1 009 mg/kg bw per day
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	1 052 mg/kg bw per day[c]	–
		Parental toxicity	1 052 mg/kg bw per day[c]	–
		Offspring toxicity	1 052 mg/kg bw per day[c]	_
	Developmental toxicity study[a]	Maternal toxicity	13 500 ppm, equal to 1 036 mg/kg bw per day[c]	_
		Embryo and fetal toxicity	13 500 ppm, equal to 1 036 mg/kg bw per day[c]	_
Rabbit	Developmental toxicity study[a]	Maternal toxicity	1 500 ppm, equal to 52.8 mg/kg bw per day	5 000 ppm, equal to 177 mg/kg bw per day
		Embryo and fetal toxicity	15 000 ppm, equal to 495 mg/kg bw per day[c]	_
Dog	One-year study of toxicity[a]	Toxicity	3 000 ppm, equal to 80 mg/kg bw per day	10 000 ppm, equal to 273 mg/kg bw per day

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

Acceptable daily intake (ADI)

0–0.05 mg/kg bw

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 fenpicoxamid

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid, but low
Dermal absorption	No data
Distribution	Wide; higher concentrations in plasma, liver, kidney and urinary bladder
Potential for accumulation	No evidence of accumulation
Rate and extent of excretion	Rapid and complete; primarily via the faeces; 58–90% eliminated within 24 hours
Metabolism in animals	Large percentage not absorbed and bound within faeces; otherwise extensively metabolized
Toxicologically significant compounds in animals and plants	Fenpicoxamid
Acute toxicity	
Rat, LD_{50}, oral	>2 000 mg/kg bw
Rat, LD_{50}, dermal	>5 000 mg/kg bw
Rat, LC_{50}, inhalation	>0.53 mg/L
Rabbit, dermal irritation	Non-irritating
Rabbit, ocular irritation	Minimally irritating
Mouse, dermal sensitization	Not sensitizing
Short-term studies of toxicity	
Target/critical effect	Thin appearance, decreased body weight and liver changes
Lowest relevant oral NOAEL	80 mg/kg bw per day (dog)
Lowest relevant dermal NOAEL	No data
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Liver changes and adenomas
Lowest relevant NOAEL	5.3 mg/kg bw per day (male mouse)

Carcinogenicity	Carcinogenic in male mice and female rats; not carcinogenic in female mice or male rats[a]
Genotoxicity	
	No evidence of genotoxicity in vivo[a]
Reproductive toxicity	
Target/critical effect	None
Lowest relevant parental NOAEL	1 052 mg/kg bw per day, highest dose tested (rat)
Lowest relevant offspring NOAEL	1 052 mg/kg bw per day, highest dose tested (rat)
Lowest relevant reproductive NOAEL	1 052 mg/kg bw per day, highest dose tested (rat)
Developmental toxicity	
Target/critical effect	Decreased feed consumption, body weight gain and faecal output
Lowest relevant maternal NOAEL	52.8 mg/kg bw per day (rabbit)
Lowest relevant embryo/fetal NOAEL	495 mg/kg bw per day, highest dose tested (rabbit)
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	
X642188	LD_{50} > 2 000 mg/kg bw No evidence of genotoxicity
Human data	
	No adverse health effects noted in manufacturing plant personnel

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

Summary

	Value	**Study**	**Safety factor**
ADI	0–0.05 mg/kg bw	Eighteen-month carcinogenicity study in mice	100
ARfD	Unnecessary	–	–

References

Boverhof DR, Sosinski LK (2012). XR-777: local lymph node assay in CBAJ mice. Unpublished report no. 101154. Toxicology & Environmental Research and Consulting, The Dow Chemical Company. Submitted to WHO by The Dow Chemical Company.

Dakoulas EW, Divi K (2010). *Salmonella–Escherichia coli*/mammalian-microsome reverse mutation assay preincubation method with a confirmatory assay with XR-777. Unpublished report no. 100088. BioReliance. Submitted to WHO by The Dow Chemical Company.

Dalal V (2013). Acute oral toxicity study of X642188 in rats. Unpublished report no. 120874. JAI Research Foundation. Submitted to WHO by The Dow Chemical Company.

Dow (date unknown). Toxicological evaluation of metabolites (EFSA request no. 7, reporting table 2(36)). Unpublished report (number unknown). The Dow Chemical Company. Submitted to WHO by The Dow Chemical Company.

Dow (2016). Fenpicoxamid M-CA section 5, Toxicology and metabolism, March 16 update. Unpublished report no. SANCO/11802/2010. The Dow Chemical Company. Submitted to WHO by The Dow Chemical Company.

Durando J (2011a). Acute oral toxicity up and down procedure in rats. Unpublished report no. 101555. Eurofins PSL. Submitted to WHO by The Dow Chemical Company.

Durando J (2011b). Acute dermal toxicity study in rats. Unpublished report no. 101664. Eurofins PSL. Submitted to WHO by The Dow Chemical Company.

Durando J (2011c). Primary skin irritation study in rabbits. Unpublished report no. 101665. Eurofins PSL. Submitted to WHO by The Dow Chemical Company.

Durando J (2011d). Primary eye irritation study in rabbits. Unpublished report no. 101666. Eurofins PSL. Submitted to WHO by The Dow Chemical Company.

Ellis-Hutchings RG, Bell MP, McCoy AT (2013). XDE-777: dietary developmental toxicity study in New Zealand white rabbits. Unpublished report no. 121070. Toxicology & Environmental Research and Consulting, The Dow Chemical Company. Submitted to WHO by The Dow Chemical Company.

Ellis-Hutchings RG, Zablotny CL, Hukkanen RR, Yano BL (2013). XDE-777: two generation dietary reproduction toxicity study in Crl:CD(SD) rats. Unpublished report no. 111186. Toxicology & Environmental Research and Consulting, The Dow Chemical Company. Submitted to WHO by The Dow Chemical Company.

Hansen SC, Clark AJ, Staley JL (2012). XDE-777: tissue distribution in F344DuCrl rats. Unpublished report no. 111150. Toxicology & Environmental Research and Consulting, The Dow Chemical Company. Submitted to WHO by The Dow Chemical Company.

Hansen SC, Clark AJ, Markham DA, Staley JL (2012). XDE-777: probe study to determine absorption, metabolism and elimination in F344NTac rats, Crl:CD1(ICR) mice and New Zealand white rabbits (revision). Unpublished report no. 101038. Toxicology & Environmental Research and Consulting, The Dow Chemical Company. Submitted to WHO by The Dow Chemical Company.

Hansen SC, Clark AJ, Douglass L, Markham DA, Staley JL (2013). XDE-777: pharmacokinetics and metabolism in F344DuCrl rats. Unpublished report no. 111149. Toxicology & Environmental Research and Consulting, The Dow Chemical Company. Submitted to WHO by The Dow Chemical Company.

Heward J (2012). XDE-777: a preliminary palatability study in beagle dogs. Unpublished report no. 110033. MPI Research, Inc. Submitted to WHO by The Dow Chemical Company.

Heward J (2013a). XDE-777: a 28-day dietary toxicity study in beagle dogs. Unpublished report no. 111034. MPI Research, Inc. Submitted to WHO by The Dow Chemical Company.

Heward J (2013b). XDE-777: a 90-day dietary toxicity study in beagle dogs. Unpublished report no. 111035. MPI Research, Inc. Submitted to WHO by The Dow Chemical Company.

Heward J (2014). XDE-777: a one-year dietary toxicity study in beagle dogs. Unpublished report no. 121002. MPI Research, Inc. Submitted to WHO by The Dow Chemical Company.

Krieger SM, Garlinghouse CR (2012). XR-777: acute dust aerosol inhalation toxicity study in F344DuCrl rats. Unpublished report no. 101136. Toxicology & Environmental Research and Consulting, The Dow Chemical Company. Submitted to WHO by The Dow Chemical Company.

O'Connell B, Hopkins J (2017). Ethyl acetate, 2-butanol, 18-crown-6 and 15-crown-5: a review of their genotoxicity. Unpublished report (number not available). Bibra Toxicology Advice and Consulting. Submitted to WHO by The Dow Chemical Company.

Pant K (2014). XDE-777: in vivo unscheduled DNA synthesis (UDS) test in mouse liver cells. Unpublished report no. 140628. BioReliance Corporation. Submitted to WHO by The Dow Chemical Company.

Patel NN (2012). Bacterial reverse mutation test of X642188 using *Salmonella typhimurium*. Unpublished report no. 120873. JAI Research Foundation. Submitted to WHO by The Dow Chemical Company.

Press R, Reynolds I (2013). Elimination of radioactivity in bile, urine, and feces following oral administration of [^{14}C]-labeled XDE-777 to rats. Unpublished report no. 130007. Covance Laboratories Inc. Submitted to WHO by The Dow Chemical Company.

Rasoulpour RJ, Marshall VA, McCoy AT (2012). XDE-777: dietary developmental toxicity study in Crl:CD(SD) rats. Unpublished report no. 111184. Toxicology & Environmental Research and Consulting, The Dow Chemical Company. Submitted to WHO by The Dow Chemical Company.

Rasoulpour RJ, Zablotny CL, McCoy AT, Murray JA, Thomas J (2012a). XR-777: dietary reproduction/developmental toxicity screening test in Crl:CD(SD) rats. Unpublished report no. 101200. Toxicology & Environmental Research and Consulting, The Dow Chemical Company. Submitted to WHO by The Dow Chemical Company.

Rasoulpour RJ, Brooks KJ, Zablotny CL, Clark AJ, McCoy AT, Stebbins KE (2012b). XR-777: dietary developmental toxicity probe study in Crl:CD(SD) rats. Unpublished report no. 101099. Toxicology & Environmental Research and Consulting, The Dow Chemical Company. Submitted to WHO by The Dow Chemical Company.

Rasoulpour RJ, Bell MP, Hukkanen RR, McCoy AT (2012c). XDE-777: dietary developmental toxicity probe study in New Zealand white rabbits. Unpublished report no. 121001. Toxicology & Environmental Research and Consulting, The Dow Chemical Company. Submitted to WHO by The Dow Chemical Company.

Roth M (2015). XDE-777: cytotoxicity assay in vitro with BALB/c 3T3 cells: neutral red (NR) test during simultaneous irradiation with artificial sunlight. Unpublished report no. 150039. Harlan Cytotest Cell Research GmbH. Submitted to WHO by The Dow Chemical Company.

Schisler MR (2011a). Evaluation of XR-777 in an in vitro chromosomal aberration assay utilizing rat lymphocytes. Unpublished report no. 101069. Toxicology & Environmental Research and Consulting, The Dow Chemical Company. Submitted to WHO by The Dow Chemical Company.

Schisler MR (2011b). Evaluation of XR-777 in the Chinese hamster ovary cell hypoxanthine–guanine–phosphoribosyl transferase (CHOHGPRT) forward mutation assay. Unpublished report no. 101089. Toxicology & Environmental Research and Consulting, The Dow Chemical Company. Submitted to WHO by The Dow Chemical Company.

Schisler MR (2011c). Evaluation of XR-777 in the mouse peripheral blood micronucleus test. Unpublished report no. 101061. Toxicology & Environmental Research and Consulting, The Dow Chemical Company. Submitted to WHO by The Dow Chemical Company.

Stebbins KE, Murray JA, McCoy AT (2012). XR-777: 28-day dietary toxicity study in F344DuCrl rats. Unpublished report no. 101053. Toxicology & Environmental Research and Consulting, The Dow Chemical Company. Submitted to WHO by The Dow Chemical Company.

Stebbins KE, Brooks KJ, Andrus AK, Clark AJ, Hukkanen RR, Markham DA et al. (2012). XR-777: 90 day dietary toxicity study in F344DuCrl rats. Unpublished report no. 101110. Toxicology & Environmental Research and Consulting, The Dow Chemical Company. Submitted to WHO by The Dow Chemical Company.

Stebbins KE, Golden RM, Hukkanen RR, McCoy AT (2014). XDE-777: two-year dietary chronic toxicity/oncogenicity study in F344/DuCrl rats. Unpublished report no. 111064. Toxicology & Environmental Research and Consulting, The Dow Chemical Company. Submitted to WHO by The Dow Chemical Company.

Sura R, Murray JA (2010). XR-777: palatability probe study in F344DuCrl rats. Unpublished report no. 100041. Toxicology & Environmental Research and Consulting, The Dow Chemical Company. Submitted to WHO by The Dow Chemical Company.

Thomas J, Murray JA, McCoy AT (2012). XR-777: 28-day dietary toxicity study in Crl:CD1(ICR) mice. Unpublished report no. 101052. Toxicology & Environmental Research and Consulting, The Dow Chemical Company. Submitted to WHO by The Dow Chemical Company.

Thomas J, Murray JA, McCoy AT (2013). XR-777: 18-month dietary oncogenicity study in Crl:CD1(ICR) mice. Unpublished report no. 111068. Toxicology & Environmental Research and Consulting, The Dow Chemical Company. Submitted to WHO by The Dow Chemical Company.

Thomas J, Murray JA, McCoy AT (2014). XR777: 90-day dietary toxicity study with a 28-day recovery in Crl:CD1(ICR) mice (revision). Unpublished report no. 101103. Toxicology & Environmental Research and Consulting, The Dow Chemical Company. Submitted to WHO by The Dow Chemical Company.

Thomas J, Murray JA, Sura R (2010). XR-777: palatability probe study in Crl:CD1(ICR) mice. Unpublished report no. 100043. Toxicology & Environmental Research and Consulting, The Dow Chemical Company. Submitted to WHO by The Dow Chemical Company.

Thomas JA (2012). A probe study to investigate the metabolism and excretion of ^{14}C-labeled XDE-777 in beagle dogs following a single oral (gavage) administration. Unpublished report no. 111004. Toxicology & Environmental Research and Consulting, The Dow Chemical Company. Submitted to WHO by The Dow Chemical Company.

Zhang F, McClymont EL, Fiting JA, Erskine TC, Clark AJ (2014). XDE-777: in vitro comparative metabolism study. Unpublished report no. 130798. Toxicology & Environmental Research and Consulting, The Dow Chemical Company. Submitted to WHO by The Dow Chemical Company.

FLUXAPYROXAD (addendum)

First draft prepared by
G. Wolterink[1] *and J. Zarn*[2]

[1] *Centre for Nutrition, Prevention and Health Services, National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands*
[2] *Federal Food Safety and Veterinary Office (FSVO), Bern, Switzerland*

Explanation....107
Evaluation for acceptable intake....107
1. Toxicological studies....107
1.1 Special studies....107
(a) Mode of action studies....107
Comments....126
Toxicological evaluation....126
References....126

Explanation

Fluxapyroxad was evaluated in 2012 by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR), which concluded that high doses of fluxapyroxad caused hepatocellular adenomas and carcinomas and thyroid follicular cell adenomas and carcinomas in rats.

Following a request for additional maximum residue levels by the Codex Committee on Pesticide Residues, fluxapyroxad was placed on the agenda of the present Meeting, which assessed additional toxicological information on fluxapyroxad available since the last review.

Five in vitro studies were provided that investigated the potential of fluxapyroxad to activate nuclear hormone receptors and to stimulate cell proliferation using rat or human hepatocytes or rat liver microsomes to support the mode of action proposed for rodent liver tumours and its lack of relevance to humans.

Evaluation for acceptable intake

1. Toxicological studies

1.1 Special studies

(a) Mode of action studies

The potential of fluxapyroxad to activate nuclear hormone receptors – i.e. constitutive androstane receptor (CAR), pregnane X receptor (PXR), aryl hydrocarbon receptor (AhR) and peroxisome proliferator–activated receptor alpha (PPARα) – and to stimulate cell proliferation/ replicative DNA synthesis was investigated in five in vitro studies using rat hepatocytes, rat liver microsomes or human hepatocytes.

In the first study, designed as a range-finding study for the subsequent main study (Elcombe, 2016c), cultured primary hepatocytes isolated from male wild-type and CAR knockout Sprague Dawley rats were incubated either with fluxapyroxad (batch no. COD-001049; purity 99.2%) at concentrations ranging from 10 to 300 µmol/L or with vehicle control (0.1% volume per volume [v/v] dimethyl sulfoxide [DMSO]). Concentrations were based on a preliminary cytotoxicity evaluation. Phenobarbital (PB) (100 and 1000 µmol/L) and epidermal growth factor (EGF) (25 ng/mL) were used

as positive controls for the induction of the enzyme activities and replicative DNA synthesis, respectively. The medium, with fluxapyroxad or control substances, was replenished daily for a further 3 days.

Cell toxicity was assessed after 96 hours of culture by measurement of ATP depletion. Cellular ATP was determined by luminometry. CAR-mediated activation of cytochrome P450 2B (CYP2B) in hepatocytes was determined spectrofluorometrically by the formation of resorufin from pentoxyresorufin (pentoxyresorufin *O*-depentylation [PROD]) or benzyloxyresorufin (benzyloxyresorufin *O*-debenzylation [BROD]). PXR-mediated activation of CYP2B and CYP3A in cultured hepatocytes was determined by BROD. PXR-mediated activation of CYP3A1 in cultured hepatocytes was determined spectrofluorometrically by the formation of 7-hydroxyquinoline from benzyloxyquinoline (benzyloxyquinoline *O*-debenzylation [BQ]). The number of cells undergoing replicative DNA synthesis (S-phase) was determined immunocytochemically following the incorporation of 5-bromo-2′-deoxyuridine (BrdU) into hepatocyte nuclei over the last 3 days of culture. The number of hepatocytes undergoing DNA synthesis was recorded as the labelling index [(no. of labelled hepatocytes / total no. of hepatocytes) × 100].

The effects of fluxapyroxad and PB on cytochrome P450 activity in wild-type and CAR knockout hepatocytes are presented in Tables 1 and 2.

Table 1. Induction of CYP2B and CYP3A activities in wild-type rat hepatocytes treated with fluxapyroxad or PB[a]

Treatment and concentration	PROD (pmol resorufin/min per milligram)	BROD (pmol resorufin/min per milligram)	BQ (nmol 7-hydroxyquinoline/min per milligram)
Vehicle control (0.1% [v/v] DMSO)	0.223 ± 0.040 (100.0 ± 17.9)	0.643 ± 0.009 (100.0 ± 1.4)	0.032 ± 0.001 (100.0 ± 3.0)
PB			
100 µmol/L	1.286 ± 0.168*** (576.7 ± 75.1)	5.920 ± 0.415*** (920.4 ± 64.4)	0.064 ± 0.005*** (201.5 ± 14.4)
1 000 µmol/L	1.827 ± 0.308*** (819.6 ± 138.3)	9.496 ± 0.552*** (1 476.4 ± 85.8)	0.158 ± 0.008*** (495.4 ± 23.6)
Fluxapyroxad			
10 µmol/L	1.612 ± 0.199*** (723.2 ± 89.1)	7.494 ± 0.717*** (1 165.1 ± 111.5)	0.130 ± 0.004*** (407.3 ± 12.6)
30 µmol/L	1.354 ± 0.169*** (607.3 ± 76.0)	8.380 ± 0.114*** (1 302.9 ± 17.7)	0.194 ± 0.018*** (607.4 ± 55.2)
100 µmol/L	0.800 ± 0.248* (359.1 ± 111.4)	5.288 ± 0.315*** (822.1 ± 49.0)	0.260 ± 0.010*** (811.8 ± 31.5)
300 µmol/L	0.356 ± 0.462 (159.8 ± 207.5)	2.633 ± 2.369 (409.4 ± 368.3)	0.104 ± 0.117 (326.2 ± 364.3)

BQ: benzyloxyquinoline *O*-debenzylation; BROD: benzyloxyresorufin *O*-debenzylation; DMSO: dimethyl sulfoxide; PB: phenobarbital; PROD: pentoxyresorufin *O*-depentylation; v/v: volume per volume; *: $P < 0.05$; ***: $P < 0.001$ (Student's *t*-test, two-sided)

[a] Values are mean ± standard deviation. Values in parentheses are mean % of control ± standard deviation; $n = 3$ per group.

Source: Elcombe (2016a)

In wild-type rat hepatocytes, fluxapyroxad caused increases of up to 7.2-, 13- and 8.1-fold in PROD, BROD and BQ activities, respectively. PROD and BROD activities peaked at 10 and 30

µmol/L, respectively, declining at higher concentrations, whereas the CYP3A-mediated metabolism of BQ was highest at 100 µmol/L and decreased at 300 µmol/L (Table 1). The decrease in CYP2B activity at 100 µmol/L was attributed to an inhibition of CYP2B by fluxapyroxad, as was demonstrated in a separate study using rat liver microsomes (Elcombe, 2016b). The lower levels of induction of PROD, BROD and BQ at 300 µmol/L (Table 1) were probably the result of cytotoxicity, as ATP levels were reduced by about 99% at this dose.

Table 2. Induction of CYP2B and CYP3A activities in CAR knockout rat hepatocytes treated with fluxapyroxad or PB[a]

Treatment and concentration	**PROD (pmol resorufin/min per milligram)**	**BROD (pmol resorufin/min per milligram)**	**BQ (nmol 7-hydroxyquinoline/min per milligram)**
Vehicle control (0.1% [v/v] DMSO)	0.212 ± 0.017 (100.0 ± 8.0)	0.771 ± 0.097 (100.0 ± 12.6)	0.030 ± 0.002 (100.0 ± 7.7)
PB			
100 µmol/L	0.220 ± 0.043 (103.6 ± 20.3)	0.902 ± 0.074 (117.0 ± 9.6)	0.027 ± 0.004 (89.7 ± 12.5)
1 000 µmol/L	0.363 ± 0.032** (171.0 ± 15.3)	2.121 ± 0.058*** (275.2 ± 7.5)	0.076 ± 0.002*** (252.7 ± 8.2)
Fluxapyroxad			
10 µmol/L	0.350 ± 0.011*** (165.3 ± 5.3)	2.278 ± 0.099*** (295.6 ± 12.8)	0.089 ± 0.007*** (297.5 ± 21.8)
30 µmol/L	0.434 ± 0.062** (204.7 ± 29.3)	2.659 ± 0.333*** (345.0 ± 43.2)	0.143 ± 0.003*** (479.3 ± 11.1)
100 µmol/L	0.364 ± 0.012*** (171.8 ± 5.7)	1.865 ± 0.412* (242.0 ± 53.5)	0.206 ± 0.022*** (688.9 ± 73.3)
300 µmol/L	0.428 ± 0.026*** (202.1 ± 12.3)	1.156 ± 0.031** (150.0 ± 4.0)	0.166 ± 0.036** (554.4 ± 119.5)

BQ: benzyloxyquinoline *O*-debenzylation; BROD: benzyloxyresorufin *O*-debenzylation; DMSO: dimethyl sulfoxide; PB: phenobarbital; PROD: pentoxyresorufin *O*-depentylation; v/v: volume per volume; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ (Student's *t*-test, two-sided)

[a] Values are mean ± standard deviation. Values in parentheses are mean % of control ± standard deviation; $n = 3$ per group.

Source: Elcombe (2016a)

Fluxapyroxad treatment of CAR knockout rat hepatocytes resulted in a non-dose-dependent increase of up to only 2.0-fold in CYP2B activity when measured as PROD. BROD levels showed up to a 3.5-fold increase at 30 µmol/L. The higher induction was considered to be the result of a co-metabolism of benzyloxyresorufin by CYP3A, as the induction of this cytochrome is PXR mediated, which is not knocked out in the rat model used. The lower levels of induction of BROD and BQ at 300 µmol/L (Table 2) were probably the result of cytotoxicity, as ATP levels were reduced by about 99% at this dose.

PB treatment of wild-type and knockout hepatocytes resulted in dose-dependent increases in CYP2B and CYP3A enzyme activities, as indicated by increases of up to 8.2-, 15- and 5.0-fold in PROD, BROD and BQ activities (Tables 1 and 2).

Fluxapyroxad treatment of wild-type rat hepatocytes resulted in an increase of up to 1.5-fold in replicating cells (Table 3). Similar to the induction of PROD activity, the greatest induction of cell proliferation was noted at 10 µmol/L, with a gradual decrease in the S-phase labelling index with

increasing concentrations. At 300 µmol/L, cytotoxicity was noted, which was visually evident by fewer and less confluent hepatocytes. PB and EGF caused 1.8- and 4.1-fold increases in the S-phase labelling index, respectively, in wild-type hepatocytes.

Table 3. Effects of fluxapyroxad, PB and EGF on replicative DNA synthesis in wild-type and CAR knockout rat hepatocytes[a]

	S-phase labelling index (%)	
Test item and concentration	**Wild-type rat hepatocytes**	**CAR knockout rat hepatocytes**
Vehicle control (0.1% [v/v] DMSO)	10.88 ± 1.48 (100.0 ± 13.6)	4.68 ± 0.56 (100.0 ± 11.9)
PB		
100 µmol/L	19.80 ± 2.24*** (182.1 ± 20.6)	4.59 ± 0.43 (98.1 ± 9.2)
1 000 µmol/L	19.59 ± 1.65*** (180.1 ± 15.1)	5.14 ± 0.56 (109.9 ± 11.9)
Fluxapyroxad		
10 µmol/L	15.81 ± 1.07*** (145.3 ± 9.8)	4.87 ± 0.80 (104.0 ± 17.1)
30 µmol/L	15.16 ± 1.39** (139.4 ± 12.8)	4.07 ± 0.90 (87.0 ± 19.2)
100 µmol/L	11.92 ± 1.29 (109.6 ± 11.8)	1.60 ± 0.39*** (34.2 ± 8.4)
300 µmol/L	9.31 ± 4.30[b] (85.6 ± 39.5)	Significant cytotoxicity[b]
EGF		
25 ng/mL	44.17 ± 3.51*** (406.1 ± 32.3)	20.32 ± 4.54*** (434.2 ± 97.0)

CAR: constitutive androstane receptor; DMSO: dimethyl sulfoxide; DNA: deoxyribonucleic acid; EGF: epidermal growth factor; PB: phenobarbital; v/v: volume per volume; **: $P < 0.01$; ***: $P < 0.001$ (Student's *t*-test, two-sided)

[a] Values are mean ± standard deviation. Values in parentheses are mean % of control ± standard deviation; $n = 5$ per group.

[b] Cytotoxicity at this concentration.

Source: Elcombe (2016a)

Treatment of CAR knockout rat hepatocytes with fluxapyroxad resulted in a dose-dependent decrease in cell proliferation. Severe cytotoxicity was observed at 300 µmol/L. Phenobarbital did not affect cell proliferation. EGF led to a 4.3-fold increase in the labelling index in CAR knockout hepatocytes (Table 3).

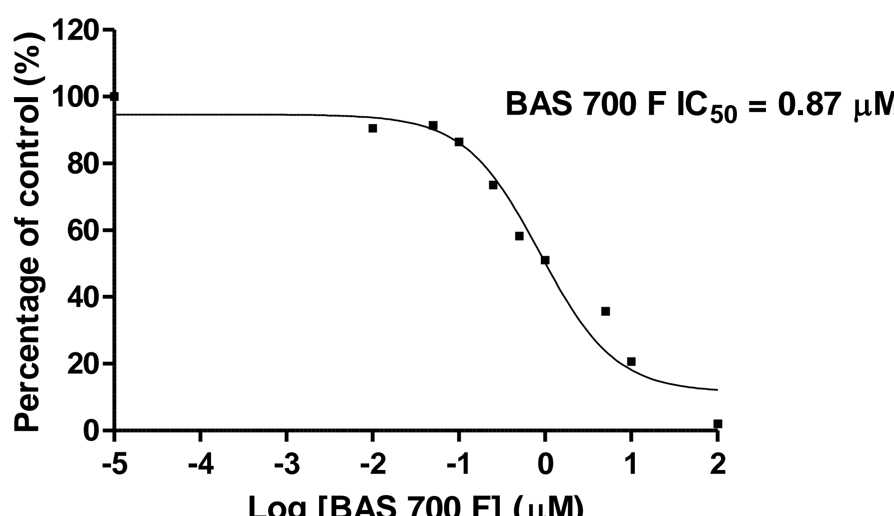
This study indicates that fluxapyroxad induces S-phase (replicative DNA synthesis) and CYP2B activity through CAR activation. Additionally, the experiment with CAR knockout hepatocytes showed that fluxapyroxad can activate PXR and subsequently induce CYP3A (Elcombe, 2016a).

In the second study, the potential of fluxapyroxad (batch no. COD-001049; purity 99.2%) to inhibit CYP2B and CYP3A activities was assessed in Sprague Dawley rat liver microsomes. The activity of CYP2B in PB-induced rat liver microsomes was determined spectrofluorometrically by the

formation of resorufin from pentoxyresorufin (PROD). PROD activity was measured in PB-induced rat liver microsomes following incubation with fluxapyroxad at a concentration of 0, 0.01, 0.05, 0.1, 0.25, 0.5, 1, 5, 10 or 100 µmol/L. The activity of CYP3A in 5-pregnen-3β-ol-20-one 16α-carbonitrile–induced rat liver microsomes was determined spectrofluorometrically by the formation of 7-hydroxyquinoline from BQ. BQ activity was measured following incubation with fluxapyroxad at a concentration of 100 µmol/L.

Fluxapyroxad inhibited PROD (CYP2B) activity, with a median inhibitory concentration (IC_{50}) of 0.87 µmol/L (95% confidence limits: 0.42–1.79), decreasing activity to 2.0% of the control value at the top concentration of 100 µmol/L (Fig. 1).

Fig. 1. PROD (CYP2B) inhibition by fluxapyroxad



Note: The −5 log (µmol/L) concentration is the full activity of the control and not the actual concentration. This data point was included to aid with the curve fit, as recommended by the Prism software.

Fluxapyroxad at 100 µmol/L decreased BQ (CYP3A) activity to 69% of control levels.

This study shows that fluxapyroxad potently inhibits PROD activity and only mildly reduces BQ activity at a high concentration (Elcombe, 2016b).

In the third study, the potential of fluxapyroxad (batch no. COD-001049; purity 99.2%) to activate AhR, CAR, PXR and PPARα and stimulate cell proliferation/replicative DNA synthesis was investigated in cultured primary hepatocytes isolated from male wild-type and CAR knockout Sprague Dawley rats at fluxapyroxad concentrations ranging from 1 to 16 µmol/L. PB (500 µmol/L) was tested in parallel for comparison with fluxapyroxad. EGF (25 ng/mL) was used as a positive control for the induction of replicative DNA synthesis. No positive controls for the induction of AhR or PPARα were included. Cultured hepatocytes were exposed to fluxapyroxad, PB or EGF for 3 days.

AhR-mediated activation of CYP1A in hepatocytes was determined spectrofluorometrically by the formation of resorufin from ethoxyresorufin (ethoxyresorufin *O*-deethylation [EROD]), as well as by measurement of CYP1A1 and CYP1A2 mRNA levels. CAR-mediated activation of CYP2B was determined spectrofluorometrically by the formation of resorufin from pentoxyresorufin (PROD) or benzyloxyresorufin (BROD), as well as by measurement of CYP2B1 and CYP2B2 mRNA levels. PXR-mediated activation of CYP2B and CYP3A was determined spectrofluorometrically by the formation of resorufin from benzyloxyresorufin (BROD) and the formation of 7-hydroxyquinoline

from BQ, as well as by measurement of the CYP3A1 mRNA level. PPARα-mediated activation of CYP4A was measured as the formation of 12-hydroxylauric acid from lauric acid (lauric acid ω-hydroxylase [LAH]) using liquid chromatography with tandem mass spectrometry. In addition, the activity of PPARα-induced peroxisome proliferation was determined by the peroxisomal acyl-coenzyme A (CoA) oxidase (Acox1)–mediated desaturation of acyl-CoA using palmitoyl CoA as substrate. In addition, CYP4A1 mRNA levels and Acox1 mRNA levels were measured. Cell toxicity was assessed after 96 hours of culture by measurement of ATP depletion. Cellular ATP was determined by luminometry. The number of cells undergoing replicative DNA synthesis (S-phase) was determined immunocytochemically following the incorporation of BrdU into hepatocyte nuclei over the last 3 days of culture. The number of hepatocytes undergoing DNA synthesis was recorded as the labelling index [(no. of labelled hepatocytes / total no. of hepatocytes) × 100].

The effects of fluxapyroxad on cytochrome P450 activity in wild-type and CAR knockout hepatocytes are presented in Tables 4 and 5.

In wild-type hepatocytes incubated with fluxapyroxad, dose-dependent increases in CYP1A, CYP2B and CYP3A enzyme activities were noted, as indicated by 3.8-, 3.8-, 10- and 4.0-fold increases in EROD, PROD, BROD and BQ activities, respectively (Tables 4 and 5). The effects of fluxapyroxad and PB on enzyme activities were comparable. Compared with classical AhR activators such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 2,3,7,8-tetrachlorodibenzofuran (TCDF), which induce about 18- and 35-fold increases in CYP1A enzyme activity, respectively (Budinsky et al., 2010), the increase in CYP1A activity induced by fluxapyroxad and PB was small.

The dose-dependent induction of CYP1A activity (EROD) in CAR knockout hepatocytes by fluxapyroxad was similar to that in wild-type hepatocytes. In CAR knockout hepatocytes treated with fluxapyroxad, the induction of PROD metabolism by CYP2B was much lower than in wild-type hepatocytes, reaching a maximum 1.5-fold increase at a fluxapyroxad concentration of 16 µmol/L. The increase was comparable with the 1.3-fold increase seen with 500 µmol/L PB. Also, the BROD metabolism (maximum 3.5-fold at 16 µmol/L) was considerably lower in CAR knockout hepatocytes than in wild-type hepatocytes (10-fold). The higher metabolism for BROD compared with PROD is considered to be partly due to the co-metabolism of BROD by CYP3A. CYP3A enzyme activity (BQ) was not affected in CAR knockout hepatocytes (maximum 5.8-fold increase) compared with wild-type hepatocytes (maximum 4.0-fold increase), as CYP3A activity is regulated via the PXR receptor, which is not knocked out in the CAR knockout hepatocytes (Tables 4 and 5).

In wild-type rat hepatocytes, fluxapyroxad induced a slight, non-dose-dependent increase in CYP4A-catalysed lauric acid 12-hydroxylation (maximum 1.8-fold). PB (500 µmol/L) induced a similar increase. The peroxisomal acyl-CoA oxidase (PCoA) activity was slightly reduced by fluxapyroxad at concentrations at and above 8 µmol/L, whereas PB had no effect on PCoA activity. PCoA activity in CAR knockout hepatocytes tended to increase (maximum 2.3-fold) at and above 4 µmol/L.

The effects of fluxapyroxad on cytochrome P450 mRNA levels in wild-type and CAR knockout hepatocytes are presented in Tables 6 and 7.

Fluxapyroxad induced a slight increase in CYP1A1 and CYP1A2 mRNA in wild-type (up to 2.5-fold) and CAR knockout hepatocytes (up to 1.9-fold). PB induced similar increases in mRNA. Dose-dependent inductions of CYP2B1 (up to about 200-fold) and CYP2B2 mRNA (up to about 300-fold) were observed in rat wild-type hepatocytes. Induction by 500 µmol/L PB was about 400-fold for CYP2B1 and 670-fold for CYP2B2 mRNA. In CAR knockout hepatocytes, the mRNA induction by fluxapyroxad was only up to 6-fold for CYP2B1 and up to 2.8-fold for CYP2B2.

Fluxapyroxad dose-dependently increased CYP3A1 mRNA levels in wild-type and CAR knockout rat hepatocytes, up to about 50-fold. It is noted that the PXR receptor is functional in both types of hepatocytes. CYP3A1 mRNA induction was 32- and 8.5-fold after treatment of wild-type and CAR knockout hepatocytes, respectively, with 500 µmol/L PB. Fluxapyroxad induced no increase in CYP4A1 mRNA levels in wild-type or CAR knockout hepatocytes. In fact, CYP4A1 mRNA levels tended to decrease with increasing fluxapyroxad levels. A 2.3-fold increase was noted in wild-type

Table 4. Induction of CYP1A, CYP2B and CYP3A activities in wild-type and CAR knockout Sprague Dawley rat hepatocytes treated with fluxapyroxad or PB[a]

Test item and concentration	EROD (pmol resorufin/min per milligram)		PROD (pmol resorufin/min per milligram)		BROD (pmol resorufin/min per milligram)	
	Wild-type rat hepatocytes	**CAR knockout rat hepatocytes**	**Wild-type rat hepatocytes**	**CAR knockout rat hepatocytes**	**Wild-type rat hepatocytes**	**CAR knockout rat hepatocytes**
Vehicle control (0.1% [v/v] DMSO)	14.07 ± 0.83 (100.0 ± 5.9)	9.72 ± 0.65 (100.0 ± 6.7)	0.320 ± 0.059 (100.0 ± 18.4)	0.343 ± 0.044 (100.0 ± 12.9)	0.939 ± 0.140 (100.0 ± 14.9)	0.814 ± 0.125 (100.0 ± 15.3)
Fluxapyroxad						
1 µmol/L	22.60 ± 0.28*** (160.6 ± 2.0)	20.70 ± 0.63*** (213.0 ± 6.5)	0.548 ± 0.032** (171.5 ± 10.1)	0.346 ± 0.016 (100.7 ± 4.5)	3.931 ± 0.134*** (418.6 ± 14.3)	1.273 ± 0.048** (156.4 ± 5.9)
2 µmol/L	29.26 ± 0.36*** (208.0 ± 2.5)	30.14 ± 0.27*** (310.2 ± 2.8)	0.835 ± 0.058*** (261.1 ± 18.2)	0.384 ± 0.013 (111.8 ± 3.7)	5.739 ± 0.226*** (611.0 ± 24.0)	1.565 ± 0.070*** (192.2 ± 8.6)
4 µmol/L	35.87 ± 2.05*** (254.9 ± 14.6)	38.76 ± 3.12*** (398.9 ± 32.1)	0.943 ± 0.057*** (295.1 ± 17.8)	0.494 ± 0.031** (143.9 ± 9.1)	7.206 ± 0.335*** (767.2 ± 35.7)	2.106 ± 0.056*** (258.7 ± 6.9)
8 µmol/L	45.19 ± 1.66*** (321.2 ± 11.8)	47.32 ± 3.846*** (487.0 ± 39.6)	1.109 ± 0.103*** (347.1 ± 32.1)	0.475 ± 0.011** (138.3 ± 3.1)	8.677 ± 0.860*** (923.8 ± 91.5)	2.423 ± 0.154*** (297.6 ± 18.9)
16 µmol/L	53.85 ± 3.34*** (382.8 ± 23.7)	56.75 ± 3.62*** (584.1 ± 37.3)	1.208 ± 0.015*** (377.8 ± 4.7)	0.502 ± 0.071* (146.2 ± 20.6)	9.540 ± 0.767*** (1 015.8 ± 81.6)	2.876 ± 0.069*** (353.3 ± 8.5)
PB						
500 µmol/L	39.72 ± 2.15*** (282.3 ± 15.3)	29.91 ± 1.14*** (307.8 ± 11.7)	1.437 ± 0.080*** (449.7 ± 24.9)	0.440 ± 0.030* (128.0 ± 8.7)	9.927 ± 0.461*** (1 056.9 ± 49.1)	1.699 ± 0.078*** (208.7 ± 9.5)

BROD: benzyloxyresorufin *O*-debenzylation; CAR: constitutive androstane receptor; CYP: cytochrome P450; DMSO: dimethyl sulfoxide; EROD: ethoxyresorufin *O*-deethylation; PB: phenobarbital; PROD: pentoxyresorufin *O*-depentylation; v/v: volume per volume; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ (Student's *t*-test, two-sided)

[a] Values are mean ± standard deviation. Values in parentheses are mean % of control ± standard deviation; $n = 3$ per group.

Source: Elcombe (2016c)

Table 5. Induction of CYP3A and CYP4A activities and PPARα-induced peroxisomal acyl-CoA oxidase in wild-type and CAR knockout Sprague Dawley rat hepatocytes treated with fluxapyroxad or PB

Test item and concentration	BQ (nmol 7-OH quinoline/min per milligram)		LAH (nmol 12-OH lauric acid/h per milligram)		PCoA (nmol NADH/min per milligram)	
	Wild-type rat hepatocytes	CAR knockout rat hepatocytes	Wild-type rat hepatocytes	CAR knockout rat hepatocytes	Wild-type rat hepatocytes	CAR knockout rat hepatocytes
Vehicle control (0.1% [v/v] DMSO)	0.055 ± 0.002 (100.0 ± 4.4)	0.025 ± 0.002 (100.0 ± 7.9)	0.540 ± 0.111 (100.0 ± 20.5)	0.702 ± 0.019 (100.0 ± 2.7)	0.427 ± 0.095 (100.0 ± 22.2)	0.176 ± 0.035 (100.0 ± 20.2)
Fluxapyroxad						
1 µmol/L	0.075 ± 0.007* (137.2 ± 13.6)	0.038 ± 0.001*** (151.2 ± 2.8)	0.751 ± 0.084* (139.0 ± 15.6)	1.049 ± 0.363 (149.4 ± 51.7)	0.363 ± 0.144 (85.0 ± 33.8)	0.177 ± 0.041 (100.7 ± 23.4)
2 µmol/L	0.089 ± 0.006*** (162.8 ± 10.7)	0.041 ± 0.001*** (162.6 ± 5.7)	0.907 ± 0.062** (167.9 ± 11.5)	1.193 ± 0.086*** (170.0 ± 12.3)	0.392 ± 0.146 (91.8 ± 34.2)	0.251 ± 0.141 (142.6 ± 80.4)
4 µmol/L	0.113 ± 0.009*** (206.1 ± 16.1)	0.060 ± 0.005*** (238.1 ± 18.4)	0.881 ± 0.146** (163.2 ± 27.0)	1.146 ± 0.004*** (163.3 ± 0.6)	0.357 ± 0.135 (83.7 ± 31.7)	0.393 ± 0.101* (224.0 ± 57.7)
8 µmol/L	0.156 ± 0.008*** (284.0 ± 15.0)	0.093 ± 0.007*** (370.1 ± 26.4)	0.717 ± 0.057* (132.8 ± 10.6)	1.116 ± 0.023*** (159.1 ± 3.3)	0.220 ± 0.116 (51.4 ± 27.1)	0.413 ± 0.059** (235.0 ± 33.6)
16 µmol/L	0.219 ± 0.011*** (399.3 ± 20.5)	0.146 ± 0.002*** (577.6 ± 8.7)	0.950 ± 0.049** (176.0 ± 9.2)	1.074 ± 0.027*** (153.1 ± 3.8)	0.200 ± 0.110 (46.9 ± 25.8)	0.405 ± 0.006*** (230.4 ± 3.5)
PB						
500 µmol/L	0.152 ± 0.001*** (278.0 ± 2.3)	0.043 ± 0.003*** (172.5 ± 10.7)	0.893 ± 0.154** (165.4 ± 28.6)	0.898 ± 0.033*** (128.0 ± 4.8)	0.567 ± 0.170 (132.8 ± 39.9)	0.229 ± 0.030 (130.2 ± 17.3)

BQ: benzyloxyquinoline *O*-debenzylation; CAR: constitutive androstane receptor; CoA: coenzyme A; CYP: cytochrome P450; DMSO: dimethyl sulfoxide; LAH: lauric acid ω-hydroxylase; NADH: nicotinamide adenine dinucleotide (reduced); PB: phenobarbital; PCoA: peroxisomal acyl-coenzyme A oxidase; PPARα: peroxisome proliferator–activated receptor alpha; v/v: volume per volume; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ (Student's *t*-test, two-sided)

[a] Values are mean ± standard deviation. Values in parentheses are mean % control ± standard deviation; $n = 3$ per group.

Source: Elcombe (2016c)

Table 6. Induction of CYP1A1/1A2 and CYP2B1/2B2 mRNA in wild-type and CAR knockout rat hepatocytes treated with fluxapyroxad or PB[a]

	CYP1A1 mRNA		**CYP1A2 mRNA**		**CYP2B1 mRNA**		**CYP2B2 mRNA**	
Test item and concentration	**Wild-type rat hepatocytes**	**CAR knockout rat hepatocytes**	**Wild-type rat hepatocytes**	**CAR knockout rat hepatocytes**	**Wild-type rat hepatocytes**	**CAR knockout rat hepatocytes**	**Wild-type rat hepatocytes**	**CAR knockout rat hepatocytes**
Vehicle control (0.1% [v/v] DMSO)	1.00 ± 0.00	1.00 ± 0.17	1.00 ± 0.11	1.00 ± 0.36	1.00 ± 0.76	1.00 ± 0.54	1.00 ± 0.17	1.00 ± 0.20
Fluxapyroxad								
1 µmol/L	1.29 ± 0.21	1.26 ± 0.20	1.00 ± 0.17	1.01 ± 0.24	48.65 ± 25.56*	1.42 ± 0.86	44.38 ± 30.34	1.26 ± 1.15
2 µmol/L	0.82 ± 0.17	1.21 ± 0.04	1.39 ± 0.23	1.01 ± 0.10	69.56 ± 16.64**	2.49 ± 2.61	58.80 ± 14.55**	1.33 ± 0.62
4 µmol/L	0.83 ± 0. 04**	1.28 ± 0.02*	1.57 ± 0.22*	1.20 ± 0.08	79.69 ± 7.61***	3.23 ± 4.05	113.16 ± 30.74**	1.36 ± 0.59
8 µmol/L	1.12 ± 0.09	1.67 ± 0.05**	1.43 ± 0.70	1.32 ± 0.21	150.41 ± 57.65*	2.67 ± 1.09	296.33 ± 77.37**	2.83 ± 1.00*
16 µmol/L	2.01 ± 0.30**	1.25 ± 0.14	2.54 ± 0.53**	1.91 ± 0.14*	212.07 ± 33.99***	6.26 ± 3.79	287.06 ±105.16**	2.10 ± 0.79
PB								
500 µmol/L	2.51 ± 0.25***	1.46 ± 0.31	1.88 ± 0.22**	1.24 ± 0.37	395.71 ± 8.50***	1.49 ± 0.48	668.30 ± 66.47***	1.84 ± 0.22**

CAR: constitutive androstane receptor; CYP: cytochrome P450; DMSO: dimethyl sulfoxide; mRNA: messenger ribonucleic acid; PB: phenobarbital; v/v: volume per volume; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ (Student's *t*-test, two-sided)

[a] Values are mean ± standard deviation ($n = 3$ per group). Results are expressed as fold change relative to controls, where control values were normalized to 1.00. Rat β-actin was employed as the internal control.

Source: Elcombe (2016c)

Table 7. Induction of CYP3A1, CYP4A1 and Acox1 mRNA in wild-type and CAR knockout rat hepatocytes treated with fluxapyroxad or PB[a]

	CYP3A1 mRNA		**CYP4A1 mRNA**		**Acox1 mRNA**	
Test item and concentration	**Wild-type rat hepatocytes**	**CAR knockout rat hepatocytes**	**Wild-type rat hepatocytes**	**CAR knockout rat hepatocytes**	**Wild-type rat hepatocytes**	**CAR knockout rat hepatocytes**
Vehicle control (0.1% [v/v] DMSO)	1.00 ± 0.01	1.00 ± 0.57	1.00 ± 0.27	1.00 ± 0.24	1.00 ± 0.10	1.00 ± 0.14
Fluxapyroxad						
1 µmol/L	4.38 ± 0.82**	6.09 ± 4.49	0.82 ± 0.30	0.88 ± 0.25	1.03 ± 0.17	0.93 ± 0.05
2 µmol/L	5.39 ± 0.81***	5.53 ± 0.56***	0.49 ± 0.10*	0.48 ± 0.10*	0.72 ± 0.07*	0.94 ± 0.09
4 µmol/L	11.14 ± 1.35***	14.35 ± 0.49***	0.53 ± 0.08*	0.42 ± 0.10*	0.76 ± 0.09*	0.95 ± 0.03
8 µmol/L	21.51 ± 3.02***	33.73 ± 6.25***	0.57 ± 0.34	0.31 ± 0.09*	0.86 ± 0.11	1.04 ± 0.05
16 µmol/L	49.26 ± 4.44***	52.76 ± 3.51***	0.76 ± 0.11	0.15 ± 0.04**	1.59 ± 0.21*	0.89 ± 0.11
PB						
500 µmol/L	32.18 ± 1.73***	8.48 ± 4.24*	2.28 ± 0.17**	0.88 ± 0.20	1.51 ± 0.05**	0.99 ± 0.04

Acox1: acyl-coenzyme A oxidase 1; CAR: constitutive androstane receptor; CYP: cytochrome P450; mRNA: messenger ribonucleic acid; PB: phenobarbital; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ (Student's *t*-test, two-sided)

[a] Values are mean ± standard deviation ($n = 3$ per group). Results are expressed as fold change relative to controls, where control values were normalized to 1.00. Rat β-actin was employed as the internal control.

Source: Elcombe (2016c)

hepatocytes treated with 500 µmol/L PB. In addition, there was no indication for PPARα-induced peroxisome proliferation, as no biologically relevant changes in Acox1 mRNA levels were observed after fluxapyroxad or PB treatment in wild-type or CAR knockout hepatocytes.

The effects of fluxapyroxad, PB and EGF on replicative DNA synthesis in rat hepatocytes are presented in Table 8.

Table 8. Effects of fluxapyroxad, PB and EGF on replicative DNA synthesis in wild-type and CAR knockout rat hepatocytes[a]

	S-phase labelling index (%)	
Test item and concentration	**Wild-type hepatocytes**	**CAR knockout hepatocytes**
Vehicle control (0.1% [v/v] DMSO)	8.15 ± 0.60 (100.0 ± 7.4)	5.63 ± 0.66 (100.0 ± 11.7)
Fluxapyroxad		
1 µmol/L	9.89 ± 1.55* (121.4 ± 19.0)	5.56 ± 0.85 (98.9 ± 15.2)
2 µmol/L	13.18 ± 1.28*** (161.7 ± 15.7)	6.03 ± 0.72 (107.1 ± 12.7)
4 µmol/L	15.65 ± 2.00*** (192.1 ± 24.6)	5.96 ± 0.67 (106.0 ± 12.0)
8 µmol/L	16.53 ± 0.64*** (202.8 ± 7.8)	5.97 ± 0.68 (106.1 ± 12.1)
16 µmol/L	15.53 ± 1.49*** (190.5 ± 18.2)	5.58 ± 0.47 (99.3 ± 8.3)
PB		
500 µmol/L	11.32 ± 0.52*** (138.9 ± 6.3)	5.63 ± 0.49 (100.0 ± 8.7)
EGF		
25 ng/mL	40.16 ± 3.52*** (492.7 ± 43.2)	23.43 ± 1.84*** (416.3 ± 32.8)

CAR: constitutive androstane receptor; DMSO: dimethyl sulfoxide; DNA: deoxyribonucleic acid; EGF: epidermal growth factor; PB: phenobarbital; v/v: volume per volume; *: $P < 0.05$; ***: $P < 0.001$ (Student's *t*-test, two-sided)

[a] Values are mean ± standard deviation ($n = 5$ per group). Values in parentheses are mean % of control ± standard deviation.

Source: Elcombe (2016c)

Fluxapyroxad dose-dependently increased replicative DNA synthesis up to 2.0-fold, as indicated by an increased percentage of cells in replication (S-phase labelling index). PB (500 µmol/L) induced a 1.4-fold increase in replicative DNA synthesis, whereas EGF induced a 4.9-fold increase in S-phase labelling index. Fluxapyroxad and PB treatment did not affect replicative DNA synthesis in CAR knockout rat hepatocytes, indicating that induction of cell proliferation by fluxapyroxad and PB is mediated by the CAR receptor. EGF induced a 4.2-fold increase in replicative DNA synthesis in CAR knockout rat hepatocytes.

This study shows that fluxapyroxad increases S-phase (replicative DNA synthesis) and CYP2B activity through CAR activation. The induction profile of fluxapyroxad was similar to that of PB. Fluxapyroxad and PB also activated PXR and subsequently induced CYP3A, as BQ activities were still elevated in the CAR knockout rat hepatocytes. There was only a slight activation of AhR, as

shown by a small induction of CYP1A mRNA expression and enzyme activity. There was no evidence of PPARα-mediated activation, as CYP4A and peroxisome proliferation markers were generally unchanged (Elcombe, 2016c).

As the rat carcinogenicity study was conducted in Wistar rats, a fourth study was conducted to demonstrate similar CAR-mediated effects in this rat strain. In this study, the potential of fluxapyroxad (batch no. COD-001049; purity 99.2%) to activate AhR, CAR, PXR and PPARα and stimulate cell proliferation/replicative DNA synthesis was investigated in cultured primary hepatocytes isolated from male Wistar rats at fluxapyroxad concentrations ranging from 1 to 100 µmol/L. PB (500 µmol/L) was tested in parallel for comparison with fluxapyroxad. EGF (25 ng/mL) was used as a positive control for the induction of replicative DNA synthesis. No positive controls for the induction of AhR or PPARα were included. Cultured hepatocytes were exposed to fluxapyroxad, PB or EGF for 3 days.

The methods to assess activation of AhR, CAR, PXR and PPARα and stimulation of cell proliferation/replicative DNA synthesis were the same as described in Elcombe (2016c) above.

The effects of fluxapyroxad on cytochrome P450 activity in Wistar rat hepatocytes are presented in Table 9.

Fluxapyroxad induced a dose-dependent increase in CYP1A enzyme activity (EROD), up to about 6.5-fold, in Wistar rat hepatocytes. PB treatment at 500 µmol/L resulted in a 2.7-fold increase in CYP1A enzyme activity. CYP2B enzyme activity measured as PROD and BROD peaked at 10 µmol/L fluxapyroxad (13- and 12-fold increases, respectively), but declined at 30 and 100 µmol/L to about a 5.0-fold increase. This decrease was probably due to an inhibition of CYP2B by fluxapyroxad (IC_{50} = 0.87 µmol/L), as was demonstrated by Elcombe (2016b). PB at 500 µmol/L induced similar increases in PROD (12-fold) and BROD (9.5-fold) (Table 9).

Fluxapyroxad induced a dose-dependent increase in CYP3A enzyme activity (BQ), up to 8.6-fold. PB treatment at 500 µmol/L resulted in a 2.5-fold increase in CYP3A activity (Table 9).

The induction of CYP4A1 enzyme activity, measured by 12-hydroxylation of lauric acid (LAH), was marginally affected, as indicated by a maximum 2.8-fold increase. Treatment with PB resulted in a 1.7-fold increase in lauric acid hydroxylation (Table 9).

PPARα-induced peroxisomal proliferation, as measured by palmitoyl-CoA oxidation, was not affected by fluxapyroxad or PB.

The effects of fluxapyroxad on cytochrome P450 mRNA levels in Wistar rat hepatocytes are presented in Table 10.

Fluxapyroxad induced a small increase in CYP1A1 (up to 2.5-fold) and CYP1A2 mRNA (up to 4.6-fold) in Wistar rat hepatocytes. PB induced 1.8- and 1.9-fold increases in CYP1A1 and CYP1A2 mRNA, respectively (Table 10).

Dose-dependent inductions of CYP2B1 (up to about 46-fold) and CYP2B2 mRNA (up to about 19-fold) were observed in wild-type rat hepatocytes at fluxapyroxad concentrations up to 10 µmol/L. At higher fluxapyroxad concentrations, induction of CYP2B1 and CYP2B2 mRNA decreased to 11- and 7.2-fold at 100 µmol/L, respectively. Induction by 500 µmol/L PB was 100-fold for CYP2B1 and 56-fold for CYP2B2 mRNA (Table 10).

Fluxapyroxad dose-dependently increased CYP3A1 mRNA levels in Wistar rat hepatocytes up to about 70-fold. It is noted that the PXR receptor is functional in both types of hepatocytes. CYP3A1 mRNA induction was 23-fold after treatment with 500 µmol/L PB (Table 10).

Fluxapyroxad and PB induced no increase in CYP4A1 and Acox1 mRNA levels in Wistar rat hepatocytes. In fact, CYP4A1 mRNA levels tended to decrease with increasing fluxapyroxad levels (Table 10).

Table 9. Induction of CYP1A, CYP2B, CYP3A and CYP4A as well as of PPARα-induced PCoA in Wistar rat hepatocytes treated with fluxapyroxad or PB[a]

Test item and concentration	EROD (pmol resorufin/min per milligram)	PROD (pmol resorufin/min per milligram)	BROD (pmol resorufin/min per milligram)	BQ (nmol 7-OH quinoline/min per milligram)	LAH (nmol 12-OH lauric acid/h per milligram)	PCoA (nmol NADH/min per milligram)
Vehicle control (0.1% [v/v] DMSO)	10.73 ± 0.43 (100.0 ± 4.0)	0.143 ± 0.025 (100.0 ± 17.6)	1.21 ± 0.09 (100.0 ± 7.3)	0.062 ± 0.003 (100.0 ± 4.5)	0.411 ± 0.035 (100.0 ± 8.6)	0.563 ± 0.038 (100.0 ± 6.8)
Fluxapyroxad						
1 µmol/L	15.33 ± 3.01 (142.9 ± 28.0)	0.759 ± 0.075*** (532.0 ± 52.2)	5.48 ± 0.44*** (454.2 ± 36.8)	0.100 ± 0.002*** (162.6 ± 3.4)	0.660 ± 0.210 (160.7 ± 51.2)	0.427 ± 0.078 (75.9 ± 13.9)
3 µmol/L	27.17 ± 2.22*** (253.2 ± 20.7)	1.408 ± 0.203*** (986.7 ± 141.9)	10.09 ± 0.66*** (835.5 ± 55.0)	0.149 ± 0.008*** (241.5 ± 12.4)	0.845 ± 0.121** (205.6 ± 29.4)	0.493 ± 0.133 (87.6 ± 23.7)
10 µmol/L	41.74 ± 6.99** (389.0 ± 65.1)	1.897 ± 0.082*** (1 329.2 ± 57.2)	14.40 ± 0.23*** (1 192.7 ± 19.3)	0.305 ± 0.017*** (492.8 ± 27.4)	0.915 ± 0.091*** (222.7 ± 22.2)	0.478 ± 0.194 (84.9 ± 34.4)
30 µmol/L	57.27 ± 5.12*** (533.9 ± 47.7)	1.649 ± 0.243*** (1 155.5 ± 170.3)	13.17 ± 0.30*** (1 090.7 ± 25.0)	0.533 ± 0.030*** (861.8 ± 49.0)	1.136 ± 0.217** (276.4 ± 52.9)	0.371 ± 0.003*** (65.8 ± 0.5)
100 µmol/L	70.12 ± 4.89*** (653.6 ± 45.6)	0.715 ± 0.030*** (501.1 ± 21.2)	6.12 ± 0.27*** (507.2 ± 22.7)	0.491 ± 0.013*** (794.3 ± 20.6)	1.076 ± 0.091*** (261.9 ± 22.1)	0.483 ± 0.137 (85.8 ± 24.4)
PB						
500 µmol/L	29.29 ± 2.72*** (273.0 ± 25.4)	1.664 ± 0.043*** (1 165.8 ± 30.3)	11.49 ± 0.35*** (951.9 ± 28.8)	0.157 ± 0.004*** (253.3 ± 6.6)	0.695 ± 0.045*** (169.3 ± 10.9)	0.444 ± 0.136 (78.8 ± 24.2)

BQ: benzyloxyquinoline *O*-debenzylation; BROD: benzyloxyresorufin *O*-debenzylation; DMSO: dimethyl sulfoxide; EROD: ethoxyresorufin *O*-deethylation; LAH: 12-hydroxylation of lauric acid; NADH: nicotinamide adenine dinucleotide (reduced); PB: phenobarbital; PCoA: peroxisomal acyl-coenzyme A oxidase; PPARα: peroxisome proliferator–activated receptor alpha; PROD: pentoxyresorufin *O*-depentylation; v/v: volume per volume; **: $P < 0.01$; ***: $P < 0.001$ (Student's *t*-test, two-sided)

[a] Values are mean ± standard deviation ($n = 3$ per group). Values in parentheses are mean % control ± standard deviation.

Source: Elcombe (2016d)

Table 10. Induction of CYP1A1/1A2, CYP2B1/2B2, CYP3A1, CYP4A1 and Acox1 mRNA in Wistar rat hepatocytes treated with fluxapyroxad or PB[a]

Test item and concentration	CYP1A1 mRNA	CYP1A2 mRNA	CYP2B1 mRNA	CYP2B2 mRNA	CYP3A1 mRNA	CYP4A1 mRNA	Acox1 mRNA
Vehicle control (0.1% [v/v] DMSO)	1.00 ± 0.10	1.00 ± 0.03	1.00 ± 0.41	1.00 ± 0.36	1.00 ± 0.26	1.00 ± 0.23	1.00 ± 0.13
Fluxapyroxad							
1 µmol/L	1.12 ± 0.05	1.16 ± 0.10	31.30 ± 3.40***	15.00 ± 1.03***	5.77 ± 0.22***	0.87 ± 0.14	0.98 ± 0.05
3 µmol/L	1.05 ± 0.07	1.85 ± 0.39*	44.89 ± 20.48*	18.02 ± 3.31***	12.63 ± 0.67***	0.47 ± 0.14*	0.88 ± 0.05
10 µmol/L	1.11 ± 0.15	2.08 ± 0.30**	46.44 ± 4.98***	18.61 ± 2.26***	33.57 ± 3.93***	0.40 ± 0.05*	0.94 ± 0.03
30 µmol/L	1.34 ± 0.29	2.52 ± 0.39**	27.30 ± 13.59*	12.71 ± 2.85**	70.26 ± 6.91***	0.25 ± 0.12**	1.00 ± 0.17
100 µmol/L	2.46 ± 0.11***	4.59 ± 0.68***	10.79 ± 2.15**	7.23 ± 2.54*	69.89 ± 2.10***	0.27 ± 0.03**	1.41 ± 0.15*
PB							
500 µmol/L	1.82 ± 0.16**	1.85 ± 0.06***	99.82 ± 6.37***	56.23 ± 2.31***	23.00 ± 1.61***	1.13 ± 0.10	1.06 ± 0.10

Acox1: acyl-coenzyme A oxidase 1; CYP: cytochrome P450; DMSO: dimethyl sulfoxide; mRNA: messenger ribonucleic acid; PB: phenobarbital; v/v: volume per volume; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ (Student’s *t*-test, two-sided)

[a] Values are mean ± standard deviation ($n = 3$ per group). Results are expressed as fold change relative to controls, where control values were normalized to 1.00. Rat β-actin was employed as the internal control.

Source: Elcombe (2016d)

The effects of fluxapyroxad, PB and EGF on replicative DNA synthesis in rat hepatocytes are presented in Table 11.

***Table 11. Effects of fluxapyroxad, PB and EGF on replicative DNA synthesis in Wistar rat hepatocytes*[a]**

Test item and concentration	S-phase labelling index (%)
Vehicle control (0.1% [v/v] DMSO)	10.40 ± 1.23 (100.0 ± 11.9)
Fluxapyroxad	
1 µmol/L	16.51 ± 1.44*** (158.8 ± 13.8)
3 µmol/L	24.45 ± 0.78*** (235.2 ± 7.5)
10 µmol/L	27.11 ± 2.15*** (260.7 ± 20.7)
30 µmol/L	26.83 ± 1.75*** (258.0 ± 16.9)
100 µmol/L	15.15 ± 2.62** (145.7 ± 25.2)
PB	
500 µmol/L	16.09 ± 2.11*** (154.7 ± 20.3)
EGF	
25 ng/mL	52.37 ± 6.68*** (503.6 ± 64.2)

DMSO: dimethyl sulfoxide; DNA: deoxyribonucleic acid; EGF: epidermal growth factor; PB: phenobarbital; v/v: volume per volume; **: $P < 0.01$; ***: $P < 0.001$ (Student's *t*-test, two-sided)

[a] Values are mean ± standard deviation ($n = 5$ per group). Values in parentheses are mean % of control ± standard deviation.

Source: Elcombe (2016d)

Fluxapyroxad treatment of Wistar rat hepatocytes induced a dose-dependent, up to 2.6-fold increase in replicative DNA synthesis. Replicative DNA synthesis peaked at 10 and 30 µmol/L and decreased at 100 µmol/L, which was considered by the study author as probably being due to cytotoxicity at the highest dose. PB at 500 µmol/L induced a 1.5-fold increase in replicative DNA synthesis. EGF induced a 5.0-fold increase in replicative cell proliferation (Table 11).

This study showed that fluxapyroxad activates the nuclear hormone receptors CAR and PXR, inducing both S-phase (replicative DNA synthesis) and CYP2B/CYP3A mRNA levels and activity in Wistar rat primary hepatocytes. Inhibition of CYP2B was evident. A slight activation of AhR was observed, as shown by a small induction of CYP1A mRNA expression and enzyme activity. There was no evidence of PPARα activation by fluxapyroxad (Elcombe, 2016d).

In the fifth study, the potential of fluxapyroxad (batch no. COD-001049; purity 99.2%) to activate CAR and PXR and stimulate cell proliferation/replicative DNA synthesis was investigated in cultured primary human hepatocytes from two male donors. Donor A had no history of smoking, drug use or medication, whereas donor B was a smoker with a history of drug abuse and medication. The

effects of fluxapyroxad at concentrations ranging from 1 to 100 µmol/L were investigated. PB (500 µmol/L) was tested in parallel for comparison with fluxapyroxad. EGF (25 ng/mL) was used as a positive control for the induction of replicative DNA synthesis. Cultured hepatocytes were exposed to fluxapyroxad, PB or EGF for 3 days.

Activation of CAR was detected by induction of PROD and BROD (CYP2B) activities and CYP2B6 mRNA levels. Activation of PXR was detected by induction of BQ (CYP3A) activity and CYP3A4 mRNA levels. Cell proliferation was determined by replicative DNA synthesis, whereas cytotoxicity was assayed via determination of the ATP content relative to the vehicle control, DMSO.

The effects of fluxapyroxad and PB on CYP2B and CYP3A activities in human hepatocytes are presented in Table 12 for donor A and Table 13 for donor B.

Table 12. Induction of CYP2B and CYP3A activities in human hepatocytes treated with fluxapyroxad or PB: donor A[a]

Test item and concentration	PROD (pmol resorufin/min per milligram)	BROD (pmol resorufin/min per milligram)	BQ (nmol 7-hydroxyquinoline/min per milligram)
Vehicle control (0.1% [v/v] DMSO)	0.191 ± 0.022 (100.0 ± 11.8)	1.490 ± 0.200 (100.0 ± 13.4)	0.178 ± 0.009 (100.0 ± 5.1)
Fluxapyroxad			
1 µmol/L	0.220 ± 0.025 (115.3 ± 13.1)	2.491 ± 0.190** (167.2 ± 12.7)	0.260 ± 0.005*** (146.1 ± 3.0)
3 µmol/L	0.275 ± 0.011** (144.3 ± 5.9)	3.218 ± 0.036*** (216.0 ± 2.4)	0.314 ± 0.025*** (176.6 ± 13.9)
10 µmol/L	0.332 ± 0.027** (174.2 ± 14.0)	3.488 ± 0.080*** (234.1 ± 5.3)	0.455 ± 0.013*** (256.1 ± 7.3)
30 µmol/L	0.344 ± 0.041** (180.4 ± 21.6)	4.380 ± 0.406*** (294.0 ± 27.2)	0.399 ± 0.053** (224.6 ± 30.1)
100 µmol/L	0.230 ± 0.017 (120.5 ± 8.8)	2.821 ± 0.087*** (189.3 ± 5.8)	0.066 ± 0.018*** (37.3 ± 10.4)
PB			
500 µmol/L	0.463 ± 0.019*** (242.7 ± 10.2)	8.158 ± 0.441*** (547.5 ± 29.6)	0.939 ± 0.036*** (528.4 ± 20.2)

BQ: benzyloxyquinoline *O*-debenzylation; BROD: benzyloxyresorufin *O*-debenzylation; DMSO: dimethyl sulfoxide; PB: phenobarbital; PROD: pentoxyresorufin *O*-depentylation; v/v: volume per volume; **: $P < 0.01$; ***: $P < 0.001$ (Student's *t*-test, two-sided)

[a] Values are mean ± standard deviation ($n = 3$ replicate analyses at each concentration). Values in parentheses are mean % of control ± standard deviation.

Source: Elcombe (2016e)

Table 13. Induction of CYP2B and CYP3A activities in human hepatocytes treated with fluxapyroxad or PB: donor B[a]

Test item and concentration	PROD (pmol resorufin/min per milligram)	BROD (pmol resorufin/min per milligram)	BQ (nmol 7-hydroxyquinoline/min per milligram)
Vehicle control (0.1% [v/v] DMSO)	0.086 ± 0.009 (100.0 ± 10.8)	0.579 ± 0.080 (100.0 ± 13.8)	0.321 ± 0.034 (100.0 ± 10.5)

Test item and concentration	PROD (pmol resorufin/min per milligram)	BROD (pmol resorufin/min per milligram)	BQ (nmol 7-hydroxyquinoline/min per milligram)
Fluxapyroxad			
1 µmol/L	0.049 ± 0.014* (57.4 ± 16.6)	0.670 ± 0.104 (115.7 ± 18.0)	0.404 ± 0.005 (125.8 ± 1.4)
3 µmol/L	0.042 ± 0.006** (49.1 ± 7.0)	0.679 ± 0.044 (117.2 ± 7.7)	0.508 ± 0.025** (158.4 ± 7.8)
10 µmol/L	0.051 ± 0.010* (60.1 ± 11.3)	0.908 ± 0.147* (156.7 ± 25.3)	0.743 ± 0.019*** (231.8 ± 6.0)
30 µmol/L	0.057 ± 0.009* (66.5 ± 10.2)	0.959 ± 0.130* (165.6 ± 22.4)	0.796 ± 0.027*** (248.1 ± 8.5)
100 µmol/L	0.105 ± 0.032 (122.2 ± 37.4)	1.019 ± 0.203* (176.0 ± 35.1)	0.107 ± 0.017*** (33.3 ± 5.5)
PB			
500 µmol/L	0.111 ± 0.008* (129.3 ± 9.7)	1.789 ± 0.091*** (309.0 ± 15.6)	1.236 ± 0.117*** (385.3 ± 36.4)

BQ: benzyloxyquinoline *O*-debenzylation; BROD: benzyloxyresorufin *O*-debenzylation; DMSO: dimethyl sulfoxide; PB: phenobarbital; PROD: pentoxyresorufin *O*-depentylation; v/v: volume per volume; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ (Student's *t*-test, two-sided)

[a] Values are mean ± standard deviation ($n = 3$ replicate analyses at each concentration). Values in parentheses are mean % of control ± standard deviation.

Source: Elcombe (2016e)

In hepatocytes from donor A (Table 12), CYP2B activity was increased by fluxapyroxad, as shown by increased PROD (1.8-fold) and BROD (2.9-fold) activities at 30 µmol/L. CYP3A (BQ) activity peaked at 10 µmol/L, with a 2.6-fold increase. The lower enzyme activities at 100 µmol/L may be due to cytotoxicity or cytochrome P450 inhibition. Basal activity of PROD was average, whereas those of BROD and BQ were at the upper end of the historical control ranges observed in the laboratory (Table 14).

Table 14. Historical control data for PROD, BROD and BQ activities in rat and human hepatocytes

	PROD	BROD	BQ
Rat ($n = 18$)			
Vehicle[a]	0.30 (0.04–1.33)	1.11 (0.27–2.53)	0.05 (0.03–0.08)
500 µmol/L PB[b]	6.2 (2.2–11.7)	10.3 (6.7–14.3)	2.5 (2.0–2.8)
Human ($n = 19$)			
Vehicle[a]	0.19 (0.05–0.52)	0.84 (0.27–1.91)	0.11 (0.02–0.24)
500 µmol/L PB[b]	1.5 (0.9–2.4)	4.4 (2.9–6.0)	5.3 (3.9–8.5)

BQ: benzyloxyquinoline *O*-debenzylation; BROD: benzyloxyresorufin *O*-debenzylation; *n*: number of studies conducted between 2010 and 2016; PB: phenobarbital; PROD: pentoxyresorufin *O*-depentylation

[a] Basal activity in pmol substrate/minute per milligram protein; data are mean, with minimum–maximum in parentheses.

[b] Fold induction above controls; data are mean, with minimum–maximum in parentheses.

Source: Elcombe (2016e)

In hepatocytes from donor B (Table 13), PROD was slightly increased only at the highest concentration (1.2-fold), whereas BROD was dose-dependently increased from 10 µmol/L onwards,

with a maximum 1.8-fold increase at 100 µmol/L. The study author noted that BROD activity is considered a superior marker of CYP2B activity in human hepatocytes compared with PROD (Mudra & Parkinson, 2004). Furthermore, the observed CYP2B6 activity induction is consistent with the induction of CYP2B6 mRNA (see below). CYP3A4 activity as determined by BQ peaked at 30 µmol/L, with a 2.5-fold increase, and substantially declined to one third of the control activity at 100 µmol/L. Basal activities of PROD and BROD in hepatocytes of donor B were at the lower end of the historical control ranges, whereas the BQ turnover was almost 1.5-fold higher than the maximum observed in previous studies (Table 14).

PB treatment resulted in 2.4-, 5.5- and 5.3-fold increases in PROD and BROD activities and BQ turnover in donor A (Table 12) and 1.3-, 3.1- and 3.9-fold increases in donor B (Table 13), respectively. Generally, the magnitude of all responses to PB treatment was within the historical control range.

The effects of fluxapyroxad and PB on CYP2B6 and CYP3A4 mRNA levels in hepatocytes of donors A and B are presented in Table 15.

Table 15. Induction of CYP2B6 and CYP3A4 mRNA in human hepatocytes treated with fluxapyroxad or PB[a]

	Donor A		**Donor B**	
Test item and concentration	**CYP2B6 mRNA**	**CYP3A4 mRNA**	**CYP2B6 mRNA**	**CYP3A4 mRNA**
Basal Ct[b]	24.73 ± 0.59	21.75 ± 0.65	25.88 ± 0.19	19.57 ± 0.42
Vehicle control (0.1% [v/v] DMSO)	1.00 ± 0.20	1.00 ± 0.20	1.00 ± 0.41	1.00 ± 0.37
Fluxapyroxad				
1 µmol/L	1.57 ± 0.03**	1.23 ± 0.11	1.27 ± 0.13	1.27 ± 0.27
3 µmol/L	3.39 ± 2.41	1.66 ± 0.86	0.87 ± 0.22	0.98 ± 0.27
10 µmol/L	3.49 ± 0.32***	3.70 ± 0.93**	2.00 ± 0.84	1.58 ± 0.09
30 µmol/L	4.41 ± 0.95**	3.26 ± 1.37*	3.33 ± 0.54**	1.75 ± 0.42
100 µmol/L	3.57 ± 0.12***	1.18 ± 0.09	1.20 ± 0.11	0.23 ± 0.06*
PB				
500 µmol/L	7.84 ± 0.27***	7.52 ± 1.31**	8.18 ± 3.74*	5.86 ± 0.23***

Ct: cycle threshold; CYP: cytochrome P450; DMSO: dimethyl sulfoxide; mRNA: messenger ribonucleic acid; PB: phenobarbital; v/v: volume per volume; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ (Student's *t*-test, two-sided)

[a] Values are mean ± standard deviation ($n = 3$ per group). Results are expressed as fold change relative to controls, where control values were normalized to 1.00. Rat β-actin was employed as the internal control.

[b] Calculated from individual Taqman® mRNA data.

Source: Elcombe (2016e)

For donor A, basal levels of CYP2B6 mRNA were about average, whereas CYP3A4 mRNA levels were in the upper third of the historical range (Table 16; the lower the Ct value, the higher the mRNA concentration). For donor B, the CYP3A4 basal levels in particular were at the uppermost end of the historical control range, whereas CYP2B6 mRNA basal levels tended towards the lower third of the historical control range. Induction of CYP2B6 and CYP3A4 by 500 µmol/L PB was average compared with the historical control range. Induction of CYP3A4 mRNA in donor B by 500 µmol/L PB was at the low end of the historical control range. It seems that the already high basal level of CYP3A4 mRNA was somehow difficult to increase further.

Table 16. Historical control data for basal Ct values and fold induction values for CYP2B6 and CYP3A4 in human hepatocytes after treatment with 500 µmol/L PB[a]

	CYP2B6		CYP3A4	
	Basal Ct	**500 µmol/L PB**	**Basal Ct**	**500 µmol/L PB**
Human (n = 10)	24.72 (23.0–26.9)	8.33 (7.67–9.62)[b]	23.24 (19.6–27.5)	7.85 (5.86–41.7)[b]

Ct: cycle threshold; CYP: cytochrome P450; PB: phenobarbital; *n*: number of studies performed between 2010 and 2016; the data were provided by CXR Biosciences Ltd on request

[a] Data are mean, with minimum–maximum in parentheses.

[b] Fold induction above control.

Source: Elcombe (2016e)

The 400- to 700-fold induction of CYP2B mRNA in wild-type hepatocytes of Sprague Dawley rats by 500 µmol/L PB (Elcombe, 2016c) was considerably higher than in human hepatocytes. However, it is noted that the maximum mRNA induction by fluxapyroxad was about half that of PB in rat hepatocytes as well as in human hepatocytes. Thus, with respect to CYP2B induction, human hepatocytes of both donors responded as expected.

The effects of fluxapyroxad, PB and EGF on replicative DNA synthesis in human hepatocytes are presented in Table 17.

Table 17. Effects of fluxapyroxad, PB and EGF on replicative DNA synthesis in human hepatocytes[a]

	S-phase labelling index (%)	
Test item and concentration	**Donor A**	**Donor B**
Vehicle control (0.1% [v/v] DMSO)	0.33 ± 0.09 (100.0 ± 28.0)	0.48 ± 0.07 (100.0 ± 15.5)
Fluxapyroxad		
1 µmol/L	0.34 ± 0.11 (102.1 ± 32.8)	0.47 ± 0.19 (97.9 ± 38.5)
3 µmol/L	0.38 ± 0.16 (114.1 ± 49.5)	0.50 ± 0.21 (102.8 ± 43.4)
10 µmol/L	0.33 ± 0.06 (99.6 ± 18.7)	0.55 ± 0.11 (113.6 ± 23.0)
30 µmol/L	0.30 ± 0.11 (90.0 ± 33.6)	0.48 ± 0.17 (98.8 ± 36.3)
100 µmol/L	0.07 ± 0.07***[b] (21.6 ± 19.7)	0.13 ± 0.09*** (26.6 ± 18.9)
PB		
500 µmol/L	0.41 ± 0.09 (122.1 ± 26.4)	0.64 ± 0.19 (132.6 ± 40.1)
EGF		
25 ng/mL	4.50 ± 0.89*** (1 352.2 ± 268.6)	3.47 ± 0.38*** (719.7 ± 77.9)

DMSO: dimethyl sulfoxide; DNA: deoxyribonucleic acid; EGF: epidermal growth factor; PB: phenobarbital; v/v: volume per volume; ***: $P < 0.001$ (Student's *t*-test, two-sided)

[a] Values are mean ± standard deviation (n = 5 per group). Values in parentheses are mean % control ± standard deviation.

[b] Cytotoxicity observed.

Source: Elcombe (2016e)

Fluxapyroxad did not increase the S-phase labelling index. Actually, at 100 µmol/L, a decreased labelling index was noted for both donors, which was considered to be due to cytotoxicity. At least for donor A, cytotoxicity was visually evident by fewer hepatocytes, resulting in poorer confluency. Furthermore, PB also did not increase replicative DNA synthesis, whereas EGF caused a 14- and 7.2-fold increase of the S-phase labelling index in hepatocytes from donors A and B, respectively, indicating that the human hepatocytes were able to proliferate.

The study indicates that fluxapyroxad and PB increase CAR/PXR-mediated CYP2B6 and CYP3A4 mRNA levels and enzyme activity in human hepatocytes. However, fluxapyroxad and PB did not induce proliferation of human hepatocytes, whereas human hepatocytes could be stimulated to proliferation by the positive control substance, EGF (Elcombe, 2016e).

Comments

Toxicological data

In vitro studies in CAR wild-type and knockout rat hepatocytes show that fluxapyroxad can activate the nuclear receptors CAR and PXR, leading to the induction of the P450 enzymes CYP2B and CYP3A and hepatocellular proliferation. Qualitatively, these effects of fluxapyroxad are similar to those of PB (Elcombe, 2016a,b,c,d).

An in vitro study using human hepatocytes showed that, similar to PB, fluxapyroxad can activate human CAR and PXR, but does not stimulate proliferation of these cells (Elcombe, 2016e).

Toxicological evaluation

The new in vitro studies support the conclusion of the 2012 Meeting that high doses of fluxapyroxad cause hepatocellular adenomas and carcinomas in rats through a mitogenic mode of action associated with induction of CYP2B-type P450, and that this mode of action does not occur in humans.

The Meeting concluded that the new studies support the existing acceptable daily intake (ADI) of 0–0.02 mg/kg body weight (bw) and have no impact on the acute reference dose (ARfD) of 0.3 mg/kg bw established in 2012.

References

Budinsky RA, LeCluyse EL, Ferguson SS, Rowlands JC, Simon T (2010). Human and rat primary hepatocyte CYP1A1 and 1A2 induction with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, 2,3,7,8-tetrachlorodibenzofuran, and 2,3,4,7,8-pentachlorodibenzofuran. Toxicol Sci. 118:224–35.

Elcombe B (2016a). BAS 700 F – enzyme and DNA-synthesis induction in cultured Sprague Dawley male wild-type (WT) and CAR knock-out (CARKO) rat hepatocytes. Unpublished report no. CXR1445. CXR Biosciences Ltd, Dundee, United Kingdom. Submitted to WHO by BASF Crop Protection, Limburgerhof, Germany.

Elcombe B (2016b). Assessment of rat cytochrome P450 2b and 3a inhibition by BAS 700 F (fluxapyroxad). Unpublished report no. CXR1507. CXR Biosciences Ltd, Dundee, United Kingdom. Submitted to WHO by BASF Crop Protection, Limburgerhof, Germany.

Elcombe B (2016c). BAS 700 F (fluxapyroxad) – enzyme and DNA-synthesis induction in cultured male Sprague Dawley wild-type (WT) and CAR knock-out (CAR KO) rat hepatocytes Unpublished report no. CXR1498. CXR Biosciences Ltd, Dundee, United Kingdom. Submitted to WHO by BASF Crop Protection, Limburgerhof, Germany.

Elcombe B (2016d). BAS 700 F (fluxapyroxad) – enzyme and DNA-synthesis induction in cultured male Wistar Han rat hepatocytes. Unpublished report no. CXR1497. CXR Biosciences Ltd, Dundee, United Kingdom. Submitted to WHO by BASF Crop Protection, Limburgerhof, Germany.

Elcombe B (2016e). BAS 700 F (fluxapyroxad) – enzyme and DNA-synthesis induction in cultured male human hepatocytes; assessment of two separate donors. Unpublished report no. CXR1495. CXR Biosciences Ltd, Dundee, United Kingdom. Submitted to WHO by BASF Crop Protection, Limburgerhof, Germany.

Mudra DR, Parkinson A (2004). In vitro CYP induction in human hepatocytes. In: Kang YJ, editor. Methods in pharmacology and toxicology. New York (NY): Humana Press; 203–14.

IMAZALIL

First draft prepared by
P.V. Shah[1] *and Angelo Moretto*[2]

[1] *Office of Pesticide Programs, Environmental Protection Agency, Washington, DC, United States of America (USA)*
[2] *Department of Biomedical and Clinical Sciences, University of Milan, and International Centre for Pesticides and Health Risk Prevention, ASST Fatebenefratelli Sacco, Milan, Italy*

Explanation 129
Evaluation for acceptable intake 130
1. Biochemical aspects 130
1.1 Absorption, distribution and excretion 130
1.2 Biotransformation 131
2. Toxicological studies 132
2.1 Acute toxicity 132
(a) Oral administration 135
(b) Dermal application 135
(c) Exposure by inhalation 136
(d) Dermal irritation 136
(e) Ocular irritation 137
(f) Dermal sensitization 137
(g) Single-dose toxicity study to derive an ARfD 138
2.2 Short-term studies of toxicity 139
(a) Oral administration 139
(b) Dermal application 144
2.3 Long-term studies of toxicity and carcinogenicity 144
2.4 Genotoxicity 156
2.5 Reproductive and developmental toxicity 158
(a) Multigeneration studies 158
(b) Developmental toxicity 159
2.6 Special studies 164
(a) Neurotoxicity 164
(b) Mechanistic studies on liver and thyroid toxicity 166
(c) Endocrine disruption 179
(d) Studies on metabolites 179
3. Observations in humans 183
3.1 Medical surveillance on manufacturing plant personnel 183
3.2 Case reports 183
3.3 Experimental studies 184
Comments 184
Toxicological evaluation 188
References 191
Appendix 1. IPCS framework for the preliminary analysis of the relevance of a cancer mode of action for humans: liver and thyroid tumours 199

Explanation

Imazalil (synonym: enilconazole, a pharmaceutical) is the International Organization for Standardization–approved common name for 1-[2-(2,4-dichlorophenyl)-2-(2-propenyloxy)ethyl]-1*H*-imidazole (International Union of Pure and Applied Chemistry), for which the Chemical Abstracts Service number is 33586-44-0.

Imazalil belongs to the group of imidazole fungicides used to control a wide range of fungi on fruits, vegetables and ornamentals. Its fungicidal mode of action is by inhibition of sterol biosynthesis.

Imazalil was previously evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 1977, 1980, 1985, 1986, 1991, 2000, 2001 and 2005. In 1991, JMPR established an acceptable daily intake (ADI) of 0–0.03 mg/kg body weight (bw), based on a no-observed-adverse-effect level (NOAEL) for clinical signs, decreased body weight gain and feed consumption, decreased serum concentrations of calcium, increased alkaline phosphatase (ALP) activity and increased liver weight in a 12-month toxicity study in dogs. In 2000, JMPR reaffirmed the ADI and concluded that an acute reference dose (ARfD) was unnecessary.

In 2005, the Meeting established an ARfD of 0.05 mg/kg bw, using a NOAEL of 5 mg/kg bw per day for maternal and fetal toxicity in a study of developmental toxicity in rabbits and a safety factor of 100.

Imazalil was reviewed by the present Meeting under the periodic review programme of the Codex Committee on Pesticide Residues. New studies included a single-dose toxicity study in rats, an acute neurotoxicity study in rats, a 28-day toxicity study in rats, a 90-day toxicity study in mice, mechanistic studies to evaluate the human relevance of liver and thyroid tumours, published studies on evaluation of endocrine effects and neurodevelopmental effects, and toxicity studies on metabolites.

The evaluation of the biochemical and toxicological aspects of imazalil was based on previous JMPR evaluations, updated as necessary with additional information. All critical studies contained statements of compliance with good laboratory practice (GLP) and were conducted in accordance with relevant national or international test guidelines, unless otherwise specified. A search of the open literature did not reveal any relevant publications that would have an impact on the evaluation.

Evaluation for acceptable intake

1. Biochemical aspects

Imazalil is rapidly absorbed, distributed, metabolized and excreted by rats.

1.1 Absorption, distribution and excretion

Wistar rats (five of each sex) were given ^{3}H-labelled imazalil sulfate in a single oral dose of 20 mg/kg bw. Almost 90% of the administered radioactivity was excreted within 96 hours, with approximately equal quantities detected in the urine and faeces. Tissue residues ranged from 5.4% to 6.1% of the administered radioactivity within 48 hours after dosing and from 1.8% to 3.5% within 96 hours after dosing. The highest levels of radioactivity were found in the liver, lung and kidneys. Analysis of the urine from dosed animals indicated that 4% of the tritium was volatile by lyophilization. Thus, tritium exchange apparently occurred to a minor extent (Heykants, Meuldermans & Hurkmans, 1975).

Five male rats were given ^{3}H-labelled imazalil sulfate in a single oral dose of 20 mg/kg bw to study the absorption, distribution, metabolism and excretion of imazalil. Approximately 90% of the administered radioactivity was excreted within 96 hours, with comparable quantities excreted in the urine and faeces. Similar results were found when a single oral dose of α-(2,4-diochlorophenyl)-1*H*-imidazole-1-ethanol (a metabolite of imazalil) was administered. In both these experiments, up to approximately 2% of the administered radioactivity was identified as tritiated water, indicating exchange of the tritium label. The relative amount of tritiated water found in the urine increased with time (Meuldermans et al., 1977b).

[^{14}C]Imazalil (purity 99.9%; Fig. 1) and unlabelled technical-grade imazalil (purity 98.63%) were administered to groups of five male and five female Wistar rats. A single dose of [^{14}C]imazalil at 1.25 mg/kg bw was administered intravenously to the first group. The second group received [^{14}C]imazalil at a single dose of 1.25 mg/kg bw by gavage. The third group received unlabelled imazalil at 1.25 mg/kg bw per day for 14 days by gavage followed by a single oral dose of

[^{14}C]imazalil at 1.25 mg/kg bw. The fourth group received [^{14}C]imazalil at a single dose of 20 mg/kg bw by gavage. The final specific activities of the labelled formulations were 1.1 MBq/mg at the low dose and 74 kBq/mg at the high dose. The animals were observed before dosing and at the end of each faecal collection. Urine was collected over the periods 0–4, 4–8, 8–24, 24–48, 48–72 and 72–96 hours. Faeces were collected 0–24, 24–48, 48–72 and 72–96 hours after dosing with radiolabelled imazalil. The animals were killed at 96 hours, and selected organs were removed.

Fig. 1. Structure of radiolabelled imazalil

Source: Mannens, Van Leemput & Heykants (1993)

After the single intravenous dose, 84% of the label was excreted by males and females within 24 hours and 89% within 96 hours. When imazalil was given at a single gavage dose of 1.25 mg/kg bw, 90% was excreted within 24 hours by males and 93% by females. After imazalil was given for 14 days at a dose of 1.25 mg/kg bw per day by gavage followed by a single oral dose of [^{14}C]imazalil, males excreted 94% within 24 hours and females excreted 84%; nearly all the label had been excreted in the urine and faeces of animals of both sexes by 96 hours. When imazalil was given at a single dose of 20 mg/kg bw by gavage, 90% and 89% were excreted within 24 hours by males and females, respectively; more than 95% was excreted in the faeces and urine by 96 hours. Somewhat more imazalil appeared in the urine than in the faeces with all dosing regimens, whereas there was no significant sex difference (Table 1).

At 96 hours after oral administration of [^{14}C]imazalil, tissue concentrations (including carcass) of radioactivity were about 1% of the administered dose. There was a dose-linear increase in the tissue levels after single oral low and high doses. There was no indication that accumulation occurred in any tissue after multiple dosing. Nearly 50% of the radiolabel retained in the body was found in the liver. Comparison of the excretion patterns after oral and intravenous dosing suggests that the bioavailability, and therefore the absorption, of imazalil given orally is high (Mannens, Van Leemput & Heykants, 1993).

1.2 Biotransformation

Studies involving administration of ^{3}H-labelled imazalil sulfate to rats in a single oral dose of 20 mg/kg bw indicate that extensive metabolism of imazalil occurs. In one study, α-(2,4-dichlorophenyl)-1*H*-imidazole-1-ethanol and 2,4-dichloromandolic acid were identified as the major metabolites in the urine. In this study, 10% of the radioactivity in the urine and 3% of the radioactivity in the faeces were identified as unchanged imazalil (Heykants, Meuldermans & Hurkmans, 1975).

The major metabolites identified in a more extensive metabolic study are shown in Fig. 2. The compound shown in brackets in Fig. 2 was not identified in the study. In contrast to the study described above, no unchanged imazalil was detected in the urine, and only 0.1% of the administered radioactivity was identified as unchanged imazalil in the faeces (Meuldermans al., 1977b).

Table 1. Distribution of radioactivity following administration of imazalil to rats

	% of administered radioactivity (standard deviation)							
	Single IV dose of 1.25 mg/kg bw		Single oral dose of 1.25 mg/kg bw		Repeated oral dose of 1.25 mg/kg bw for 14 days		Single oral dose of 20 mg/kg bw	
Sample	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
Urine								
0–24 h	44.88 (3.34)	53.52 (6.44)	46.55 (8.60)	58.52 (4.02)	49.20 (5.70)	52.63 (4.99)	51.40 (2.75)	54.54 (8.88)
0–96 h	46.31 (3.39)	55.17 (6.72)	48.66 (8.76)	59.96 (3.77)	50.91 (6.01)	55.10 (6.01)	53.14 (2.97)	56.86 (8.84)
Faeces								
0–24 h	39.04 (5.05)	30.48 (5.34)	43.41 (8.32)	34.22 (2.72)	44.67 (4.78)	31.50 (15.09)	38.56 (2.37)	34.06 (4.09)
0–96 h	41.74 (5.00)	32.41 (5.93)	46.07 (8.36)	36.11 (2.29)	47.55 (5.59)	41.67 (3.83)	42.77 (2.59)	37.71 (5.40)
Cage wash	0.55 (0.44)	0.52 (0.23)	0.85 (0.55)	0.61 (0.16)	0.37 (0.17)	1.03 (0.58)	0.74 (0.42)	1.25 (1.18)
Total	88.60 (2.20)	89.01 (1.14)	95.83 (1.00)	96.77 (1.72)	99.02 (0.39)	97.95 (0.47)	96.68 (0.51)	95.89 (3.26)
Tissues	–	–	1.05 (0.12)	0.85 (0.13)	1.01 (0.13)	0.89 (0.3)	1.20 (0.31)	1.03 (0.33)

bw: body weight; IV: intravenous
Source: Mannens, Van Leemput & Heykants (1993)

In a study described in section 1.1 above, little imazalil was excreted unchanged: less than 1% of the administered dose in the faeces, and trace amounts in the urine. The compound was metabolized to at least 25 metabolites. The metabolic profiles in the urine and faeces were largely comparable. Moreover, identical metabolites were recovered in the excreta from both sexes, and the intergroup similarities were remarkable. Three major metabolites were identified: (±)-1-[2-(2,4-dichlorophenyl)-2-(2,3-dihydroxypropyloxy)ethyl]-imidazolidine-2,5-dione (metabolite 8), (±)-1-[2-(2,4-dichlorophenyl)-2-(2,3-dihydroxypropyloxy)ethyl]-1*H*-imidazole (metabolite 10) and (±)-1-(2,4-dichlorophenyl)-2-imidazol-1-ylethanol (metabolite 11; see Fig. 2). The main routes of metabolism were epoxidation, epoxide hydration, oxidative *O*-dealkylation, imidazole oxidation and scission, and oxidative *N*-dealkylation. The metabolic pattern was similar after oral and intravenous administration and in animals of both sexes (Mannens, Van Leemput & Heykants, 1993).

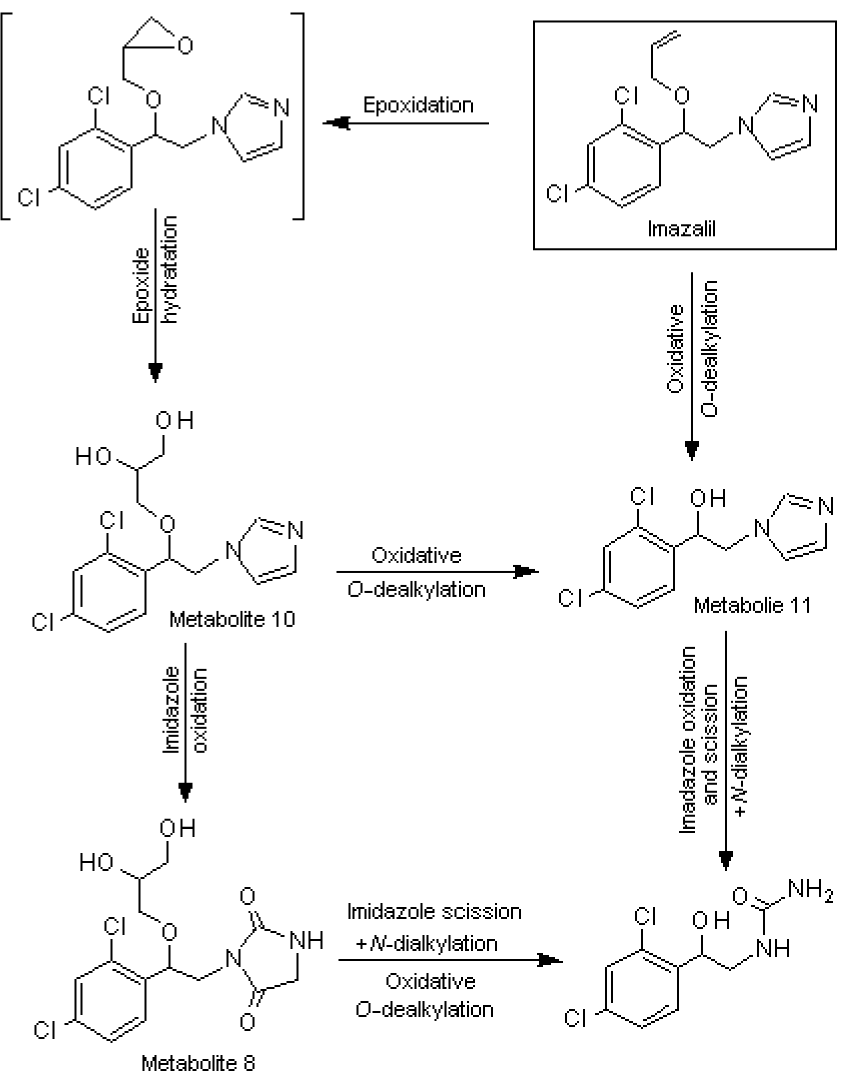
Tritium-labelled imazalil sulfate was incubated with rat liver homogenates to study the in vitro metabolism of imazalil. The two metabolites identified were α-(2,4-dichlorophenyl)-1*H*-imidazole-1-ethanol and 1-[2-(2,4-dichlorophenyl)-2-(2,3-dihydroxypropyloxy)ethyl]-1*H*-imidazole (Meuldermans et al., 1977a).

2. Toxicological studies

2.1 Acute toxicity

The results of studies on the acute toxicity of imazalil in rats and rabbits are summarized in Table 2.

Fig. 2. Metabolic pathways of imazalil in rats



Source: Mannens, Van Leemput & Heykants (1993)

Table 2. Acute toxicity of imazalil

Route	Species	Strain	Sex	LD_{50} (mg/kg bw)[a] (95% CI)	Purity (%)	Reference
Oral	Rat	Wistar	Male	343 (262–448)	>95 (nitrate)	Niemegeers (1979)
			Female	288 (221–377)		
			Male	343 (262–448)	100.4 (technical)	Niemegeers (1979); Goodwine (1990)
			Female	227 (174–297)		
			Male	355 (272–464)	102.7 (sulfate)	Niemegeers (1979)
			Female	309 (237–404)		
			Male	371 (284–485)	99.1 (acetate)	Niemegeers (1979)
			Female	309 (237–404)		

Route	Species	Strain	Sex	LD_{50} (mg/kg bw)[a] (95% CI)	Purity (%)	Reference
		Sprague Dawley	Male and female	664 (595–742)	NS (technical)	Dreher (1990a)
			Female	720 (212–2 160)	97.5 (technical)	Moore (2005a)
Dermal	Rat	Wistar	Male	4 200 (2 966–5 948)	NS	Marsboom & Van Ravestyn (1975)
			Female	4 880 (3 144–7 575)		
	Rat	Sprague Dawley	Male and female	>2 000	NS (technical)	Dreher (1990b)
	Rabbit	New Zealand white	Male and female	>2 000	97.6 (technical)	Teuns et al. (1990a)
Inhalation[b]	Rat	Wistar	Male and female	LC_{50} > 2 000 mg/m^3	Smoke from a candle containing 5.0 g imazalil	Appelman & Woutersen (1983)
Inhalation (dust, 27.3–39.6%; <4 µm, nose only)	Rat	Sprague Dawley	Male	LC_{50} = 2.88 (2.34–3.54) mg/L	NS (technical)	Blagden (1990)
			Female	LC_{50} = 1.84 (1.04–3.26) mg/L		
Primary skin irritation	Rabbit	New Zealand white	NS	Not irritating	98.9	Teuns & Marsboom (1987)
			Male and female	Mildly irritating	NS (technical)	Dreher (1990c)
			Male and female	Mildly irritating	97.46	Durando (2006)
Eye irritation	Rabbit	New Zealand white	Male and female	Severely irritating	97.6 (technical)	Teuns et al. (1990b)
			Male and female	Moderately irritating	NS (technical)	Dreher (1990d)
			Male and female	Moderately irritating	NS (technical)	Glaza (1996)
Skin sensitization	Guinea-pig	Dunkin-Hartley	Male	Not sensitizing (Magnusson & Kligman maximization)	NS (technical)	Dreher (1990e)
		Pirbright	Male	Slightly sensitizing (Magnusson & Kligman maximization)	98.5	Teuns et al. (1990c)
		Hartley albino	Male	Not sensitizing (Buehler method)	98.94	Wnorowski (1997)

bw: body weight; CI: confidence interval; LC_{50}: median lethal concentration; LD_{50}: median lethal dose; NS, not stated
[a] Except where specified otherwise.
[b] Deployed as smoke from a smoke generator.

(a) Oral administration

The oral toxicities of the nitrate, sulfate and acetate salts of imazalil, as well as imazalil base, were investigated. Each compound was orally administered by gavage at 160, 320 or 640 mg/kg bw as an aqueous suspension at a dosing volume of 1 mL/100 g bw to groups of 10 male and 10 female Wistar rats. The median lethal dose (LD_{50}) values for males ranged from 343 to 371 mg/kg bw, and for females from 227 to 309 mg/kg bw. There was no mortality for any of the compounds at 160 mg/kg bw (although there were signs of toxicity, which included ataxia, piloerection, hypotonia, hypothermia and ptosis). Mortality started at 320 mg/kg bw (for all compounds) (Niemegeers, 1979).

Sprague Dawley rats (five of each sex per dose) were dosed with imazalil technical, administered as a suspension in polyethylene glycol, at 400, 438, 480 or 679 mg/kg bw. The only mortalities that occurred were at 679 mg/kg bw. The oral LD_{50} was calculated to be 664 mg/kg bw for both males and females (Dreher, 1990a).

In an up-and-down procedure, seven female Sprague Dawley rats were orally dosed with imazalil technical (purity 97.46%) administered as a 50% weight per weight (w/w) mixture in corn oil. Doses were 72 (one rat), 227 (two rats), 720 (three rats) and 2000 (one rat) mg/kg bw. Mortality occurred in 2/3 rats dosed at 720 mg/kg bw as well as the one rat dosed at 2000 mg/kg bw. Signs of toxicity (hypoactivity, hunched posture, piloerection) occurred only at 720 and 2000 mg/kg bw. Decedents had red intestines. The oral LD_{50} was calculated to be 720 mg/kg bw, with a 95% profile likelihood–based confidence interval of 212.4– 2160 mg/kg bw (Moore, 2005a).

(b) Dermal application

Rats

In a dermal toxicity study with Wistar albino rats, groups of 10 males and 10 females received 24-hour occluded exposure to test material at either 2560 or 5120 mg/kg bw. The post-exposure observation period was 7 days. At 2560 mg/kg bw, one male and one female died; at 5120 mg/kg bw, seven males and five females died. The report gives no information as to signs of toxicity, dermal irritation or gross necropsy results. The LD_{50} values are reported as 4200 (2966–5948) mg/kg bw for males and 4880 (3144–7575) mg/kg bw for females (Marsboom & Van Ravestyn, 1975).

In a dermal toxicity study, five male and five female Sprague Dawley rats were semi-occlusively exposed for 24 hours to imazalil at a dose of 2000 mg/kg bw. The application site was moistened with distilled water before the test material was applied. There were no mortalities, no signs of toxicity and no dermal irritation. All rats gained weight on days 0–7 and 7–14. No abnormalities were detected at gross necropsy. The dermal LD_{50} was greater than 2000 mg/kg bw (Dreher, 1990b).

Rabbits

In a dermal toxicity study with New Zealand white rabbits, five males and five females received 24-hour occluded exposure to undiluted technical imazalil (purity 97.6%) at 2000 mg/kg bw. There was no mortality. There was slight sedation in six (three of each sex) rabbits on day 1, but this was gone the following day. There was slight dermal (grade 1 erythema and/or oedema) irritation, which was gone by day 4. All rabbits gained weight from day −1 to day 7 and again from day 7 to day 14. At necropsy, one female had slightly thickened skin at the application site, and another had a slightly swollen and pale liver with pronounced lobulation; the report stated that this has also been observed in controls and therefore is of no relevance. The dermal LD_{50} was greater than 2000 mg/kg bw (Teuns et al., 1990a).

(c) *Exposure by inhalation*

Rats

In an inhalation study involving whole body exposure to imazalil-containing smoke generated from a candle containing 5.0 g imazalil, five male and five female specific pathogen–free (SPF)–reared Wistar rats were exposed for 4 hours to imazalil at a nominal concentration of 2 g/m^3 (calculated by dividing 5.0 g by the 2.5 m^3 volume of the chamber). According to the report: "During the exposure the following levels of Imazalil were measured by the sponsor: 20.67 g/m^3, 6.30 g/m^3, 8.55 g/m^3 and 7.39 g/m^3. All these values were much higher than the nominal concentration. An explanation for this discrepancy was not found." From the particle size distribution, 99.7% by weight had an aerodynamic diameter less than 3.6 µm. There was no mortality. During the first 30 minutes of exposure, the rats could not be observed because of the smoke. During the remainder of the exposure period, the rats were somewhat restless and had their eyes half closed. During the first 4 days of the observation period, all rats had laboured respiration. They recovered completely during the second week. All rats lost weight from the day of exposure to day 2, but all had gains from their pre-exposure weight by day 7, and all had good weight gains from day 7 to day 14. Gross necropsy did not reveal any abnormalities that could be ascribed to treatment (Appelman & Woutersen, 1983).

In an inhalation study, groups of five male and five female Sprague Dawley rats received 4-hour nose-only exposure to imazalil technical at 1.97, 3.15 or 4.57 mg/L. The respective mass median aerodynamic diameters were 6.2, 5.1 and 6.2 µm, and the respirable fractions (% particles < 4 µm) were 33.9%, 39.6% and 27.3%. All rats exposed to 4.57 mg/L died during the 4-hour exposure period. Eight (three males and five females) of the 10 rats exposed to 3.15 mg/L were found dead on the day following exposure, whereas at 1.97 mg/L, two of the five females died, with one euthanized in extremis following exposure and the other found dead the next day. The LC_{50} values were 2.88 (2.34–3.54) mg/L for males, 1.84 (1.04–3.26) mg/L for females and 2.43 (1.99–2.96) mg/L for the two sexes combined (Blagden, 1990).

In an inhalation feasibility study with imazalil (purity 97.46%), it was stated that the test substance (described as a yellow to brown crystalline mass of solidified oil) was extremely difficult to aerosolize. Attempts to generate an atmosphere with standard dust generation equipment were unsuccessful. The test substance was ground in a ball mill for 24 hours. These attempts to grind the test substance resulted in the test substance adhering to the grinding media, rendering it useless. The test substance was then ground in a coffee mill, but again this resulted in an unsuccessful atmosphere generation. An effort was made to aerosolize the test material as a 33% w/w suspension in distilled water, but all attempts to generate the desired atmosphere with this suspension were unsuccessful. It was concluded that the overall results of the pre-test trials indicate that the physical properties of the test substance prevent the achievement of the required testing concentration and mass median aerodynamic diameter (Moore, 2005b).

(d) *Dermal irritation*

The potential of imazalil to cause primary irritation of the skin was studied in three New Zealand white rabbits that received a single application of technical-grade imazalil (purity 98.9%) to 6 cm^2 of skin for 4 hours at a total dose of 0.5 mL under a gauze patch. The animals were observed at 1 hour and then daily for 14 days, and irritation was scored according to the Draize method. Slight (grade 1) erythema was observed in one animal at 1 hour after treatment, but this animal scored zero at 24 hours and subsequently. Otherwise, all animals were free (all scores were zero) of dermal irritation for the entire 14-day observation period. Imazalil was considered to be non-irritating to the skin of rabbits (Teuns & Marsboom, 1987).

In a dermal irritation study, three New Zealand white rabbits were dermally exposed to 0.5 g imazalil technical moistened with 0.5 mL distilled water applied under a 2.5 cm × 2.5 cm gauze patch. After 4 hours, the patches and residual test material were removed, with irritation scoring at 1, 24, 48 and 72 hours. Erythema scores ranged from 0 to 2, and oedema scores ranged from 0 to 1. All scores

were zero at 72 hours. The primary dermal irritation score was 0.46, and the test material was designated as mildly irritating to the skin of rabbits (Dreher, 1990c).

The potential of imazalil to cause primary irritation of the skin was studied in three New Zealand white rabbits that were exposed to a single application of technical-grade imazalil (purity 97.46%) on 6 cm^2 of skin for 4 hours at a total dose of 0.5 g moistened with water. The animals were observed and irritation was scored (according to the Draize method) at 1, 24, 48 and 72 hours. At 1 and 24 hours after patch removal, all three treated sites exhibited well-defined (grade 2) erythema and very slight (grade 1) oedema. The overall incidence and severity of irritation decreased thereafter. All animals were free of dermal irritation by 72 hours. The primary dermal irritation index (mean score for 1, 24, 48 and 72 hours) was 1.8. Imazalil was mildly irritating to the skin of rabbits (Durando, 2006). This study was not previously evaluated by JMPR.

(e) Ocular irritation

In an eye irritation study with imazalil technical (purity not specified), 0.1 mL (approximately 96 mg) of test material was placed into the conjunctival sac of the right eye of each of three New Zealand white rabbits. All three eyes showed corneal opacity and iritis from 24 through 72 hours. All eyes had cleared (all scores zero) by day 7. These results indicate that imazalil is moderately irritating to the eyes of rabbits (Dreher, 1990d).

In an eye irritation study with imazalil (purity 97.6%), 0.1 g was instilled into the conjunctival sac of the left eye of each of three New Zealand white rabbits. All eyes showed corneal opacity from 1 hour through 14 days, and corneal opacity was still present in two eyes on day 21. Imazalil was severely irritating to the eyes of rabbits (Teuns et al., 1990b).

In an eye irritation study, 0.1 mL (65 mg) imazalil technical (purity not specified) was instilled in one eye of each of six rabbits. All six eyes showed corneal opacity from 1 to 72 hours after instillation, and one eye still showed corneal opacity on day 7, with clearing by day 14. These results indicate that imazalil is moderately irritating to the eyes of rabbits (Glaza, 1996).

(f) Dermal sensitization

In a dermal sensitization study, 20 female Dunkin-Hartley guinea-pigs were induced by intradermal injection of 1% imazalil technical (purity not stated) in arachis oil followed by epicutaneous induction with 10% w/w in arachis oil and epicutaneous challenge with 10% w/w in arachis oil. Groups of 10 controls given distilled water and vehicle were included. The animals were scored for sensitization 24 and 48 hours after challenge, according to the Magnusson and Kligman scale. No adverse reactions were noted at the test material sites of the test animals or the vehicle control sites of the control animals at 24 and 48 hours of observation. It was concluded that imazalil technical was non-sensitizing when tested according to the Magnusson and Kligman method (Dreher, 1990e).

In a dermal sensitization study, 20 male Pirbright guinea-pigs were induced by intradermal injection of 1% imazalil technical (purity 98.5%) in sesame seed oil followed by epicutaneous induction with 10% powder in petrolatum and epicutaneous challenge with 5% powder in petrolatum. Groups of 20 controls given the solvent and vehicle were included. The animals were scored for sensitization 48 and 72 hours after challenge according to the Magnusson and Kligman scale. The sensitization rate was 5% (one of 20 induced guinea-pigs showed a positive response at 48 hours following challenge; the same guinea-pig also had a positive response at 72 hours after challenge). It was concluded that imazalil technical had only a slight sensitizing potential when tested according to the Magnusson and Kligman method (Teuns et al., 1990c).

In a dermal sensitization study using the Buehler protocol, 0.4 g imazalil technical (purity 98.94%) was moistened with 95% ethanol and topically applied to the left side of each of 10 Hartley albino guinea-pigs. Exposure was for 6 hours, and this procedure was repeated on a once-a-week basis

for 3 consecutive weeks. Following a 2-week rest period, challenge doses of the test material moistened with acetone were applied to the right side of each of these 10 previously exposed guinea-pigs and an additional group of five previously unexposed guinea-pigs. None of the 15 guinea-pigs showed a positive response (Wnorowski, 1997).

(g) Single-dose toxicity study to derive an ARfD

In a newly submitted single-dose oral toxicity study conducted to facilitate the derivation of an ARfD, two parallel sets of Wistar rats (five of each sex per dose) were gavaged with a single dose of imazalil (purity 98.8%) at 0, 25, 100 or 400 mg/kg bw. The vehicle was polyethylene glycol 400. One set of animals (main group: control group and three treated dose groups) was terminated at 24 hours for examination of acute effects, whereas the parallel set (recovery group: control group and three treated dose groups) was subjected to termination at 7 days post-administration for examination of delayed effects or recovery. Mortality was assessed at least twice daily. Clinical signs were assessed on all animals at 1–2 and 4–5 hours after dosing and daily up to and including the day of necropsy. Necropsy was performed on day 2 for the main group animals and on day 8 for the recovery group animals. Body weights were recorded on days 1, 2, 4, 7 and 8. Feed consumption was recorded for days 1–2, 2–5 and 5–7. Neurobehavioural examinations were made on all recovery group males prior to treatment and at 4 hours, 24 hours and 7 days after treatment. Clinical laboratory investigations were made on day 2 for the main group animals and on day 8 for the recovery group animals. Haematology included assessment of white blood cells, differential leukocyte count, red blood cells, reticulocytes, red blood cell distribution width, haemoglobin, haematocrit, mean cell volume, mean cell haemoglobin, mean cell haemoglobin concentration and platelet count. Prothrombin time and activated partial thromboplastin time were determined. Clinical biochemistry included determination of liver enzymes (alanine aminotransferase [ALT], aspartate aminotransferase [AST], ALP), total protein, albumin, bilirubin, bile acids, urea, creatinine, glucose, cholesterol, triglycerides, phospholipids, electrolytes and inorganic phosphate. At necropsy, all animals were investigated for macroscopic abnormalities, and samples of all organs were subjected to histopathological examination. Organ weights were recorded for adrenal glands, ovaries, brain, prostate, epididymides, seminal vesicles, heart, spleen, kidneys, testes, liver, thymus, lungs and thyroid.

No preterminal mortalities were observed. No toxicologically relevant changes were noted in general clinical observations, body weights, feed consumption or macroscopic examination. Formulation analyses confirmed that formulations of test substance in polyethylene glycol 400 were prepared accurately and distributed homogeneously and were stable over at least 6 hours. At the high dose of 400 mg/kg bw, neurobehavioural observations revealed abnormal posture, piloerection, loss of righting reflex, hunched posture and slow breathing at 4 hours post-dosing. Body temperature was decreased by 1.1 °C compared with controls. At 100 mg/kg bw, one animal showed piloerection. No other signs were noted at any dose tested during later observations. Owing to the transient and incidental character of these findings, they were not considered to be toxicologically relevant. No toxicologically relevant effects on haematological parameters were observed that could be attributed to treatment. Decreased AST activity was noted at the end of the recovery period for both sexes at the high dose but was considered to be not relevant, as liver toxicity would be expected to have the opposite effect. At 400 mg/kg bw, clinical biochemistry changes consisting of lower glucose and urea levels and higher triglyceride and sodium levels in both sexes were seen 24 hours post-dosing. Cholesterol and potassium levels were decreased in males only. At the middle dose (100 mg/kg bw), changes at 24 hours post-dosing were confined to lower urea levels in females and decreased cholesterol and phospholipids in males. These alterations were not considered to be toxicologically relevant, as they were transient, not dose related or just naturally variable (as was apparent in the recovery groups). Statistically significant increases in absolute and relative liver weights were noted at the end of the entire study and were considered to be treatment related. Relative liver weight in high-dose males was increased by 10%. Absolute and relative liver weights in females were increased by 16% and 19% at 100 and 400 mg/kg bw, respectively. At the end of recovery, relative liver weight in high-dose females was still statistically significantly increased by 13%. Minimal or slight

hepatocellular vacuolation was found in most females at 100 and 400 mg/kg bw at both the 24-hour and 7-day terminations. Similar findings were made in high-dose males after 7 days. All other findings were considered to be within the normal background ranges encountered for Wistar-Han strain rats.

The NOAEL was 100 mg/kg bw, based on the increased incidence of slight hepatocellular vacuolation in males and females and increased liver weights in females at 400 mg/kg bw (Teunissen, 2013).

2.2 *Short-term studies of toxicity*

(a) Oral administration

Mice

In a 90-day toxicity study not previously reported by JMPR, imazalil (purity not specified) was administered in the diet to groups of 10 albino Swiss mice of each sex per dose at a concentration of 0, 200, 400 or 800 parts per million (ppm) (equivalent to 0, 30, 60 and 120 mg/kg bw per day, respectively). General health and behaviour were checked daily, body weight was measured weekly and feed intake was measured weekly. Blood for haematological and clinical chemical investigations was collected at the end of the study. At termination of the study, the animals were examined postmortem, organ weights were recorded and gross and micropathological examinations were carried out on livers from all animals after dissection.

There were no treatment-related effects on mortality or clinical signs of toxicity. At termination, decreased body weights (by 12% compared with controls) and body weight gains (30% of controls) were observed in female mice at 800 ppm. Feed consumption was decreased in females at 800 ppm. Statistically significant changes in haematological parameters were observed in high-dose females, but these changes were minor and considered to be toxicologically not relevant. The following clinical chemistry parameters were statistically significantly different compared with controls: decreased cholesterol in females at 400 ppm and in both sexes at 800 ppm; decreased albumin and phospholipids in males at 400 ppm and in both sexes at 800 ppm; decreased bilirubin in both sexes at 400 and 800 ppm; and decreased AST in females at 800 ppm. Increases in relative liver weights were observed in males at 400 ppm ($P < 0.05$) and in females at 800 ppm ($P < 0.001$), and increases in absolute and relative liver weights were observed in males at 800 ppm. Vacuolar degeneration (small and large vacuolation) of hepatocytes was observed in male mice at 200 ppm and in both sexes at 400 and 800 ppm.

No NOAEL could be identified in this study, as hepatocytic vacuolation was seen at all doses in males (Verstraeten et al., 1993a).

Rats

In a 4-week range-finding toxicity study not previously evaluated by JMPR, imazalil (purity 98%) was administered via the diet to Fischer 344 rats (10 of each sex per dose) at a concentration of 0, 100, 1000, 2000 or 3000 ppm (equal to 0, 11.5, 116.7, 232.1 and 351.3 mg/kg bw per day for males and 0, 11.7, 124.0, 229.0 and 351.6 mg/kg bw per day for females, respectively). Haematological and clinical chemistry parameters were not evaluated. Urine analysis was not performed.

No clinical signs of toxicity were observed. No mortality occurred during the study. Reduced body weights were observed in males (10–12%, 14–19% and 22–28% at 1000, 2000 and 3000 ppm, respectively) and in females (8–10% and 15–20% at 2000 and 3000 ppm, respectively). Feed consumption was also reduced in males at 1000, 2000 and 3000 ppm and in females at 2000 and 3000 ppm. The absolute weights of the heart, spleen, kidneys and testes were lower in males at 1000, 2000 and 3000 ppm. In females, the absolute weights of all organs except the liver were lower at 3000 ppm, and the absolute weights of the spleen, kidneys, adrenals and ovaries were lower at 2000 ppm. The

absolute weights of the liver in females were increased at 1000, 2000 and 3000 ppm. At necropsy, reduced testes size was observed in males at 2000 and 3000 ppm.

The NOAEL was 100 ppm (equal to 11.5 mg/kg bw per day), based on decreased body weight, feed consumption and heart, spleen, kidneys and testes weights in males and increased liver weights in females at 1000 ppm (equal to 116.7 mg/kg bw per day) (Gur, Nyska & Crown, 1990).

In a 13-week dietary toxicity study, imazalil (purity 98%) was administered to Fischer 344 rats (20 of each sex per dose) at a concentration of 0, 40, 200 or 1000 ppm (equal to 0, 4.02, 18.78 and 94.58 mg/kg bw per day for males and 0, 4.05, 20.28 and 99.34 mg/kg bw per day for females, respectively). General health and behaviour were checked daily, and body weight and feed consumption were measured weekly. Water consumption was measured in weeks 1, 2, 7 and 10. Ophthalmology was performed on 10 rats of each sex per dose prior to commencement of treatment and then at 12 weeks. Blood for clinical chemistry and haematological investigations was collected in week 11 from 10 rats of each sex per dose, as was urine for urine analysis. At termination of the study, the animals were examined postmortem, and organs were processed and examined histologically.

No treatment-related mortality occurred. No clinical signs related to treatment were observed. Rats of the high-dose group (1000 ppm) showed a statistically significantly lower body weight than the controls from weeks 2 to 7 in the males (8–12%) and from weeks 2 to 5 in the females (6–7%) and consumed less feed than the control rats during weeks 1 and 8 in the males and sporadically in the females. Water intake was depressed in both sexes of the high-dose group. Ophthalmic examination showed no differences between the high-dose and control groups. The high-dose females excreted more urine and slightly alkaline urine compared with controls. No treatment-related effects on haematological parameters were observed. Among the females, declines in the activities of ALT (at 1000 ppm) and AST (at 200 and 1000 ppm) were detected. Gamma-glutamyl transferase (GGT) activity was increased in females at the high and intermediate doses, whereas bilirubin concentration was increased at the high dose only. Potassium levels were decreased in all treated groups of females. Among high-dose males, there were increases in GGT activity and urea concentration (Table 3).

Liver weights in both males and females at 1000 ppm were statistically significantly higher than in the controls (absolute: approximately 12% and 15% in males and females, respectively; relative: approximately 16% and 17% in males and females, respectively). The kidney weights (absolute and relative to body weight) were higher in females at 1000 ppm (6–8%), but there were no macroscopic or microscopic findings in the kidneys. No treatment-related lesions were detected at macroscopic examination. Hepatic dose-related lesions associated with treatment with imazalil were detected microscopically in males at 200 and 1000 ppm and in females at 1000 ppm. These lesions consisted of slight centrilobular hepatocytic hypertrophy associated with an increase in cytoplasmic eosinophilia (most probably representing an adaptive proliferative response of the smooth endoplasmic reticulum), focal or midzonal fine and large fatty vacuolation and a decrease in the severity of the cytoplasmic anoxic vacuolation. The maximum tolerated dose was not achieved in this study.

The NOAEL was 40 ppm (equal to 4.02 mg/kg bw per day), on the basis of liver histopathology and associated clinical chemistry changes at 200 ppm (equal to 18.78 mg/kg bw per day) (Gur, Nyska & Waner, 1991).

In a 14-week, non-GLP-compliant toxicity study, groups of Wistar rats (10 of each sex per group) were fed imazalil nitrate (purity not specified) at a concentration of 0, 5, 20 or 80 mg/100 g feed (equal to 0, 4.2, 16.0 and 64.0 mg/kg bw per day for males and 0, 4.4, 17.4 and 69.4 mg/kg bw per day for females, respectively).

Table 3. Changes in selected clinical chemistry parameters in rats following 13-week exposure to imazalil in the diet[a]

Dietary concentration (ppm)	ALP (IU/L)	ALT (IU/L)	AST (IU/L)	GGT (IU/L)	CPK (IU/L)	Urea (mmol/L)	Bilirubin (µmol/L)	Potassium (mmol/L)
Males								
0	141	29	52	0.28	295	7.25	3.48	3.4
	(11)	(3)	(6)	(0.39)	(109)	(0.83)	(0.72)	(0.2)
40	140	29	45	0.23	247	7.06	4.12	3.5
	(8)	(2)	(4)	(0.34)	(68)	(0.43)	(0.88)	(0.2)
200	135	27	51	0.54	279	7.20	3.37	3.5
	(4)	(3)	(9)	(0.60)	(98)	(0.59)	(1.05)	(0.40
1 000	134	27	45	1.78***	367	8.18***	3.19	3.3
	(15)	(3)	(8)	(0.76)	(77)	(0.76)	(0.85)	(0.2)
Females								
0	98	33	64	1.64	404	7.98	2.34	3.8
	(5)	(11)	(10)	(1.11)	(108)	(0.49)	(0.32	(0.3)
40	94	31	63	1.42	335	7.68	2.22	3.5***
	(7)	(8)	(12)	(1.08)	(43)	(0.89)	(0.54)	(0.2)
200	92	28	47***	3.41**	223***	7.81	2.23	3.6
	(8)	(8)	(6)	(0.94)	(49)	(0.69)	(0.47)	(0.2)
1 000	93	22*	47***	5.11***	430	8.22	3.35***	3.4**
	(6)	(2)	(6)	(1.16)	(101)	(0.68)	(0.77)	(0.2)

ALP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; CPK: creatine phosphokinase; GGT: gamma-glutamyl transferase; IU: international units; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$

[a] Standard deviations provided in parentheses.

Source: Gur, Nyska & Waner (1991)

Appearance, behaviour, survival, feed consumption and urine analysis were not affected at any level. Body weights of the 80 mg/100 g feed group were slightly depressed (<5%). Haematological and blood chemistry values were within normal limits, except for an increase in bilirubin in both sexes in the 20 mg/100 g feed group ($P < 0.001$) and the 80 mg/100 g feed group ($P < 0.01$). The changes in bilirubin were minor and not considered to be toxicologically relevant. Decreases in specific gravity and creatine levels were observed in males at 80 mg/100 g feed. Organ weights were not affected by the treatment. Histological examination revealed no changes that could be attributed to imazalil in any organs or tissues except the livers of the 80 mg/100 g feed group, which showed a fatty reaction localized in the midzonal to centrilobular areas and vacuolation coincident with fatty spots in Scarlet red–stained sections.

The NOAEL was 20 mg/100 g feed (equal to 16.0 mg/kg bw per day), on the basis of liver toxicity manifested as a fatty reaction localized in the midzonal to centrilobular areas and vacuolation coincident with fatty spots in Scarlet red–stained sections at 80 mg/100 g feed (equal to 64.0 mg/kg bw per day) (Marsboom et al., 1972).

In a 90-day feeding study in rats, imazalil base was administered in the diet to groups of 10 male and 10 female Wistar rats at a concentration of 0, 800, 1600, 2400 or 3200 ppm. As a result of feed wastage, most likely resulting from poor palatability of the feed, it was not possible to accurately calculate the doses in units of milligrams per kilogram of body weight per day. Nevertheless, the

following doses were estimated by the study authors: 0, 64.4, 129, 181 and 252 mg/kg bw per day for males and 0, 78.7, 150, 236 and 333 mg/kg bw per day for females, respectively. The following parameters were evaluated: mortality, clinical signs, body weight, feed consumption, haematology, clinical chemistry, urine analysis, gross necropsy and organ weights. Livers and macroscopic lesions were examined for all animals in all treatment groups, but no other organs or tissues were histologically examined. Electron microscopy of liver sections was also conducted.

No treatment-related effects on mortality or clinical signs were observed. Compared with control values, dose-related decreased body weights and body weight gains were observed in treated animals of both sexes throughout the study at all doses. At 13 weeks for 3200 ppm animals, body weights were decreased by 19% in males and 11% in females, and body weight gains were decreased by 26% in males and 19% in females. In males, body weights were 9%, 11% and 19% lower than the control values at 800, 1600 and 2400 ppm, respectively. In females, decreased body weights were 4%, 8% and 10% lower than the control values at 800, 1600 and 2400 ppm, respectively. Decreases in feed consumption were noted at all doses, but the decrease in feed consumption was minor at 800 ppm in both sexes. Haematological parameters were not affected. The following minor changes in clinical chemistry parameters observed at 3 months were possibly related to the test material: decreased triglycerides in males at all treatment levels and in females at 3200 ppm; decreased phospholipids in males at all treatment levels; decreased blood urea nitrogen in males at 1600, 2400 and 3200 ppm; and decreased albumin in females at 2400 and 3200 ppm. Urine analysis was unremarkable. Gross necropsies at 3 months revealed increased incidences of dark-coloured livers and more pronounced lobulation in the liver, particularly in males at 2400 and 3200 ppm and in females at all doses. Relative liver weights were increased in males at all doses. Histopathological examination revealed treatment-related hepatocellular hypertrophy in nearly all treated animals at all doses. This hypertrophy was mild, and there was essentially no difference between treatment groups in incidence or severity. Mild fatty vacuolation was observed in males at doses of 1600 ppm and higher and in females at all doses.

No NOAEL could be identified. The lowest-observed-adverse-effect level (LOAEL) was 800 ppm (equal to 64.4 mg/kg bw per day), based on decreased body weights and body weight gains in males and females, decreased triglycerides and phospholipids in males, dark and more pronounced lobulation of livers in females, increased relative liver weights in males, mild hepatocellular hypertrophy in males and females, and mild fatty vacuolation in the livers of females (Van Deun et al., 1996a).

Imazalil base (purity 98.1%) was administered in the diet to groups of 10 Cpb:WU Wistar rats of each sex for 6 months, as part of a lifetime study summarized below (Lina et al., 1984; Til et al., 1985). Imazalil was admixed with the diet at a concentration of 0, 25, 100 or 400 ppm (equivalent to 0, 1.25, 5.0 and 20 mg/kg bw per day, respectively). General health and behaviour were checked daily, body weight was measured weekly for 12 weeks and then once every 2 weeks, and feed intake was measured weekly. Blood for haematological investigations was collected on day 174 from males and on day 175 from females. Urine was collected on day 178 for analysis. At termination of the study (on day 183 for males and on day 184 for females), the animals were examined postmortem, blood samples for clinical chemistry were taken, and organs were processed and examined histologically.

No treatment-related deaths or abnormal behaviours were noted. There were no treatment-related effects on body weight gain or feed intake. The leukocyte count was increased in males at 400 ppm in the absence of any statistically significant change in the differential leukocyte count; the effect is thus of dubious toxicological relevance. Lactate dehydrogenase activity was increased in females at the highest dose. Blood glucose concentration was increased in females at all doses, but without a dose–response relationship. No statistically significant differences between groups were seen in urinary parameters. Males at 400 ppm had increased relative kidney weights, and females had increased absolute and relative kidney and liver weights. Females at 400 ppm also showed increased

absolute thymus weight and relative lung weight. Gross and microscopic examination of the organs showed no effects that could be ascribed to treatment.

The NOAEL was 100 ppm (equivalent to 5.0 mg/kg bw per day), on the basis of changes in liver, kidney, lung and thymus weights in females and increased relative kidney weight in males at 400 ppm (equivalent to 20 mg/kg bw per day) (Lina et al., 1983).

Dogs

Groups of four male and four female beagles were given imazalil base (purity 98.8%) orally in gelatine capsules at a dose of 1.25, 2.5 or 20 mg/kg bw per day for 1 year, whereas four controls of each sex received the capsules alone. The animals were observed twice daily and were examined for clinical signs weekly. An ophthalmic examination was carried out prior to dosing, every 6 months and at the end of the study. An electrocardiogram and heart rate were recorded before the start of the study, at weeks 6, 14, 29 and 38, and at the end of the study. Body weights were recorded weekly. Haematological and clinical chemistry parameters were measured 2 weeks after the start of dosing and monthly thereafter. Urine was analysed 1 month after the start of dosing and then every 3 months. At the end of the study, all the animals were killed, selected organs were weighed, and gross and microscopic examinations were carried out. For statistical analysis of the histopathological findings, the two sexes were treated together.

All animals survived to termination. Clinical effects consisting of soft faeces, increased salivation, vomiting and decreased appetite were observed. No treatment-related ocular changes were observed at 20 mg/kg bw per day. No adverse effects of treatment on electrocardiogram values, heart rate, urinary parameters or haematological parameters were observed. Decreased weight gain and feed consumption were found only at the high dose. The only clinical chemical abnormalities observed were decreased serum calcium ($P < 0.05$) and substantially increased serum ALP activity ($P < 0.05$) at 20 mg/kg bw per day. Liver weights (absolute and relative to body weight) were increased only in the high-dose group. No treatment-related change was seen on macroscopic or microscopic examination; in particular, the histological appearance of the liver did not differ between treated and control groups.

The NOAEL was 2.5 mg/kg bw per day, on the basis of clinical signs, decreased body weight gain and feed consumption, decreased serum calcium concentration, increased serum ALP activity and increased liver weights at 20 mg/kg bw per day (Verstraeten et al., 1989).

Groups of three male and three female beagles were given imazalil base (purity 81.6–101.4%) orally in gelatine capsules at a dose of 1.25, 5 or 20 mg/kg bw per day for 24 months, and three controls of each sex received the capsules alone. The animals were observed daily. An ophthalmic examination was carried out prior to dosing and at 3, 6, 9, 12, 18 and 24 months after dosing. An electrocardiogram and heart rate were recorded before the start of the study and at weeks 4, 8, 12, 16, 20, 24, 36, 74 and 104. Body weights were recorded weekly. Haematological, clinical chemical and urinary parameters were measured prior to dosing and again after 2, 4, 8, 12, 16, 20, 24, 36, 48, 74 and 104 weeks of dosing. At the end of the study, all the animals were killed, selected organs were weighed, and gross and microscopic examinations were carried out.

There was no mortality in the study. At the high dose, decreased appetite in all animals and frequent and abundant salivation and sporadic emesis were observed. At 5 mg/kg bw per day, a slight decrease in appetite was seen during the first 2 months of the study. Feed consumption could not be measured accurately because of wastage. However, the feed consumption was definitely lower at the high dose. Controls and the 1.25 mg/kg bw per day dosed dogs showed a normal and comparable body weight gain during the study. At 5 mg/kg bw per day, weight gain was slightly lower than in controls ($P < 0.05$; 23.9% of control gain). The 20 mg/kg bw per day group body weight gain was also lower ($P < 0.01$; 11.6% of control gain) than in the controls at 24 months. No treatment-related ocular changes were observed. No adverse effects of treatment on electrocardiogram values or heart rate, urinary parameters, haematological parameters, gross pathology or organ weights were observed.

Examination of tissues revealed no compound- or dose-related effects except for slight ground glass aspect of the cytoplasm in the centrilobular areas of the liver in the dogs receiving 5 or 20 mg/kg bw per day. At 20 mg/kg bw per day, decreased calcium levels and increased ALP were observed; however, they were not considered toxicologically relevant because the changes were very minor and within the historical range of the laboratory.

The NOAEL was 1.25 mg/kg bw per day, based on decreased body weight gain and slight ground glass aspect of the cytoplasm in the centrilobular areas of the liver at 5 mg/kg bw per day (Marsboom et al., 1977).

(b) Dermal application

Rabbits

In a dermal toxicity study, groups of five male and five female New Zealand white rabbits received imazalil (purity 98.5%) in sesame oil on the shaved back under a porous gauze dressing at a dose of 0, 10, 40 or 160 mg/kg bw per day, 6 hours per day, 5 days per week, for 3 weeks. The animals were observed daily, body weights were determined initially and then weekly, and feed consumption was determined weekly. Haematological, clinical chemistry and urinary parameters were measured at the end of the study. Irritancy was scored according to the Draize method. The animals were killed at the end of the study; the organs were weighed and examined grossly and microscopically.

No adverse effects on behaviour were observed, and there was no irritation in the controls and little in the treated groups. Erythema was observed with both the vehicle (sesame oil) and the test compound; the severity was similar in all groups. Body weight gain and feed consumption were similar in all groups. Haematological and clinical chemistry parameters were not affected by the treatment. No gross or microscopic adverse effects on the skin, kidney, liver or lung were seen at any dose. Urinary changes (reduced creatinine concentration, reduced specific gravity and reduced urobilinogen concentration) were seen only in males at the highest dose.

The NOAEL was 40 mg/kg bw per day, based on urinary changes (reduced creatinine concentration, reduced specific gravity and reduced urobilinogen concentration) seen in males at 160 mg/kg bw per day (Teuns et al., 1991).

2.3 Long-term studies of toxicity and carcinogenicity

Mice

In a study of carcinogenicity, groups of 50 male and 50 female albino Swiss mice received imazalil sulfate (purity 98.3%) via the drinking-water at a concentration of 0, 6.25, 25 or 100 ppm (equivalent to 0, 2.5, 10 and 40 mg/kg bw per day, respectively) for 18 months. All animals were examined daily for signs of waning health, abnormal behaviour, unusual appearance, occurrence of untoward clinical effects, manifestations of toxic and pharmacological responses and survival. At termination, the surviving mice were examined grossly, and the lungs, liver, pancreas, kidneys, spleen, testis, epididymides, ovary, mammary gland, adrenals, hypophysis, lymph nodes and any other organ or tissue suspected to present a neoplasia were examined microscopically.

No dose-related effects on overall survival rate or on the time at which mortalities occurred were observed. There were no dose-related effects on health, behaviour or appearance. There were no effects on gross pathology and no discernible treatment-related increases in tumour incidence. However, owing to several shortcomings in study design (e.g. overall survival <50% for most groups; fighting and cannibalism occurring during the first 8 months of the study, especially in male groups, when animals were held in groups of 25), this study is not considered to be acceptable for the current evaluation (Marsboom & Herin, 1979a).

In a study of carcinogenicity, groups of 50 male and 50 female SPF albino Swiss mice received diets containing imazalil (purity 96.9%) at a concentration of 0, 50, 200 or 600 ppm (equal to 0, 8.1, 33.4 and 105 mg/kg bw per day for males and 0, 9.9, 41.6 and 131 mg/kg bw per day for females, respectively) for approximately 23 months. The mice were observed daily, and those in extremis were killed and examined grossly and postmortem. The animals were weighed at the start of the study, weekly for the first 12 months, and then monthly until immediately before termination. Feed consumption was recorded weekly for the first 12 months and then monthly. Haematological examinations were carried out at 12 months on samples from the tail vein and in those animals killed terminally by carotid exsanguination; additional haematological examinations were undertaken, when possible, on animals killed in extremis. When the leukocyte count was above 25 000/mm^3, a differential leukocyte analysis was carried out. Clinical chemistry was undertaken on carotid blood at termination. At termination, the surviving mice were examined grossly, and selected organs were weighed and examined grossly and microscopically.

Appearance and behaviour were not affected by treatment. At the end of the study, the survival rates among controls were 30% of males and 54% of females, and no significant differences were seen among treated groups. Decreased body weight and body weight gain were seen in males at 600 ppm from week 2 to the end of the study (5–10% decrease in body weight and 15–20% decrease in body weight gain) and in males at 200 ppm from time to time early in the study (statistical significance was not achieved). Females at all doses showed decreased body weight and body weight gain from time to time, but the effects were slight, infrequent and probably biologically non-relevant. Weekly or monthly feed consumption was increased in all groups of treated males at many time intervals, and total feed consumption (throughout the study) was increased among those at 50 and 600 ppm. Females in all treated groups showed occasional increases in weekly or monthly feed consumption. There was considerable feed wastage and no clear dose–response relationship; the relevance of the findings on feed intake are thus dubious.

At termination, males at the high dose had a significant decrease in body weight, which was accompanied by increases in absolute and relative liver weights (Table 4). An increase in relative brain weight at 600 ppm in males was probably a reflection of the decrease in body weight. Other changes in organ weights observed in males (increased relative pancreatic weight at 50 ppm) and females (decreased absolute and relative lung weights at the high dose, relative lung weight at 50 ppm and absolute and relative splenic weights at 200 ppm) did not appear to be related to treatment. Increases in relative heart weight at 200 ppm, decreases in relative kidney weight at 600 ppm and decreases in absolute and relative brain weights at 50 ppm, all in females, likewise appeared to be without biological relevance.

Table 4. Mean liver weight data for mice ingesting imazalil in the diet for up to 23 months

	Mean liver weight data[a]			
Organ	**0 ppm**	**50 ppm**	**200 ppm**	**600 ppm**
Males				
Absolute weight (mg)	2 388 ± 878	2 082 ± 611 (87)	2 402 ± 712 (101)	2 812 ± 1 044* (118)
Relative weight (mg/100 g)	5 685 ± 1 826	5 112 ± 1 512 (90)	5 798 ± 1 768 (102)	7 060 ± 2 521** (124)
Females				
Absolute weight (mg)	2 143 ± 627	2 264 ± 392 (106)	2 088 ± 458 (97)	2 359 ± 1 541 (110)
Relative weight (mg/100 g)	5 509 ± 1 246	5 581 ± 664 (101)	5 580 ± 1 162 (101)	6 269 ± 3 745 (114)

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

[a] Values in parentheses are percentage of control values.

Source: Verstraeten et al. (1993b)

Some differences in haematological parameters were found between groups at 12 months. In males, the erythrocyte volume fraction was increased at 200 ppm, as was the haemoglobin at 200 and 600 ppm. In females at 200 and 600 ppm, the erythrocyte volume fraction, haemoglobin concentration and erythrocyte count were increased, and the platelet count was decreased. By 23 months, no treatment-related differences in haematological end-points were found between groups. No difference in leukocyte count was seen at any time. No differences in clinical chemistry parameters were seen that could be ascribed to the test material. Statistically non-significant gross morphological changes, including foci and nodules, were seen in the livers of males at the intermediate and high doses. No treatment-related non-neoplastic changes were observed at 50 ppm in mice of either sex or in females at 200 ppm. The livers of males at 200 and 600 ppm and those of females at 600 ppm showed focal cellular changes consisting of large and small vacuoles and pigmented, swollen sinusoidal cells. Increased incidences of males with hepatic neoplasms were found at 200 and 600 ppm, with increased incidences of neoplastic hepatic nodules; a similar increase in the incidence of females with hepatic neoplasms was found at the high dose (Table 5) (Verstraeten et al., 1993b).

Table 5. Incidence of liver tumours recorded by study pathologist

	0 ppm	50 ppm	200 ppm	600 ppm	P[a]
Males					
No. examined	*50*	*49*	*50*	*50*	–
Hepatocytic neoplasms	13	10	25*	25*	0.000 2
Hepatic neoplastic nodules	8	5	23**	17*	0.000 6
Hepatocytic carcinoma	5	7	6	11	0.068 5
Females					
No. examined	*50*	*50*	*50*	*50*	–
Hepatocytic neoplasms	4	6	2	11*	0.023 0
Hepatic neoplastic nodules	4	6	0	10	0.047 5
Hepatocytic carcinoma	0	1	2	2	0.100 0

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (chi-squared test, one-tailed)
[a] P-value computed for positive trend (Peto analysis).
Source: Verstraeten et al. (1993b)

The slides from this study were subjected to three evaluations: by the study pathologist, by Dr Stephen Sparrow, an external and qualified pathologist (Sparrow, 1993), and by a working group. The results of the first evaluation were reported by Verstraeten et al. (1993b), those of all three evaluations by Hess (1996) and those of the last two by Butler (1995).

In the first evaluation, the incidence of hepatocytic carcinoma was not found to be increased, but the incidences of hepatic neoplastic nodules and total hepatic neoplasms were increased in males at 200 and 600 ppm, with a dose-related trend. In females, the incidence of hepatic neoplasms was increased at the high dose, with a trend for neoplastic nodules and all hepatic neoplasms. The main differences between the first and second readings of the slides were in nomenclature, in particular for hepatic neoplastic nodules. Sparrow (1993) considered that the study pathologist had used the term "hepatic neoplastic nodule" to describe changes that he would have described as adenoma and some other non-neoplastic lesions. Sparrow (1993) found evidence for a trend in the incidence of hepatocellular carcinoma in males. A report on the relevance of the liver tumours prepared by Hess (1996) included a tabulation of Sparrow's (1993) findings. In a further report on the relevance of the liver tumours by Butler (1995), based on the findings of the working group, it was concluded that

there was evidence of an increased incidence of adenomas, but not carcinomas, in males at the two highest doses and that there was no statistically significant trend for carcinomas (Table 6).

Table 6. Classification of liver neoplasms reported by Verstraeten et al. (1993b) according to the findings of a working group

	Males			Females		
Dietary concentration (ppm)	**No. of animals examined**	**Adenoma**	**Carcinoma**	**No. of animals examined**	**Adenoma**	**Carcinoma**
0	50	5	5	50	4	0
50	49	3	5	50	5	1
200	50	14*	3	50	0	2
600	50	14*[a]	8	50	9	1

ppm: parts per million; *: $P < 0.05$ (pairwise comparison, one-tailed chi-squared test)
[a] $P < 0.05$ for trend (Peto) test.
Source: Butler (1995)

The NOAEL for toxicity was 50 ppm (equal to 8.1 mg/kg bw per day), on the basis of morphological changes (foci and nodules) in the livers of males at 200 ppm (equal to 33.4 mg/kg bw per day). Liver adenomas were observed in males at 200 and 600 ppm and in females at 600 ppm. The NOAEL for carcinogenicity was 50 ppm (equal to 8.1 mg/kg bw per day), based on an increased incidence of adenomas seen in males at 200 ppm (equal to 33.4 mg/kg bw per day) (Verstraeten et al., 1993b).

Rats

Groups of Wistar rats (10 of each sex per dose per termination time) were fed a 50% wettable powder of imazalil (50% imazalil, 25% aerosol and 25% starch) at 0, 5, 20 or 80 mg/100 g feed (active ingredient) for 6, 12 and 24 months. The exact doses (reported for the 6-, 12- and 24-month groups) were 0, 3.0–3.8, 12.1–14.7 and 48.0–61.7 mg/kg bw per day for males and 0, 3.8–3.9, 14.7–17.0 and 57.6–63.9 mg/kg bw per day for females, respectively.

No effects of treatment on mortality, clinical signs, body weights, feed consumption, haematology, urine analysis, clinical chemistry or gross pathology were observed. At 6 months, the livers of many 80 mg/100 g feed rats, particularly females, showed centrilobular swelling and numerous large vacuoles. No evident fatty degeneration was seen, but a tendency to some fatty surcharge was noted. At 12 months, these changes were still present. In addition, livers of the high-dose females exhibited a tendency to more concentrated glycogen in the centrilobular areas. No other effects were noted that could be attributed to the compound administration. Except for liver and kidneys, no consistent changes in absolute or relative organ weights were noted in any group at any termination. At 6 months, the absolute and relative liver weights of the 80 mg/100 g feed females were statistically significantly increased. At 12 and 24 months, the absolute liver weights of females were not statistically significantly different.

In males receiving 20 and 80 mg/100 g feed, absolute and relative liver weights were increased statistically significantly at the 12-month termination, but not at the 24-month termination. At 6 and 24 months, absolute and relative kidney weights were statistically significantly increased in the 80 mg/100 g feed female group. This trend was present at 12 months, but was not of statistical significance. Males receiving this dose exhibited, at 6 months, a trend towards increased absolute kidney weights and a statistically significant increase in relative kidney weights. At 12 months, male kidney weights were statistically significantly higher than their control values, but at 24 months, this

difference was not evident. Haematological and blood chemistry revealed no consistent changes, except that at 6 months, bilirubin values for the female 20 and 80 mg/100 g feed groups were statistically significantly elevated. This trend continued throughout the study in the 80 mg/100 g feed females, but was not statistically significant at the 12- and 24-month terminations. Males did not exhibit this trend at any level during the study.

A NOAEL was not identified in this study, because the results described are based on selected organs and various study durations (Marsboom at al., 1975).

In an 18-month study of toxicity, groups of 20 male and 20 female Wistar Cbp:WU rats were given diets containing imazalil (purity 98.1%) at a concentration of 0, 25, 100 or 400 ppm (equivalent to 0, 1.2, 5 and 20 mg/kg bw per day, respectively). The animals were observed daily and more thoroughly every 2 weeks. Body weight was recorded at the start of the study, weekly for 12 weeks, and every 2 weeks for the remainder of the study. Feed consumption was measured weekly. Blood was collected from the tail vein for haematological examination on days 538–539. Urine samples from all animals were analysed on day 541. At necropsy, blood was collected from 10 animals per group for clinical chemistry. At the end of the study, the animals were killed and examined postmortem. Selected organs were removed, weighed and examined grossly and microscopically.

General health, behaviour and survival were not affected by treatment. Females at the high dose had decreased weight, particularly in the second half of the study, and these animals showed reduced feed intake at a few times. One minor change was seen in haematological variables – namely, an increased thrombocyte count in females at the high dose. The plasma albumin concentration was decreased in males at the high dose, but no other treatment-related changes in clinical chemistry parameters were seen. Urinary parameters were unaffected. The absolute weight of the adrenals was increased in females at the intermediate and high doses, and the relative weights of the adrenals, kidneys, heart and brain were increased in females at the high dose. The relative adrenal weights were also increased in the other treated groups of females. Treatment-related pathological effects were seen in the livers of males at the high dose, involving an increase in the lobular pattern and periportal cytoplasmic vacuolation of hepatocytes. Multivacuolar hepatocytes were seen more frequently in groups at the high dose than at other doses. Intracytoplasmic inclusion bodies were seen. There was no evidence of treatment-related neoplasia. Staining with periodic acid–Schiff and oil red O showed that the vacuoles contained neither starch nor fat.

The NOAEL was 100 ppm (equivalent to 5 mg/kg bw per day), on the basis of decreased weight gain in females, decreased plasma albumin concentration in males and pathological changes in the livers of males at 400 ppm (equivalent to 20 mg/kg bw per day) (Lina et al., 1984). Although there was no evidence that imazalil was carcinogenic, the duration of the study and the number of animals used were insufficient to exclude that possibility.

Groups of Wistar rats (50 of each sex per group) were administered imazalil (purity 101.2%) in the diet at a concentration of 0, 2.5, 10 or 40 mg/100 g feed (equivalent to 0, 2.5, 10 and 40 mg/kg bw per day) for 24 months. The animals were 3–4 months of age at the initiation of the carcinogenicity study. Animals were examined daily for behavioural changes and toxicity. At the conclusion of the study, gross and microscopic examinations were conducted on all surviving animals. During the course of the study, examinations were performed on animals that had died or were terminated in extremis. Microscopic examinations were conducted on the major tissues and organs and on all lesions that were noted during the course of the gross examinations.

There was substantial mortality over the course of the study, and very few animals survived the full 24 months. It was considered that this was due to the fact that the animals were 3–4 months old at the start of the study and that they were not maintained under SPF conditions. Evaluation of the data relating to the time on study at which animals died did not suggest that imazalil contributed to the increased mortality or the accelerated death rate. There were no dose-related events noted on gross pathology.

There were no significant differences with respect to the overall tumour rate or the individual types of tumours reported in any of the dose groups. There is no evidence that imazalil was responsible for tumour formation in any organ or tissue examined in the study.

A NOAEL was not identified in the study because of the high mortality rate and accelerated death rate, unlikely due to treatment with imazalil (Marsboom & Herin, 1979b).

In a 24-month study, groups of 50 SPF Wistar (Hannover) rats of each sex received diets containing a preparation called “imazalil 50 premix”, which nominally contained 50% imazalil (in fact, 49–50% imazalil, 25% aerosol and 25% corn starch) at a concentration of 0, 50, 200, 1200 or 2400 ppm (equal to 0, 2.4, 9.7, 58 and 120 mg/kg bw per day for males and 0, 3.4, 13.5, 79 and 157 mg/kg bw per day for females, respectively). The animals were observed at least daily. Ophthalmic examinations were carried out in controls and in animals at the highest dose before dosing and towards the end of the treatment. Body weight was measured on the first day of dosing, then weekly and at the end of dosing and additionally in any animal killed in extremis. Feed consumption was measured weekly. Haematological and clinical chemistry parameters were measured in blood samples from all groups at 6, 12 and 18 months and near the end of dosing. At the same time, urine was analysed. Immediately before the terminal kill at 24 months, each rat was examined. A full necropsy was undertaken on all animals, and macroscopic abnormalities were noted. Selected organs were removed and weighed, and samples of tissue were taken and processed for histopathological examination. Blood samples were taken at necropsy for measurement of testosterone, luteinizing hormone, thyroid stimulating hormone (TSH), thyroxine (T_4) and triiodothyronine (T_3) by enzyme immunoassay, the assays for TSH and luteinizing hormone being rat specific. Toxicokinetics was determined for the first four rats at each dietary concentration other than the controls in blood samples taken on days 180, 362 and 726 of the study. Imazalil was measured in these blood samples.

The mortality rate was not affected by the treatment. Increased feed wastage was observed for males at the highest dietary concentration and for females at the two highest concentrations. Females at the highest concentration showed a decrease in subcutaneous tissue mass and alopecia. No statistically significant difference was seen between animals at the highest concentration and controls in the ophthalmic examination. The body weights of males at 50 ppm were lower than those of controls at weeks 96, 99, 101 and 102, and their body weight gain was decreased during weeks 90–94 and weeks 96–104. An increase in body weight gain was seen in males at 200 ppm in week 1, but no other effect on body weight or body weight gain was seen. Decreased body weight and body weight gain were seen in males at 1200 ppm, consistently from week 1 to week 26 and less so thereafter. In males at 2400 ppm, body weight and body weight gain were decreased throughout the study (by 15–25%). The body weights of females at 50 ppm were lower than those of controls at weeks 82, 84, 85, 87, 88, 100 and 101, and body weight gain was decreased in weeks 54, 73, 78, 81–89 and 100–104. A decrease in body weight gain was seen in females at 200 ppm in week 34, with no other statistically significant change in body weight or body weight gain at any other time. Decreases in body weight were seen in females at 1200 ppm from the third week of the study and in body weight gain from the first week. In females at 2400 ppm, body weight and body weight gain were decreased throughout the study (by 25–35%) (Table 7). Feed consumption was decreased in weeks 23, 52, 93 and 96 for males at 50 ppm and in weeks 53, 90, 93 and 95 for males at 200 ppm. Decreased feed consumption was seen at 1200 ppm intermittently throughout the study and at 2400 almost throughout the study. Total feed intake of males throughout the study was decreased at the two highest dietary concentrations. Females at 50 ppm had increased feed consumption in week 27 and decreased feed consumption in weeks 52, 83 and 88. In females at 200 ppm, increased feed consumption was seen in weeks 10, 11 and 35–37, and decreased feed consumption in weeks 51, 83 and 93. The feed consumption of females at 1200 and 2400 ppm was decreased almost throughout the study. As for males, total feed intake was decreased at the two highest dietary concentrations, and feed conversion was increased by 10% and 12% at 1200 ppm and 2400 ppm, respectively.

Table 7. Mean body weight of rats in a 24-month study

	Mean body weight (% of vehicle control)									
	Males					Females				
Week	0 ppm	50 ppm	200 ppm	1 200 ppm	2 400 ppm	0 ppm	50 ppm	200 ppm	1 200 ppm	2 400 ppm
4	100	98.9	99.6	93.8***	85.1***	100	100	98.3	94.9***	90.4***
13	100	97.7	98.5	93.7***	85.9***	100	99.6	99.6	94.2***	90.7***
26	100	98.2	99.1	95.5**	87.5***	100	99.6	98.0	93.5***	88.2***
52	100	98.8	99.4	97.2	89.6***	100	97.1	96.4	89.9***	86.0***
78	100	99.0	99.2	97.3	88.0***	100	94.6	95.9	82.2***	77.1***
104	100	96.0	97.1	96.0*	85.3***	100	94.3	95.5	81.5***	75.2***

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ (Mann-Whitney *U*-test, two-tailed)
Source: Van Deun (1999a)

In males at the two highest dietary concentrations, decreased erythrocyte volume fraction, mean cell volume and mean cell haemoglobin were found consistently, with decreased haemoglobin concentration at weeks 78–79 and 104–105. Minor changes, including decreased mean cell volumes at weeks 25 and 52, were seen in males at 200 ppm. The haematological picture in females was more complex: increases in haemoglobin concentration at weeks 25–26 and 78–79 and increases in erythrocyte count at weeks 25–26, 52–53 and 78–79 were observed in animals at dietary concentrations of 200, 1200 and 2400 ppm, respectively. Additionally, an increase in erythrocyte count was seen at weeks 104–105 in rats at the highest dietary concentration. Decreased mean cell volume and mean cell haemoglobin concentration were seen at all times in females at 1200 and 2400 ppm and at weeks 25–26 and 52–53 in females at 200 ppm. Thus, imazalil at a dietary concentration of 50 ppm did not affect haematological parameters, minor changes were seen at 200 ppm, and definite changes were seen at 1200 and 2400 ppm.

A number of differences between groups were observed in clinical chemistry parameters, many of which were transient or inconsistent. The more consistent changes included decreased activity of ALP and AST in animals of both sexes at all dietary concentrations. Of greater clinical relevance were decreases in calcium and total protein concentrations observed at most times in animals at 1200 and 2400 ppm. Increased glucose concentrations were observed at all times in males and females at the highest dietary concentration; at weeks 52–53 and 104–105 in males and at weeks 52–53, 78–79 and 104–105 in females at 1200 ppm; and at weeks 25–26, 52–53 and 78–79 and 104–105 in females at 200 ppm. Triglyceride and phospholipid concentrations were decreased in males at 2400 ppm and in females at the two highest dietary concentrations at all times. The blood urea nitrogen concentration was decreased in males at the two highest dietary concentrations at weeks 25–26, 52–53 and 78–79 and 104–105 and in females at concentrations above 200 ppm. Decreases in ALP, AST and ALT activities and blood urea nitrogen concentration are generally considered not to be adverse. However, the changes in calcium and total protein, which were observed at most times in animals at 1200 and 2400 ppm, may be clinically relevant. Furthermore, the increases in blood glucose concentration seen at 2400 ppm in both sexes, at 200 ppm in females at all times, and from time to time in both sexes at 1200 ppm are likely to be clinically relevant.

The urine of females showed decreased specific gravity at some times, with increased pH and increased urinary volume at the two highest dietary concentrations.

The testosterone and luteinizing hormone concentrations were little altered in male rats, a significant increase in testosterone being observed only at 1200 ppm. The TSH concentrations tended to be higher at 200 and 1200 ppm, the T_4 concentrations were definitely lower in males at 1200 and 2400 ppm, and the T_3 concentrations were similar in all groups. In females, the T_3 concentration was decreased at the two highest dietary concentrations, T_4 was decreased at 1200 ppm only, whereas TSH

was not consistently affected. Despite these inconsistent results, it seems likely that imazalil affected the pituitary–thyroid axis when given at a dietary concentration of 1200 or 2400 ppm.

The organ weights of animals killed at term after receiving the highest dietary concentration showed some differences from controls, but these were considered to be a reflection of changes in total body weight, with the following exceptions. In males, relative liver weight was increased at the two highest dietary concentrations, with no effect on absolute liver weight. In females, absolute liver weight was decreased at 2400 ppm, and relative liver weight was increased at 200 ppm and above. At termination, in males, absolute thyroid weight was increased at 1200 and 2400 ppm, and relative thyroid weight was increased at all dietary concentrations, whereas in females, absolute thyroid weight was decreased at 1200 and 2400 ppm, and relative thyroid weight was unaffected (Table 8). The sponsor argued that it was appropriate to include decedents in the analysis of organ weights, as the effects on the thyroid were unlikely to be fatal (Tables 9 and 10).

Table 8. Thyroid weights of rats killed at termination after receiving diets containing imazalil for 2 years

Thyroid weight	0 ppm	50 ppm	200 ppm	1 200 ppm	2 400 ppm
Males					
Absolute (mg)	44	51	101	76*	67*
Relative (mg/kg bw)	84	103*	202*	154*	145***
Terminal body weight (g)	*522*	*499*	*509*	*503*	*449****
Females					
Absolute (mg)	42	35	46	31**	26***
Relative (mg/kg bw)	128	112	153	112	104
Terminal body weight (g)	*328*	*310*	*315*	*273****	*251****

bw: body weight; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ (Mann-Whitney *U*-test, two-tailed)
Source: Van Deun (1999a)

Table 9. Thyroid weights of rats killed before the end of the study after receiving diets containing imazalil for 2 years

Thyroid weight	0 ppm	50 ppm	200 ppm	1 200 ppm	2 400 ppm
Males					
Number examined	*8*	*12*	*9*	*11*	*5*
Absolute (mg)	42	34	38	53	39
Relative (mg/kg bw)	107	85	92	130	112
Females					
Number examined	*18*	*14*	*15*	*10*	*13*
Absolute (mg)	32	28	34	70	26
Relative (mg/kg bw)	125	109	138	322	128

bw: body weight; ppm: parts per million
Source: Van Deun (1999a)

The study author concluded that the increased relative liver weight observed at 200 ppm in females, unaccompanied as it was by a decrease in absolute body weight, was an incidental finding.

Further, the increase in relative thyroid weight seen at 50 and 200 ppm in males, in the absence of absolute thyroid weight changes, is not likely to be biologically relevant.

Table 10. Thyroid weights of rats killed before and at the end of the study after receiving diets containing imazalil for 2 years

Thyroid weight	**0 ppm**	**50 ppm**	**200 ppm**	**1 200 ppm**	**2 400 ppm**
Males					
Number examined	*40*	*35*	*38*	*34*	*41*
Absolute (mg)	44	47	89	71	64*
Relative (mg/kg bw)	88	98	181*	148*	142***
Females					
Number examined	*30*	*31*	*35*	*40*	*34*
Absolute (mg)	38	33	43	38	26***
Relative (mg/kg bw)	127	111	148	154	111

bw: body weight; ppm: parts per million; *: $P < 0.05$; ***: $P < 0.001$ (Mann-Whitney *U*-test, two-tailed)
Source: Van Deun (1999a)

The pathological changes seen grossly at termination included an increased frequency of liver foci and swollen thyroid glands in males at 1200 and 2400 ppm and decreased adiposity in males at 2400 ppm. The changes seen in females included decreased adiposity and decreased inspissated secretions in the mammary glands at 1200 and 2400 ppm.

An increased prevalence of hepatocellular adenoma was seen in males at the highest dietary concentration, in those killed at termination of the study and when all animals were included in the analysis (Table 11).

Table 11. Incidence of liver adenoma and carcinoma in rats after receiving diets containing imazalil for 2 years

	Males					**Females**				
	0 ppm	**50 ppm**	**200 ppm**	**1 200 ppm**	**2 400 ppm**	**0 ppm**	**50 ppm**	**200 ppm**	**1 200 ppm**	**2 400 ppm**
No. of livers examined	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*
Hepatocellular adenoma	4	2	3	4	13*	2	1	2	1	2
Hepatocellular carcinoma	1	0	0	0	1	0	0	0	0	0

ppm: parts per million; *: $P < 0.05$
Source: Van Deun (1999a)

Males at 1200 and 2400 ppm also had an increased incidence of follicular cell neoplasia of the thyroid. In males at 2400 ppm that were killed at termination, the incidence of follicular cell adenoma was also increased (Table 12). Addition of animals that died before the end of the study had little effect on the total incidence of the tumours (Table 13). As these tumours are likely to be incidental findings and not the cause of death, the most appropriate tabulation is probably that in Table 14, showing a clear increase in follicular cell neoplasia in males at 1200 and 2400 ppm and in

females at 2400 ppm. The incidences of thyroid follicular cell adenomas and carcinomas in males at 1200 and 2400 ppm exceeded the historical control values of the laboratory (Table 15). The decreased incidences of pituitary and mammary gland hyperplasia were probably related to the reduced body weights of animals at the highest concentration in the diet. No differences in neoplastic findings were found between groups.

Table 12. Incidences of thyroid follicular cell neoplasia in animals killed at termination of the study after receiving diets containing imazalil for 2 years

	Incidence of thyroid follicular cell neoplasia				
	0 ppm	**50 ppm**	**200 ppm**	**1 200 ppm**	**2 400 ppm**
Males					
Total	3/40	7/35	6/38	9*/34	12*/41
Adenoma	3/40	7/35	5/38	7/34	10*/41
Carcinoma	0/40	0/35	2/38	2/34	2/41
Females					
Total	3/30	3/31	4/35	3/40	1/34
Adenoma	3/30	3/31	3/35	3/40	1/34
Carcinoma	0/30	0/31	1/35	0/40	0/34

ppm: parts per million; *: $P < 0.05$
Source: Van Deun (1999a)

Table 13. Incidences of thyroid follicular cell neoplasia in animals killed or dying before termination of the study after receiving diets containing imazalil for 2 years

	Incidence of thyroid follicular cell neoplasia				
	0 ppm	**50 ppm**	**200 ppm**	**1 200 ppm**	**2 400 ppm**
Males					
Total	1/10	1/15	0/12	2/16	0/9
Adenoma	1/10	1/15	0/12	2/16	0/9
Carcinoma	0/10	0/15	0/12	0/16	0/9
Females					
Total	4/20	2/19	0/15	1/10	0/16
Adenoma	4/20	2/19	0/15	0/10	0/16
Carcinoma	0/20	0/19	0/15	1/10	0/16

ppm: parts per million
Source: Van Deun (1999a)

Table 14. Numbers of thyroid follicular cell tumours in all animals killed or dying after receiving diets containing imazalil for 2 years

	Number of thyroid follicular cell tumours				
	0 ppm	**50 ppm**	**200 ppm**	**1 200 ppm**	**2 400 ppm**
Males					
Total	4	8	6	11*	12*

	Number of thyroid follicular cell tumours				
	0 ppm	50 ppm	200 ppm	1 200 ppm	2 400 ppm
Adenoma	4	8	5	9	10
Carcinoma	0	0	2	2	2
Females					
Total	7	5	4	4	1*
Adenoma	7	5	3	3	1
Carcinoma	0	0	1	1	0

ppm: parts per million; *: $P < 0.05$
Source: Van Deun (1999a)

Table 15. Historical control data on liver and thyroid tumours for the 24-month rat carcinogenicity study

End-point	Group 1	Group 2
Males		
Hepatocellular adenoma	3/50 (6%)	4/50 (8%)
Hepatocellular carcinoma	0/50 (0%)	2/50 (4%)
Thyroid follicular cell adenoma	3/50 (6%)	5/50 (10%)
Thyroid follicular cell carcinoma	0/50 (0%)	1/50 (2%)
Females		
Hepatocellular adenoma	1/50 (2%)	3/50 (6%)
Hepatocellular carcinoma	1/50 (2%)	2/50 (4%)
Thyroid follicular cell adenoma	0/50 (0%)	3/50 (6%)
Thyroid follicular cell carcinoma	0/50 (0%)	0/50 (0%)

Source: Van Deun (1999b)

The non-neoplastic findings included decreased incidences of haemorrhagic degeneration of the adrenals in female rats at the highest dietary concentration, in all animals and in the survivors to termination when the results for these animals were analysed separately. The study pathologist suggested that this lesion is commonly seen in female rats of this age. The incidence of transitional cell hyperplasia was increased in all treated males, but with no dose–response relationship; furthermore, this finding was significant only in rats at 50, 200 and 1200 ppm when the decedents were included in the analysis. Females at the highest dietary concentration that survived to termination and survivors and decedents taken together had an increased frequency of mineralization of the transitional epithelium of the renal pelvis. In the livers of animals that survived to termination and of all animals, an increased frequency of centriacinar hepatocyte hypertrophy was seen at the two highest concentrations. An increase in the frequency of periacinar hepatocyte hypertrophy was seen in females at the two highest dietary concentrations when the results for all animals were analysed together and at 1200 ppm in survivors only. Changes in the portal tract were discerned in males at the highest concentration, only when all animals were included in the analysis. Clear cell foci were observed in females at the highest concentration that survived to termination. Decreased frequencies of basophilic foci and foci of hepatocellular alteration were observed in the livers of surviving females at 200 and 2400 ppm and in survivors and decedents together at 2400 ppm. Eosinophilic foci and foci of hepatocellular alteration were seen in surviving males at the two highest concentrations and in all animals at the highest dietary concentration when the results for all animals were analysed together. Fatty vacuolation was found in the livers of surviving males and in survivors and decedents taken

together at the two highest concentrations and in female survivors at 1200 ppm at termination. Focal cystic degeneration of the liver was seen in males at 2400 ppm (both survivors separately and all animals), whereas pigment-laden hepatocytes were seen in females at the three highest dietary concentrations, whether or not the decedents were included in the analysis. In the thyroid gland, cystic focal follicular hyperplasia was seen in male survivors at the two highest concentrations and in those at the highest concentration only when all animals were included in the analysis. Few differences were found between groups with respect to pathological changes in decedents, when the findings were analysed separately; none appeared to be clearly treatment related, with the possible exception of an increased incidence of transitional cell hyperplasia in the kidney in males and periacinar hepatocyte hypertrophy and pigment-laden hepatocytes in females at the highest dietary concentration.

It might be argued that no NOAEL can be identified in this study, as effects on body weight, body weight gain and feed consumption were seen at some times in males and females at 50 ppm. However, the findings at 200 ppm suggest that the first two effects were not dose related. In females, increased feed consumption was observed at some times at 50 ppm, whereas in males, the situation was less clear, the decreases observed were small and the decreases occurred at only a few times.

The NOAEL for toxicity was therefore 50 ppm (equal to 2.4 mg/kg bw per day), on the basis of minor haematological changes in animals of both sexes and increased blood glucose concentration and liver changes (increases in relative liver weight and an increased incidence of pigmented hepatocytes) in females at 200 ppm (equal to 9.7 mg/kg bw per day). The NOAEL for carcinogenicity was 200 ppm (equal to 9.7 mg/kg bw per day), based on an increased incidence of follicular cell neoplasia of the thyroid in males at 1200 ppm (equal to 58 mg/kg bw per day) (Van Deun, 1999a).

In a 30-month study, four groups of Wistar Cbp:WU rats (50 of each sex per group) were administered imazalil base (purity 98.1%) in the diet at a concentration of 0, 25, 100 or 400 ppm (equal to 0, 1.0, 3.6 and 15.0 mg/kg bw per day for males and 0, 1.2, 4.7 and 19.7 mg/kg bw per day for females, respectively). Animals were 4–5 weeks old at the start of the study and were housed five of each sex per cage. Animals were observed daily for general condition and behaviour and examined periodically for visible/palpable tumours or signs of illness and weekly for body weight and feed consumption (per cage measurements). Ophthalmoscopic examinations were performed on control and high-dose animals initially and at weeks 52 and 104. Plasma albumin measurements were determined in 10 male and 10 female rats in each of the control and high-dose groups from blood collected, after overnight fasting, from the aorta. Macroscopic examinations were performed on all animals. Organ weights were determined on all surviving animals and included adrenals, brain, heart, kidneys, liver, lungs, ovaries, pancreas, pituitary, spleen, testes, thymus and thyroid. Histopathological examinations were performed on all control and high-dose animals for the presence of hyperplastic, preneoplastic and neoplastic changes. The same evaluations were conducted on all low- and mid-dose animals, but included only the liver, spleen, pituitary, thyroid, adrenals, brain and testes. Similarly, microscopic examinations for non-neoplastic changes in the liver and kidney were performed in control and high-dose animals only.

General cage-side observations were unremarkable, and there were no compound-related effects on mortality, with 50% mortality occurring approximately 812 days into the study for both sexes. Body weight of high-dose males was reduced for the first 13 weeks and remained lower than in controls throughout the study, but this did not adversely affect their lifespan. There were no effects on body weight in females. Mean weekly feed consumption and plasma albumin were unaffected by treatment. Absolute and relative organ weights were variable in males among all groups, with only decreased absolute brain weight in 100 and 400 ppm males being statistically significant. There were no statistically significant differences between controls and treated females regarding organ weight changes. Ophthalmoscopic examinations were unremarkable between treated and control groups, except for a marginally increased incidence of cataracts among high-dose males (i.e. 37 controls, 43 high dose).

Macroscopic examinations were limited and unremarkable. There were also no compound-related effects detected upon microscopic examination. Although imazalil was not oncogenic in this

study, there were several tumours reported among all groups, including controls, which are indicative of geriatric changes (e.g. benign pituitary tumours, fibroadenomas of the mammary gland, benign fibromatous polyp in the uterus, as well as phaeochromocytoma of the adrenal, the latter predominantly in males) (Table 16).

Table 16. Selected neoplasms in rats fed imazalil for 30 months[a]

	Males				**Females**			
Tumours	**0 ppm**	**25 ppm**	**100 ppm**	**400 ppm**	**0 ppm**	**25 ppm**	**100 ppm**	**400 ppm**
Pituitary	(47)	(49)	(48)	(47)	(48)	(49)	(50)	(46)
Solid tumour	2	0	0	1	0	0	2	1
Spongiocytic tumour	1	0	0	0	0	0	1	1
Haemorrhagic tumour	6	2	10	4	7	10	7	4
Pleomorphic haemorrhagic tumour	4	7	5	2	4	4	6	4
Para intermedia tumour	0	0	0	0	0	0	1	0
Uterus	–	–	–	–	(50)	(23)	(24)	(50)
Multiple polyps	–	–	–	–	13	12	12	18
Adenocarcinoma	–	–	–	–	1	4	5	3
Epidermoid carcinoma	–	–	–	–	0	1	0	1
Mammary glands	(46)	(1)	(2)	(45)	(49)	(27)	(23)	(49)
(Multiple) fibroma	–	–	–	–	2	0	1	0
Adenoma	–	–	–	–	0	1	0	1
(Multiple) fibroadenoma	1	0	1	0	22	16	16	13
Papillary carcinoma	–	–	–	–	2	2	0	1
Carcinoma	0	0	0	0	0	0	0	1
Adrenals	(30)	(49)	(48)	(50)	(50)	(48)	(50)	(49)
Phaeochromocytoma	15	7	8	9	0	2	0	3

[a] Figures in parentheses represent the number of animals from which this tissue was examined microscopically. In this table, a benign tumour is ignored if a malignant tumour of the same histogenetic origin is also present in the same tissue. Absence of a numeral (marked with a dash) indicates that the lesion specified was not identified or that the organ is not relevant to the sex of the animal examined.

Source: Til et al. (1985)

The NOAEL for toxicity was 100 ppm (equal to 3.6 mg/kg bw per day), on the basis of decreased weight gain in males at 400 ppm (equal to 15.0 mg/kg bw per day). Imazalil was not carcinogenic at doses up to and including 400 ppm (equal to 15.0 mg/kg bw per day) (Til et al., 1985).

2.4 Genotoxicity

The results of studies of genotoxicity of imazalil are summarized in Table 17.

Table 17. Results of assays for the genotoxicity of imazalil

End-point	Test object	Concentration/dose	Purity (%)	Results	Reference
In vitro					
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537, TA1538	0.5–30 µg/plate in DMSO (±S9)	Not reported	Negative	Steelman & Schreiner (1977)
Reverse mutation	*S. typhimurium* TA97, TA98, TA1535, TA1538	5–500 µg/plate in DMSO (±S9)	98.7	Negative	Vanparys & Marsboom (1988a)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537; *Escherichia coli* WP2*uvr*A	9.8–313 µg/plate in DMSO (±S9)	>98	Negative	Watabe (1992)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	31.25–500 µg/plate in DMSO (±S9)	96.4	Negative	Jenkinson (1990a)
Chromosomal aberration	Human lymphocytes	9–145 µg/mL in DMSO (±S9)	99.7	Negative	Vanparys et al. (1990)
Unscheduled DNA synthesis	Male Wistar (WU) rat primary hepatocytes	0.9–30 µg/mL in DMSO (±S9)	97.6	Negative	Fautz (1990)
Forward mutation	Chinese hamster lung V79 cells; *Hprt* locus	10–100 µg/mL in DMSO (±S9)	98.3	Negative	Van Gompel, Vanparys & Van Cauteren (1995)
In vivo					
Micronucleus formation	Male Wistar rats	0, 10, 40, 160 mg/kg bw (ip)	Not reported	Negative	Vanparys & Marsboom (1979)
Micronucleus formation	Male and female albino Swiss mice	0, 20, 80, 320 mg/kg bw (orally)	98.7	Negative	Vanparys & Marsboom (1988b)
Micronucleus formation	Male and female albino BKW mice	100 mg/kg bw (orally)	96.4	Negative	Jenkinson (1990b)
Unscheduled DNA synthesis	CD-1 mouse hepatocytes ex vivo	125, 250 mg/kg bw (gavage)	97–98.1	Negative	Clare (1996)
Sex-linked recessive lethal test	*Drosophila melanogaster* Berlin K males	250, 1 000 ppm	Not reported	Negative	Vanparys & Marsboom (1982)
Dominant lethal test	Male and female albino Swiss mice	Single oral dose 0, 10, 40, 160 mg/kg bw	Not reported	Negative	Vanparys & Marsboom (1987a,b)

bw: body weight; DMSO: dimethyl sulfoxide; DNA: deoxyribonucleic acid; *Hprt*: hypoxanthine–guanine phosphoribosyltransferase; ip: intraperitoneally; ppm: parts per million: S9: 9000 × *g* supernatant fraction from rat liver homogenate

In an in vivo comet assay not previously evaluated by JMPR, groups of five Swiss mice of each sex were given daily oral doses of technical-grade imazalil (purity 95%) at 0 or 10 mg/kg bw for 28 days. Imazalil was suspended in 0.2 mL corn oil. Tail moment was calculated from measurements of comet tail length and tail intensity; long tail nuclei were also analysed. A statistically significantly increased tail moment was apparent in the liver cells of treated animals of both sexes compared with

controls (accompanied by statistically significant increases in tail length, long tail nuclei and tail intensity). Statistically significant sex differences in all measured parameters, with the exception of tail length, were also noted. The study authors concluded that, over time, the low concentrations of imazalil present in the environment and cypermethrin in food, and especially their mixtures with carbendazim, have genotoxic properties that could be particularly dangerous in mammalian organisms (Đikić et al., 2012).

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

In a non-GLP-compliant fertility study, Wistar rats (20 of each sex per dose) received imazalil (purity 98.8%) at 0, 5, 20 or 80 mg/100 g feed (equivalent to 0, 7.5, 30 and 120 mg/kg bw per day, respectively) as follows: males for 60 days prior to pairing with females and through copulation; females for 14 days prior to pairing and throughout pregnancy.

Pregnancy rate was not affected at any level, regardless of treatment of either sex. Test group data for all other measured or observed parameters were comparable with the control data (Marsboom, 1977).

In a two-generation study, groups of 24 male and 24 female Wistar rats were given diets containing imazalil (purity 98.0%) at a nominal dose of 0, 5, 20 or 80 mg/kg bw per day for 60 days before mating (both sexes), during a 3-week mating period (both sexes), during gestation and until weaning of the first generation. The F_0 males were killed after mating. The first generation (F_1) received imazalil during growth, mating, gestation and weaning of the second generation (F_2). The F_1 males were killed after mating. The doses during the premating period were equal to 0, 4.2, 17.6 and 70.5 mg/kg bw per day for males and 0, 5.0, 21.5 and 104.3 mg/kg bw per day for females, respectively. During the F_0 gestation, the intakes were 0, 4.0, 16.3 and 86.3 mg/kg bw per day, respectively, and those during the F_1 gestation were 0, 4.3, 18.6 and 87.8 mg/kg bw per day, respectively. Thus, the intakes tended to be slightly lower than the nominal doses. The animals were observed daily for general health, appearance and behaviour. Body weights were recorded weekly before mating and, for females, on days 1 and 22 after copulation. Body weights were also recorded during the 3-week lactation period. Feed consumption was recorded weekly before mating for both sexes and during gestation and lactation for females. The body weights of both F_1 and F_2 pups in each litter were recorded 8–12 hours after parturition and on days 4, 14 and 21. The kidneys, liver and reproductive organs of the F_0 and F_1 generations were examined histologically.

Two F_1 females at the high dose were killed in extremis, and one F_2 female at the high dose and one in the control group died soon after parturition or during lactation. F_0 males at the high dose showed decreased body weight gain before mating; a similar but less pronounced effect was seen in females before mating, but an effect was also seen in F_1 and F_2 dams during gestation, at parturition and at days 4, 14 and 21 of lactation. No effects on body weight gain were seen in other treated groups, and there was no clear effect on feed consumption in any group. Histopathological changes (liver vacuoles) were seen in F_0 males at the high dose, but no lesions were seen in the male reproductive system at any dose. Decreased numbers of corpora lutea were seen in F_0 females at the high dose. At the high dose, both F_1 and F_2 generations had considerably reduced numbers of live pups and larger numbers of stillborn pups, and the F_1 generation had fewer implantations; the duration of gestation was increased in both generations, by approximately 1 day, with an associated increase in the incidence of dystocia. The survival rates of pups during lactation were decreased in all F_1 treated groups on days 4, 14 and 21; in the F_2 generation, survival was decreased at all times at the high dose and on days 14 and 21 at the low dose; at the intermediate dose, the survival was comparable with that of controls, so that there was no dose–response relationship. No differences in body weight were seen between F_1 and F_2 pups. No teratogenic effect was observed.

As noted above, the survival rates of F_1 pups during lactation were decreased in all treated groups on days 4, 14 and 21 of lactation and in the F_2 generation at 5 mg/kg bw per day (days 14 and 21) and 80 mg/kg bw per day (days 4, 14 and 21); moreover, there was no dose–response relationship for the effect in the F_1 generation at day 4, although there was at days 14 and 21. It is arguable that the data, especially for days 14 and 21, indicate a dose-related effect, but there was clearly no dose-related effect in F_2 pups. There is no biologically plausible explanation for the difference between the two generations, and a statistical re-examination on a litter basis showed that the mortality rate was statistically significantly increased only in the group at the high dose.

The NOAEL for parental toxicity was 20 mg/kg bw per day, on the basis of reduced maternal body weight gain and hepatotoxicity (vacuoles) in males at 80 mg/kg bw per day. The NOAEL for offspring toxicity was 20 mg/kg bw per day, on the basis of decreased numbers of live pups, decreased survival rate of pups and increased numbers of stillbirths at 80 mg/kg bw per day. The NOAEL for reproductive toxicity was 20 mg/kg bw per day, on the basis of increased duration of gestation for the F_0 and F_1 females and decreased gestation rate in F_1 females at 80 mg/kg bw per day (Dirkx et al., 1992b).

In a three-generation study, groups of 20 female Wistar rats received imazalil (purity not reported) on a non-continuous basis for three generations. Imazalil was administered as 50% wettable powder at a dietary concentration of 0, 5, 20 or 80 mg/100 g feed (or 0, 50, 200 and 800 ppm) (equivalent to 0, 7.5, 30 and 120 mg/kg bw per day, respectively). Prior to and after the period of organogenesis and throughout lactation, the females of the first and second generations received only the basal laboratory diet. In each of these generations, the dams received the various levels from day 6 through day 15 of pregnancy. In the third generation, the dams received the various levels from 3 weeks until 3 months of age and further from days 1 to 21 after mating.

There was a trend towards a lower number of live births at the 80 mg/100 g feed level in all generations. No other effects were noted at any other level. Waved ribs and absence of metacarpal and metatarsal bones were seen in both control and treated groups. No abnormalities were noted in any fetuses in any generation that were not within the normal range for the rats used (Marsboom, 1975a).

The submitted report did not include a GLP statement or any individual animal data, and therefore this study is of limited value.

In another three-generation study, groups of Wistar rats (10 of each sex per dose) were fed imazalil (purity 98.8%) in the diet at a concentration of 0, 50, 200 or 800 mg/100 g feed (equivalent to 0, 7.5, 30 and 120 mg/kg bw per day, respectively) and subjected to a standard three-generation, two litter per generation, reproduction study. Observations included growth, feed consumption, mortality and the standard indices of reproduction (mating, fertility, gestation, viability and lactation).

The reproductive performance of rats was unaffected by imazalil at any dose. There was no maternal mortality, and the parental generations were unaffected by imazalil. No fetal abnormalities or embryonic effects were noted. Litter size, weight and survival were normal.

This study is of limited value because all parameters were not measured and individual animal data were not provided (Marsboom, 1978).

(b) Developmental toxicity

Mice

Twenty-four female COBS mice received imazalil sulfate (purity 98.7%) orally at a dose of 0, 2.5, 10 or 40 mg/kg bw per day from days 6 through 16 of gestation. All animals were killed on day 19 of gestation.

No effects on mortality, body weight, feed consumption, pregnancy rate, number of live, dead or resorbed fetuses, pup weight or mean litter size were found. During gross observations, talipes valgus (club foot) was observed in all groups, the incidence not being statistically significantly different in any dose group when compared with the control group. No additional abnormalities were seen after radiographic examinations and Lapras' sectioning technique (Marsboom, Gillardin & Sanz, 1985).

The developmental toxicity of imazalil sulfate (purity 99.7%) was studied in groups of 30 inseminated COBS CD-1 mice given a dose of 0, 40, 80 or 120 mg/kg bw per day by gavage on days 6–16 of gestation. All animals were killed on day 19 of gestation.

Treatment did not affect the maternal mortality rate, but maternal body weight gain and feed consumption were statistically significantly decreased at the intermediate and high doses. Litter size and the number of live pups were reduced in all treated groups. The number of resorptions was increased at the high dose. No teratogenic effects were seen, and there were no differences between groups in mean pup weights.

The NOAEL for maternal toxicity was 40 mg/kg bw per day, based on decreased body weight gain and feed consumption at 80 mg/kg bw per day. A NOAEL for embryo and fetal toxicity could not be established in view of the decreases in litter size and number of live pups at all doses (Gillardin, Sanz & Marsboom, 1987).

In another study of developmental toxicity, groups of 30 inseminated COBS CD-1 mice were given imazalil (purity 99.3–99.5%) at a dose of 0, 10, 40, 80 or 120 mg/kg bw per day by gavage on days 6–16 of gestation. Deaths were recorded during the study, and the animals were examined daily for abnormal clinical signs. Body weights were determined on day 1, daily on days 6–17 and on day 19 after copulation. Feed consumption was recorded over days 1–5, days 6–16 and days 17–18. On day 19 after copulation and before killing, all survivors underwent a complete physical examination. The surviving dams were then killed, and macroscopic changes were recorded; the weights of the uteri were recorded after removal of that organ in toto. The dams were examined for numbers of live and dead fetuses, implantation sites and embryos undergoing resorption. All live fetuses were weighed, and live and dead fetuses were examined for external abnormalities. The pups were sexed, and the fetuses were examined radiographically. The fetuses were then dissected, and bones were stained with alizarin.

Fourteen mice died during the study: one control, four at 80 mg/kg bw per day and nine at 120 mg/kg bw per day. At the highest dose, piloerection, excitability, convulsions, hypothermia and prostration were observed. Tremor was observed in one female at 120 mg/kg bw per day. Body weights were decreased on days 17 and 19 in mice at the two highest doses in comparison with concurrent controls; moreover, the body weight gain of these animals was decreased during days 6–17 after copulation, and that of animals at 40 and 120 mg/kg bw per day was decreased during days 17–19 after copulation. The total body weight gain (uterine weight subtracted) over the whole period of 1–19 days was decreased at doses of 40 mg/kg bw per day and above. Feed consumption was decreased during dosing on days 6–16 in mice at 80 and 120 mg/kg bw per day and after dosing in those at 40 mg/kg bw per day and above. The pregnancy rates and number of implantations were comparable among groups, whereas fewer live fetuses were found in animals at the highest dose, and the number of resorptions was increased. The body weight of pups was decreased at the highest dose, but the sex ratio was similar in all groups. The frequency of minor changes in ribs was increased at 40 and 80 mg/kg bw per day, but not at the highest dose; there was thus no clear dose–response relationship. The number of major abnormalities was comparable in all groups.

The NOAEL for maternal toxicity was 10 mg/kg bw per day, on the basis of decreased body weight gain and reduced feed consumption after treatment at 40 mg/kg bw per day. In addition, deaths occurred at doses of 80 mg/kg bw per day and above. The NOAEL for embryo and fetal toxicity was 80 mg/kg bw per day, as the number of live fetuses was reduced, the number of resorptions was

increased and the body weights of the pups were decreased at the highest dose. There was no evidence of teratogenicity (Levron et al., 1991).

Rats

In a developmental toxicity study (non-GLP-compliant), Wistar rats (20 of each sex per dose) received imazalil (purity not specified) at 0, 5, 20 or 80 mg/kg bw per day in the diet during gestation days 6–15. Fetuses were delivered by caesarean section on day 21.

Body weights, body weight gains and feed consumption were comparable among the treated and control groups. No mortality occurred during the study. No differences in percentage of pregnancies or average duration of gestation were noted between the test and control groups. There was no difference between the control and treated groups with respect to the average number of implantations, litter size, number of live and dead fetuses, number of resorptions or fetal weights. No abnormalities were noted among the fetuses in the control and various test groups, with waved ribs in one litter of the group recorded at 80 mg/kg bw per day (Marsboom, 1970).

This study is of limited value because all parameters were not measured and individual animal data were not provided.

The developmental toxicity of imazalil sulfate (purity 99.9%) was studied in groups of 24 pregnant Sprague Dawley OFA.SD (IOPS Caw) rats treated by gavage at a dose of 0, 40, 80 or 120 mg/kg bw per day on days 6–16 of gestation. The dams were examined daily and weighed 1, 6, 17 and 22 days after copulation. Feed consumption was recorded during days 1–5 (before dosing), 6–16 (during dosing) and 17–21 (after dosing). At the end of the study, on day 22 of gestation, the dams were killed and examined for live and dead fetuses, implantation sites and resorptions. Live fetuses were weighed, and live and dead ones were examined for anomalies radiographically and by alizarin staining to visualize skeletal anomalies.

One animal at 40 mg/kg bw per day died, but this death was considered to be unrelated to treatment, as no others occurred during the study. Maternal body weight was reduced at the end of dosing at all three doses on day 17 and at the high dose on day 22, in comparison with concurrent controls. Decreased body weight gain was seen at the high dose. Decreased feed consumption was seen at all three doses throughout treatment. The rates of pregnancy and numbers of corpora lutea and implantations were similar in all groups. The number of live fetuses was decreased and the number of resorptions was increased at 120 mg/kg bw per day. At the intermediate and high doses, the birth weights of the pups were decreased. No teratogenic effects were seen.

The NOAEL for embryo and fetal toxicity was 40 mg/kg bw per day, on the basis of reduced pup weight at 80 mg/kg bw per day. No NOAEL for maternal toxicity could be identified because of decreased maternal body weight at all doses (Gillardin et al., 1988).

In an embryotoxicity and teratogenicity study, 20 female Wistar rats per group received various levels of imazalil nitrate (purity not provided) from day 16 of pregnancy through a 3-week lactation period. The doses used in this study were 0, 5, 20 and 80 mg/100 g feed.

No differences in percentage of pregnancies or average duration of gestation were noted between test and control groups. Average litter size was slightly decreased at the high dose of 80 mg/100 g feed (9) compared with the controls (11). The percentage of dead fetuses was higher in the 80 mg/100 g feed group (27%) when compared with the controls (4%), 5 mg/100 g feed (0%) and 20 mg/100 g feed (1%) groups. No abnormalities were observed in offspring from the control or test groups. No effects on pup body weight or body weight gain were observed (Marsboom, 1975b).

Rabbits

Groups of 20 female New Zealand white rabbits received imazalil nitrate (purity 99.2%) in water by gavage at 0, 0.63 or 2.5 mg/kg bw per day on days 6–18 of pregnancy. All surviving animals were killed on day 28 of pregnancy.

Four females died during the test (three in the control group and one in the 0.63 mg/kg bw per day group). Body weight gain of the dams was decreased in both dose groups, as well as average litter size, the percentages of live fetuses and 24-hour survival rates. The percentages of resorbed fetuses increased with increasing dose. Retrospective statistical analysis showed that the effects on maternal body weight, litter size and number of resorptions were not statistically significant. A small number of anomalies in all groups (hydrocephaly, fused ribs and deformed legs) were considered not to be dose related. Individual data were not available to the Meeting. In this study, embryotoxic and maternally toxic effects were observed in both dose groups. Therefore, this study was repeated at lower doses, as described below (Marsboom, 1974).

Groups of 15 female New Zealand white rabbits received imazalil nitrate (purity 97.8%) in water by gavage at 0, 0.16 or 0.63 mg/kg bw per day from days 6 to 18 of pregnancy. The surviving animals were killed on day 28 of pregnancy.

Mortality (three dams in the 0.16 mg/kg bw per day group died during the study), body weight and pregnancy rate were not statistically significantly different among the groups. Mean litter size was normal in all groups, and no statistically significant differences were seen with regard to the number of live, dead or resorbed fetuses, birth weight or 24-hour survival rate. Fetal skeletal examination and fetal sectioning revealed no compound-related abnormalities (Marsboom & Dirkx, 1981).

Groups of 15 female New Zealand white rabbits received technical-grade imazalil nitrate (purity 99%) in water by gavage at 0, 1.25, 2.5 or 5 mg/kg bw per day on days 6–18 of pregnancy. All animals were killed on day 28 of pregnancy. The dams were examined for mortality and body weight. The number of live, dead and resorbed fetuses, litter size, survival rate and pup weight were recorded, and all live and dead fetuses were examined for gross pathology (fetal skeletal examination and fetal sectioning).

One rabbit at the lowest dose and one at the highest dose died. There was no clear effect of treatment on body weight gain or pregnancy rate. No differences were seen between groups in the numbers of live or dead fetuses or resorptions, birth weights of pups or 24-hour survival rate.

The NOAEL for both maternal and embryo/fetal toxicity was 5 mg/kg bw per day, the highest dose tested (Dirkx & Marsboom, 1985).

In a developmental toxicity study, pregnant albino rabbits (15 per dose) were given imazalil sulfate (purity 98.2–100%) by gavage at a dose of 0, 5, 10 or 20 mg/kg bw per day on days 6–18 of gestation. The animals were observed daily for ill-health, abnormal behaviour or other clinical effects. Body weights were recorded on the morning of day 0 and on days 6, 19 and 28 of gestation. Feed consumption was recorded during days 0–5, 6–19 and 19–28. On day 28 of gestation, the surviving animals were killed and necropsied, the uterus was removed and the weight recorded, and the dams were examined for the numbers of dead and live fetuses and resorptions. The numbers of corpora lutea were determined, and the pups were sexed. Live fetuses were weighed, and both live and dead fetuses were examined externally by radiographic examination and alizarin staining. The fetuses were then dissected.

Eight out of 15 animals at 20 mg/kg bw per day and one out of 15 animals at 10 mg/kg bw per day died during the experiment. These deaths occurred late during the study and were not due to a single-dose effect. Emphysema and/or pneumonia were present in the majority of these animals. Five

of those at the high dose that died showed weight loss. No statistically significant differences in clinical end-points were found in the survivors. On day 19 of gestation, the body weight of animals at 20 mg/kg bw per day was decreased in comparison with that of concurrent controls. Feed consumption was reduced in animals at 10 mg/kg bw per day in comparison with concurrent controls during days 0–5 and in animals at 10 and 20 mg/kg bw per day during days 6–18. The pregnancy rates and numbers of corpora lutea were comparable between groups. Animals at 20 mg/kg bw per day had a decreased number of live pups, and those at 10 and 20 mg/kg bw per day had increased numbers of resorptions, although these changes were not statistically significant. The increase in resorptions at 10 mg/kg bw per day was within the historical control range; however, the current Meeting concluded that there was a trend towards an increase in resorptions with increasing dose and that the increase in resorptions was therefore treatment related (Table 18).

Table 18. Selected parameters in a developmental toxicity study in rabbits

Parameters	Control	5 mg/kg bw per day	10 mg/kg bw per day	20 mg/kg bw per day
No. of dead or sacrificed females	0/15	0/15	1/15	8/15**
No. of pregnant females	15/15	11/15	14/15	13/15
Body weight (g)				
Day 0	3 622	3 633	3 571	3 625
Day 6	3 774	3 774	3 711	3 757
Day 19	4 055	4 005	3 841	3 770*
Day 28	4 136	4 173	3 993	4 036
Weight of gravid uterus (g)	420	384	322	218
Weight change of pregnant females (g)	93	155	100	194
Feed consumption (g)				
Days 0–5	1 298	1 250	1 160*	1 155
Days 6–18	2 726	2 635	2 227*	2 096*
Days 19–27	1 651	1 484	1 495	1 748
Litter data				
No. of live fetuses/female	6.4	6.2	5.0	3.6
No. of dead fetuses/female	0.47	0.00	0.00	0.00
Mean litter size	6.9	6.2	5.0	3.6
No. of resorptions/female	0.67	0.64	1.46	3.71
No. of implantations/female	7.5	6.8	6.5	7.3
No. of corpora lutea/female	8.9	8.6	9.2	7.7
Mean weight of live fetuses (g)	41.3	42.4	45.2	39.0
Sex ratio (%)	46.8	34.6*	54.1	59.7
Number of malformed pups	1	2	0	0

bw: body weight; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$
Source: Dirkx et al. (1992a)

Developmental toxicity was observed at 20 mg/kg bw per day, as evidenced by a 5% decrease in fetal weight. There was a statistically significant decrease in the number of male fetuses at 5 mg/kg bw per day, but not at higher doses. Fetuses of does at 20 mg/kg bw per day had an increased

incidence of thirteenth rib and missing sternum in comparison with concurrent controls, but, especially in the latter case, there was no clear dose–response relationship, and the 2000 Meeting considered these findings to be coincidental.

The NOAEL for maternal toxicity was 5 mg/kg bw per day, on the basis of reduced feed consumption at 10 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 5 mg/kg bw per day, on the basis of an increased incidence of resorptions and a decreased number of live pups at 10 mg/kg bw per day (Dirkx et al., 1992a).

The Meeting considered the proposal newly submitted by Martens (2015) with respect to establishing an ARfD. In 2005, JMPR reviewed the available toxicological data for imazalil, and an ARfD of 0.05 mg/kg bw was established on the basis of maternal and fetal toxicity observed in the above-described study of developmental toxicity in rabbits (Dirkx et al., 1992a). Since the last review by JMPR, a single-dose toxicity study in rats and an acute neurotoxicity study in rats became available. The Meeting considered these studies for setting an ARfD for imazalil and concluded that the selected developmental toxicity study is still the most appropriate for setting the ARfD. The study suggests a trend towards increased incidence of resorptions and decreased number of live pups at 10 mg/kg bw per day, which cannot be discounted.

2.6 Special studies

(a) Neurotoxicity

Acute neurotoxicity

In a range-finding acute oral neurotoxicity study not previously evaluated by JMPR, imazalil (purity 95.5%) in polyethylene glycol (PEG) 400 was administered to groups of three Crl:CD®(SD) rats of each sex per dose by oral gavage at a dose of 40, 65, 110, 180 or 300 mg/kg bw. Owing to insufficient toxicity at the high dose, a sixth group of three rats of each sex was administered a single oral dose of 600 mg/kg bw. All animals were observed twice daily for mortality and moribundity. Clinical examinations were performed daily. Body weight was recorded daily from the first day of dosing. A modified functional observational battery (FOB) was conducted on all animals at 1, 3, 4.5, 6 and 24 hours after dose administration. All animals were euthanized following the last FOB assessment (day 1) and discarded without macroscopic examination.

There were no unscheduled deaths at any dose. Clinical observations, including piloerection, hypoactivity, unkempt appearance, red material around the nose, yellow material on the ventral abdominal area and rocking, lurching or swaying while ambulating, were noted in a single female in the 600 mg/kg bw group the day following dose administration. Yellow material on the ventral abdomen was also noted in another female at 600 mg/kg bw on the day following dosing. Body weight loss was noted for a single female in the 300 mg/kg bw group (10 g) and for all females in the 600 mg/kg bw group (8–13 g). During the FOB on the day of dosing, treatment-related findings were observed in 1–3 animals of each sex in the 300 and 600 mg/kg bw groups. The onset of these findings (soiled fur, low arousal and decreased rearing counts in males and females and piloerection in females) occurred at 3–4.5 hours following dosing; however, the incidences were highest at 4.5–6 hours following dosing. Mean rearing counts were maximally reduced in males in the 600 mg/kg bw group at 4.5 hours post-dosing. Mean rearing counts in females at 600 mg/kg bw began decreasing at 3 hours post-dosing and remained decreased for the remaining assessments. Low arousal was noted in one or two males or females at 65, 110 and/or 180 mg/kg bw; however, this finding has been reported in the historical control animals (14/208 on study day 0).

The NOAEL for systemic toxicity in this preliminary acute neurotoxicity study was 180 mg/kg bw, based on FOB findings at 300 mg/kg bw.

The time of peak effect was found to be 6 hours post-dosing, based on the increased incidence of FOB findings. Based on the results from this preliminary study, the doses selected for the main study were 0, 60, 180 and 600 mg/kg bw (Beck, 2006a).

In the main acute neurotoxicity study, which was not previously evaluated by JMPR, groups of Crl:CD®(SD) rats (12 of each sex per dose) were given imazalil (purity 95.5%) in PEG 400 orally by gavage at a dose of 0, 60, 180 or 600 mg/kg bw and observed for 14 days. Neurobehavioural assessment (FOB and motor activity testing) was performed on all animals predosing and on days 0, 7 and 14. Body weight and feed consumption were measured weekly throughout the study. At study termination, six animals of each sex per group were euthanized and perfused in situ for neuropathological examination; brain weight was recorded for these animals. Of the perfused animals, six animals of each sex from the control and high-dose groups were subjected to histopathological evaluation of brain and peripheral nervous system tissues.

At 600 mg/kg bw, four females were found dead or euthanized in extremis on study day 1 or 2. Prior to and on the day of death, clinical signs, including cool limbs, yellow material on the abdomen, hypoactivity, tremors, hunched posture, prostration and/or increased respiration, were observed in these animals. No treatment-related effects on body weight were observed in females; however, males at 600 mg/kg bw had decreased body weight gain (16%) and feed consumption (10%) in the first week post-dosing.

No changes in FOB parameters were observed in males at any time or in females on days 7 and 14. During the FOB testing, alterations observed in females at 600 mg/kg bw on day 0 at the time of peak effect included an increased incidence of the following: slightly soiled fur (5/12), crusty deposits around nose (7/12), low arousal (5/12), gait changes (hunched body [4/12] and walking on tiptoes [3/12]), no touch response (8/12), no olfactory response (5/12) and no approach response (7/12). These findings were noted in 0–2 control animals. The mean rearing count (1.7) and body temperature (35.8 °C) were decreased in females at 600 mg/kg bw on day 0 at the time of peak effect, compared with those values in controls (4.4 and 37.6 °C, respectively). During the motor activity testing, significant decreases in total and ambulatory counts (63% and 68%, respectively) compared with the control values were observed during the first 15-minute interval at the time of peak effect on day 0 in females at 600 mg/kg bw. Overall counts for total and ambulatory activity were significantly reduced on study days 0 (34–47%) and 7 (29–40%) in females at 600 mg/kg bw. At necropsy, no treatment-related effects on brain weight or measurements (length and width) were observed. The Meeting noted that the highest dose used was above the LD_{50}, and therefore the FOB results are due to general toxicity. On microscopic examination of the central and peripheral nervous systems, minimal axonal degeneration in the peripheral and spinal nerves and spinal nerve roots was observed in both treated and control groups of both sexes, with no consistent increase in the treated groups.

The NOAEL for systemic toxicity of imazalil in rats was 180 mg/kg bw, based on deaths in females, decreased body weight gain in males and FOB and motor activity alterations at the time of peak effect on day 0 in females at 600 mg/kg bw. The NOAEL for acute neurotoxicity was 600 mg/kg bw, the highest dose tested (Beck, 2006b).

Developmental neurotoxicity

A published study of reproductive toxicity with measurement of neurobehavioural end-points was reported in which imazalil (purity >99%) was administered in the diet to groups of 10 Crj:CD-1 mice of each sex from 5 weeks of age in the F_0 generation to 9 weeks of age in the F_1 generation. The compound was given at a concentration of 0%, 0.012%, 0.024% or 0.048% (equal to 0, 120, 240 and 480 ppm), resulting in respective intakes for the F_0 generation of 0, 19, 39 and 79 mg/kg bw per day for males and 0, 26, 45 and 102 mg/kg bw per day for females before conception; 0, 18, 34 and 74 mg/kg bw per day during mating; 0, 21, 38 and 83 mg/kg bw per day during gestation; and 0, 65, 152 and 262 mg/kg bw per day during lactation. The respective intakes in the F_1 generation were 0, 21, 41 and 87 mg/kg bw per day for males and 0, 24, 49 and 93 mg/kg bw per day for females.

In the F_0 generation, there was no effect on the body weight of either sex before conception or on the body weights of dams during gestation or lactation. Feed intake was increased in females at the low and high doses before conception, but not in those at the intermediate dose. A number of indicators of exploratory behaviour were increased in males at the high dose. In the F_1 generation, litter size and weight and sex ratio at birth were unaffected by treatment. The mean body weights of animals at the intermediate and high doses were reduced in early lactation. Indicators of behavioural development were affected, as follows. In males, surface righting was affected at the intermediate and high doses on postnatal day 4 and at the intermediate dose on postnatal day 7. Swimming head angle was affected in males at the high dose on postnatal day 4. In females, surface righting was affected in all treated F_1 offspring on postnatal day 7, without a clear dose–response relationship, and swimming head angle was affected in females at the high dose on postnatal day 4. No convincing dose–response relationships were seen for other observations, such as on multiple water T-maze performance in week 7. By week 8, there were no effects on the exploratory behaviour of either sex.

The doses used in this study were higher than those used in other studies reviewed in this monograph. Furthermore, although the results suggest that neurobehavioural end-points can be adversely affected in mice exposed to imazalil in their diet during development, comparison with the study of Levron et al. (1991), described in section 2.5(b) above, suggests that the lowest dose was maternally toxic. The NOAEL for developmental neurotoxicity was 120 ppm (equal to 19 mg/kg bw per day), based on effects on surface righting in males at 240 ppm (equal to 39 mg/kg bw per day) (Tanaka, 1995).

In another published study, which was not previously evaluated by JMPR, designed to investigate the effects of imazalil on birth outcome and neurobehavioural effects in offspring, groups of 10 pregnant Crlj:CD1 mice were given diets containing 6, 18 or 54 ppm imazalil (purity >99%) throughout gestation and lactation. This resulted in respective intakes of about 0, 0.85, 2.49 and 7.87 mg/kg bw per day during gestation and of 0, 3.2, 8.4 and 26.8 mg/kg bw per day during lactation.

No overt effects were noted in the dams. In offspring, there were no convincing test substance–related effects on behaviour, other than a transient and inconsistent, but dose-related, delay in surface righting ability in males on postnatal day 4, but not on postnatal day 7.

Regarding exploratory behaviour in the F_1 generation, there was no treatment-related difference ($P < 0.05$) in movement time at 3 weeks of age, but movement time was statistically significantly longer ($P = 0.0206$) in the low-dose group of males at 8 weeks of age. Spontaneous behaviour examination indicated that movement time increased in males, but decreased in females, in the low-dose groups in the F_1 generation.

The results of the study showed that some neurobehavioural parameters were inconsistently affected at all doses (Tanaka et al., 2013).

(b) Mechanistic studies on liver and thyroid toxicity

Liver pathology: mice

The effects of imazalil on liver morphology, enzymes and metabolism were studied in SPF albino Swiss mice. Imazalil (purity 98.5%) was administered in the diet to groups of 40 male and 40 female mice at a concentration of 0, 50, 200 or 600 ppm (equal to 0, 13, 53 and 160 mg/kg bw per day for males and 0, 16, 64 and 200 mg/kg bw per day for females for the first month and 0, 12, 46 and 140 mg/kg bw per day for males and 0, 14, 55 and 170 mg/kg bw per day for females for 3 months) for 1 or 3 months. An interim termination of 15 mice of each sex per group was carried out at 1 month, with 10 being assigned for toxicological and toxicokinetics evaluation and five for biochemistry. The remaining animals were killed at 3 months, 20 being used for toxicological and toxicokinetics evaluation and five for biochemistry. The mice were examined daily, and body weight and feed consumption were measured weekly. ALT, AST and ALP activities were measured in serum at both termination times, and selected organs were weighed and examined grossly. Only the livers

were examined, but they were examined microscopically, and the livers of one control mouse and one mouse of each sex at the high dose at the interim kill were examined ultramicroscopically.

No deaths were observed during the study, and imazalil had no effects on appearance or behaviour. No difference in body weight was observed between groups. Feed consumption was increased in males at 200 and 600 ppm over weeks 1–4 and in all treated females, probably due to feed wastage. The activity of ALT was increased in animals of each sex at the high dose at 1 month, whereas the activity of ALP was increased only in males at this dose. At 3 months, ALT activity was increased in males at 600 ppm, but the level was within the normal range seen in other studies. At 1 month, the absolute weight of the liver was increased in males at 200 ppm, and absolute and relative liver weights were increased in males and females at 600 ppm. At this time, males at the intermediate dose had increased absolute kidney weights, females at the high dose had decreased absolute and relative pancreatic weights, and females at the intermediate dose had increased absolute and relative adrenal weights. At 3 months, males at the high dose had increased absolute and relative liver weights, and males at 200 ppm had increased relative heart and kidney weights. Also in males, a decrease in absolute and relative thymus weights was seen at 200 ppm, and a decrease in absolute thymus weight was observed at 600 ppm. In females at 3 months, absolute and relative liver weights were increased and relative pancreatic weight was decreased at 600 ppm. In both males and females, the increases in liver weight appeared to be dose related. The changes in liver weights were accompanied by an increased incidence of dark livers at the high dose.

Histological examination at 1 month showed an increase in the numbers of small and large vacuoles, especially in the periportal area, in males and females at the high dose and in males at the intermediate dose. Similar findings were made at 3 months, with the addition of vacuolation in females at the intermediate dose. In mice at 600 ppm, ultramicroscopic examination of the liver showed an increased number of lipid droplets in hepatocytes, mainly in the periportal area. Moreover, the rough endoplasmic reticulum of the affected hepatocytes differed morphologically from that of hepatocytes from control animals: the rough endoplasmic reticulum in hepatocytes from treated animals was not arranged in parallel stacks of cisternae, but was diffused through the cytoplasm (Van Deun et al., 1994).

Liver pathology: rats

In a published study, addition of imazalil to suspensions of isolated rat hepatocytes at a concentration of 0.75 mmol/L caused acute cell death accompanied by depletion of intracellular glutathione and protein thiols, with accumulation of intracellular malondialdehyde (Nakagawa & Moore, 1995).

The hepatotoxic potential of imazalil (purity not reported), with and without L-buthionine-*S*,*R*-sulfoximine, a glutathione synthesis inhibitor, was examined by administering the inhibitor at 900 mg/kg bw to groups of four male Fischer 344/DuCrj rats by intraperitoneal injection, followed 1 hour later by imazalil by stomach tube at a single dose of 0, 170, 255 or 340 mg/kg bw (the last dose being approximately equal to the LD_{50}). Another group received imazalil without L-buthionine-*S*,*R*-sulfoximine, and two groups received the vehicle or L-buthionine-*S*,*R*-sulfoximine. The rats were killed 24 hours after administration of imazalil. Serum was taken for measurements of ALT and AST activity and cholesterol, urea, phospholipid and triglyceride concentrations. The kidneys and livers were removed and weighed; the livers were homogenized for measurement of malondialdehyde, and sections of the two organs were stained for histopathological examination.

Imazalil, with or without L-buthionine-*S*,*R*-sulfoximine, did not affect body weight, liver weight or kidney weight. When given alone, it caused a dose-dependent decrease in ASP activity but not ALT activity. Only the high dose of imazalil alone decreased the serum concentration of triglycerides, whereas those of cholesterol and phospholipids were decreased at the intermediate and high doses. The serum concentrations of triglycerides, cholesterol and phospholipids were decreased at the intermediate and high doses when L-buthionine-*S*,*R*-sulfoximine had been given, and there was

some indication of a greater effect in these animals compared with those given imazalil alone. L-Buthionine-*S*,*R*-sulfoximine and imazalil induced fatty infiltration of the liver, the distribution of which was predominantly midzonal or periportal, but there was no evidence of hepatocellular necrosis. Malondialdehyde, an index of lipid peroxidation, was found at increased concentrations in animals given imazalil at the high dose, whether or not they also received L-buthionine-*S*,*R*-sulfoximine, but the concentration of malondialdehyde was higher when both compounds were given. No histopathological changes were observed in the kidneys, nor were urea concentrations in urine increased (Nakagawa & Tayama, 1997).

Liver enzyme induction and liver cell proliferation: mice

In the 3-month oral mechanistic toxicity study with 1-month interim termination described above (Van Deun et al., 1994), liver microsomes were prepared from 10 mice of each sex after 1 and 3 months of treatment with imazalil at 0, 50, 200 or 600 ppm. The purpose of this study was to investigate the mechanism of liver changes seen with imazalil in the mouse. The microsomal protein and cytochrome P450 (CYP) contents of the mouse livers, the uridine diphosphate–glucuronosyltransferase (UGT) activity and several monooxygenase activities were determined to detect induction and/or inhibition of cytochrome P450. Spectrophotometric or fluorimetric determinations were performed employing standard procedures for measuring cytochrome P450 activities.

The administration of imazalil through the feed at a concentration of 50, 200 or 600 ppm for 1 month caused statistically significant increases in relative liver weight and in the hepatic microsomal protein and cytochrome P450 contents of both male and female SPF albino Swiss mice. Treatment with imazalil for 1 month also induced the *N*-ethylmorphine *N*-demethylation (EMD) and 7-pentoxyresorufin *O*-dealkylase (PROD) activities, whereas aniline hydroxylation and 7-ethoxyresorufin *O*-deethylation (EROD) were decreased in both male and female mice. After dosing for 3 months, EMD was virtually unaffected, and aniline hydroxylation, PROD, lauric acid hydroxylation (LAH) and especially EROD were decreased for both sexes. 7-Ethoxycoumarin *O*-deethylation (ECOD) was induced in males after 1 month of dosing and in both males and females after 3 months of dosing. The UGT activity was not affected in male mice and was slightly induced after 1 month of treatment and inhibited after 3 months of treatment in female mice.

Imazalil was a mixed inducer and inhibitor of cytochrome P450 in the mouse (Vermeir, Lavrijsen & Van Leemput, 1994).

In the 3-month oral mechanistic toxicity study with 1-month interim termination described above (Van Deun et al., 1994), liver samples were taken to investigate cell proliferation in livers of mice treated with imazalil via the diet for up to 3 months. For each dose group, formalin-fixed liver samples embedded in paraffin wax from 20 animals were analysed. Liver sections were microwaved in a 0.1 mol/L ethylenediaminetetraacetic acid (EDTA) solution and stained using an anti-proliferating cell nuclear antigen (PCNA) antibody in conjunction with an avidin/biotin peroxidase–conjugated detection system. Approximately 1000 hepatocytes were evaluated from each liver section employing microscope-assisted cell counting. The total cell count and the PCNA-positive cell count were scored by the study pathologist. For both sexes, Bartlett's test was statistically significant at the 1% level (both cases: $P < 0.001$), using the untransformed data as well as the logarithmically transformed data. This indicated that there was evidence of heterogeneity of variance between the treatment groups. Hence, each group was compared with the vehicle control group using Wilcoxon's rank sum test. For both sexes, no statistically significant results between dosed groups and vehicle groups were found.

There was no induction of hepatic cell proliferation by imazalil administered orally to albino Swiss mice for 3 months at concentrations up to 600 ppm (Lawrence, 2001). This study was not previously evaluated by JMPR.

Imazalil (purity > 98%) administered orally to mice at 1 or 10 mg/kg bw per day for 3 days enhanced EROD and PROD activities in the microsomes of small intestinal mucosa and liver, indicating induction of CYP1A and CYP2B. Additionally, immunochemical analyses indicated induction of CYP2B, CYP2C and CYP3A (benzyloxyquinoline *O*-debenzylation [BQ]) (Muto et al., 1997).

In a more recently completed study, imazalil (purity 97.5%) was investigated for its potential to induce mouse CYP2B10, CYP3A11 and cell proliferation as measured by replicative DNA synthesis during cell cycle S-phase in isolated primary mouse hepatocytes. Following a range-finding test, freshly isolated female CD-1 mouse hepatocytes were cultivated as monolayer cultures in appropriate culture flasks and 96-well plates. Hepatocytes were exposed to imazalil at a concentration of 3, 10, 30 or 100 µmol/L or to vehicle alone (0.5% volume per volume [v/v] dimethyl sulfoxide [DMSO]). Three to six replicates, depending on the assay, were performed. Cytotoxicity was measured as change in intracellular ATP concentration. Bioluminescent determination of released ATP was carried out using the assay kit Cell-Titre Glo Luminescent Cell Viability Assay (Promega) according to the manufacturer's instructions. Levels of murine cytochrome P450 activity were determined spectrofluorometrically by formation of resorufin or 7-hydroxyquinoline from CYP2B10 or CYP3A11 activity, respectively. The activity of CYP2B in cultured hepatocytes was assessed spectrofluorometrically by the formation of resorufin from pentoxyresorufin. The activity of CYP3A in cultured hepatocytes was assessed spectrofluorometrically by the formation of 7-hydroxyquinoline. The number of hepatocytes undergoing replicative DNA synthesis was measured by incorporation of 5-bromo-2′-deoxyuridine (BrdU) in DNA and subsequent immunochemical detection; epidermal growth factor served as the positive control. Approximately 1500 hepatocytes per replicate were counted.

Treatment with imazalil at 30 or 100 µmol/L resulted in statistically significantly lower intracellular ATP, with levels being reduced to 84% and 2% of control values, respectively. The cytotoxicity observed following treatment with imazalil at 100 µmol/L was considered excessive, and therefore replicative DNA synthesis and CYP2B10 (PROD) and CYP3A11 (BQ) data either could not be measured at this concentration or were excluded from data analysis. In addition, the cytotoxicity observed following treatment with imazalil at 30 µmol/L was considered to be biologically relevant and sufficient to demonstrate that imazalil had been tested to an appropriately high concentration. Treatment with imazalil at 10 or 30 µmol/L resulted in statistically significant increases in replicative DNA synthesis of 1.8- and 1.5-fold relative to the controls, respectively. Treatment with imazalil at 3 µmol/L resulted in a statistically significant increase in CYP2B10 (PROD) activity to 117% of the control value, whereas at 30 µmol/L, a statistically significant decrease in CYP2B10 activity to 15% of the control value was observed. The decrease in cytochrome P450–mediated enzyme activity (PROD) observed in the mouse hepatocyte cultures is probably due to the inhibition of CYP2B10, in addition to the cytotoxicity described above. Treatment with imazalil at 3, 10 or 30 µmol/L exhibited a dose-dependent increase in CYP3A11 (BQ) activity of 1.3-, 2- and 2.1-fold compared with control values, respectively.

In conclusion, treatment of mouse hepatocytes with imazalil resulted in the limited induction of CYP3A11, followed by dose-dependent inhibition of CYP2B10 and increased cell proliferation (Elcombe, 2012b). This study was not previously evaluated by JMPR.

In order to investigate alterations in hepatocytes and potential proliferation induced by imazalil and, if observed, reversibility of the effect, groups of 10 male Crl:CD-1®(ICR) BR mice were treated for 2 or 13 weeks with imazalil (purity 96.2%) at a concenteration of 0, 100, 200, 300, 600 or 1200 ppm in the feed. Additional groups (10 animals) at 0 or 600 ppm were necropsied after 2- or 4-week recovery periods. Osmotic minipumps with BrdU were implanted 7 days prior to necropsy. Liver samples were analysed for cell proliferation. Slices of liver and duodenum were fixed in buffered formalin. Labelling index was determined as the percentage of cells in the DNA replicative phase or S-phase that had incorporated BrdU using immunohistochemical staining, counting at least

2000 liver cells per animal. In addition, PCNA immunostaining of hepatocytes was performed for control and high-dose mice.

Statistically significant increases in sorbitol dehydrogenase levels were noted at 600 and 1200 ppm, and a statistically significant increase in ALT was noted at 1200 ppm only. Relative or absolute and relative liver weights were increased at 600 and 1200 ppm. Increased incidences of centrilobular hypertrophy were noted at 200 ppm and above. A low incidence of minimal hepatocellular necrosis and increasing incidence and severity of hepatocellular vacuolation at weeks 2 and 13 were noted at 400 ppm and above. Cell proliferation in the liver, determined by BrdU and PCNA labelling, appeared not to be increased upon imazalil treatment.

These results indicate that there is no induction of hepatic cell proliferation following oral administration of imazalil to Crl:CD-1®(ICR) BR mice at concentrations up to 1200 ppm for 2 or 13 weeks (O'Neill, 2002). This study was not previously evaluated by JMPR.

In a similar study on the potential liver cell proliferation induced by imazalil, groups of six male Crl:CD-1®(ICR) BR mice were treated for 4 days with imazalil (purity 96.2%) at a concentration of 1200 ppm in the feed. A concurrent control received untreated diet. Osmotic minipumps with BrdU were implanted 1 day prior to commencement of treatment. Animals were observed for survival and clinical signs; body weights and feed consumption were measured weekly. At termination, blood chemistry, gross necropsy, liver weights and hepatic histopathological examinations were conducted, and slices of liver and duodenum were fixed in buffered formalin. Liver samples were analysed for cell proliferation. Labelling index was determined as the percentage of cells in the DNA replicative phase or S-phase that had incorporated BrdU using immunohistochemical staining, counting at least 2000 liver cells per animal. Quantitative data were tested for statistical significance (Student's *t*-test). Labelling index data were evaluated for normality using Shapiro-Wilk's test and then subjected to Dunnett's test.

There were no clinical findings, and all animals survived the study. For the treated group, decreased feed consumption was noted on day 1, and body weight gain and terminal body weight were reduced compared with control mice. ALT values and both absolute and relative liver weights were increased. Histopathology of the liver revealed minimal centrilobular hypertrophy, with minimal hepatocyte necrosis also observed in five out of the six treated mice. Cell proliferation in the liver determined by BrdU labelling was statistically significantly increased upon imazalil treatment.

These results indicate that there is a strong mitogenic response in the liver following acute oral administration of imazalil to CD-1 mice at a concentration of 1200 ppm (Elmore, 2004a). This study was not previously evaluated by JMPR.

Liver enzyme induction and liver cell proliferation: rats

Groups of 20 SPF Wistar rats were given diets containing imazalil (purity not specified) at a concentration of 0, 200, 400 or 800 ppm for 3 months. Over the first month, these dietary concentrations resulted in intakes of 0, 21, 42 and 82 mg/kg bw per day for males and 0, 22, 45 and 90 mg/kg bw per day for females, respectively; however, over the entire 3 months of the study, the intakes of imazalil were lower, at 0, 16, 32 and 64 mg/kg bw per day for males and 0, 19, 38 and 76 mg/kg bw per day for females, respectively. Ten animals of each sex at each dietary concentration were killed after 1 month. The rats were observed clinically and for morbidity and mortality at least daily. Ophthalmological examinations were performed at the start of the study and before the interim and terminal sacrifices in the first 10 animals in the control group and those at the high dietary concentration. Body weight was determined weekly, as was feed consumption. Blood was taken at the interim and terminal sacrifices for haematological and clinical chemical investigations; urine was analysed at the same times. Necropsies were performed at both the interim and terminal sacrifices. Selected organs were weighed in each case, and any gross changes were noted. Pieces of liver from four rats of each sex per dietary concentration were taken at both the 1-month and 3-month sacrifices.

Liver microsomes were assayed for protein and cytochrome P450 content and for certain microsomal enzyme activities – namely, aniline hydroxylation, EMD, EROD, PROD, LAH, ECOD and UGT activity towards 4-nitrophenol. The plasma concentrations of imazalil were measured.

No deaths were seen. The clinical effects were similar in the various groups, and no ophthalmological abnormalities were found. Body weight was reduced in females at 800 ppm in comparison with concurrent controls at weeks 7 and 9. Body weight gain was reduced in males at 800 ppm at weeks 1–3 and in females at 400 and 800 ppm in week 1 and at 800 ppm in week 9. No effect was observed on feed consumption, except in week 1, when there was decreased consumption by rats of each sex at the high dietary concentration. At the 1-month termination, total leukocyte and lymphocyte counts were decreased in both sexes at dietary concentrations of 400 and 800 ppm, and neutrophil and basophil counts were decreased in males at 800 ppm. In females, total leukocyte and lymphocyte counts were decreased at 200, 400 and 800 ppm, monocyte count was decreased at 800 ppm and the mean cell haemoglobin concentration was decreased at 200 ppm. At 3 months, males showed a decrease in basophil count at 400 ppm and in erythrocyte volume fraction at 200, 400 and 800 ppm. Also in males, the haemoglobin concentration was decreased at 400 ppm, the mean cell volume at 800 ppm and the mean cell haemoglobin concentration at 200 and 800 ppm. Females had a decrease in mean cell volume at 800 ppm and an increase in mean cell haemoglobin concentration at 200 and 800 ppm. The decrease in mean cell volume in animals of both sexes at 800 ppm at 3 months may have been treatment related. Otherwise, these changes were considered minor and were mostly not dose related or fell within the reference range for the strain of rats.

Some minor changes in some clinical chemistry parameters were seen at the low and intermediate doses (200 and 400 ppm), but no treatment-related effects on clinical chemistry or urine were seen at concentrations below 800 ppm. At that concentration, males had increased serum calcium, phosphate, albumin, cholesterol, triglyceride and blood urea nitrogen concentrations at 1 month, whereas females had decreased calcium, glucose and triglyceride concentrations; the total protein level was increased in both sexes. At 3 months, the serum calcium concentration was decreased in males and females. The decreases in bilirubin concentration and ALT and AST activities that were observed in the study were considered not adverse, and the inconsistent nature of the other clinical chemical changes casts doubt on their clinical relevance. Treatment-related changes in urinary parameters were not seen (Van Deun et al., 1996b).

The tissue samples from Van Deun et al. (1996b) were further evaluated for cytochrome P450 content and other enzyme activities. After 1 month, the liver microsomal protein content was increased in males at all concentrations and in females at the high concentration. The liver cytochrome P450 content was increased in animals of both sexes at the high concentration. The activities of EROD and PROD were increased in males at the intermediate and high dietary concentrations, whereas the activity of ECOD was increased at 200 and 800 ppm; UGT activity was increased at the high concentration. At 1 month in females, increased activity was seen for EMD, EROD, PROD and ECOD at all dietary concentrations and for UGT activity at the high concentration. Additional induction was not observed in males at 3 months, and increased activity of EROD was observed at dietary concentrations of 200 and 800 ppm and of PROD and ECOD only at the high concentration. However, in females, the cytochrome P450 content was further increased at 800 ppm. Increased aniline hydroxylation was seen at all dietary concentrations, EMD activity at the intermediate and high concentrations, EROD and PROD activities at all dietary concentrations and LAH at 200 and 800 ppm. Females at 3 months also showed increased activity of ECOD at 400 and 800 ppm and of UGT at 800 ppm. After 1 month, the plasma concentrations of imazalil ranged from less than 2 to 4.4 ng/mL in males and from less than 2 to 3.8 ng/mL in females at the three dietary concentrations. At 3 months, the corresponding concentrations were 1.1–2.2 ng/mL in males and 1.0–4.0 ng/mL in females.

Increased absolute and relative liver weights were observed after 1 month in males and females at 400 and 800 ppm. Increased relative liver weights were seen in males and increased absolute liver weights in females at 200 ppm. Increased relative thyroid weights were observed in males and increased absolute adrenal weights in females at 800 ppm. Decreased thymus weights, both absolute and relative, were seen in males at the high dietary concentration. At 3 months, males at 800

ppm had increased relative liver and kidney weights, whereas females had increased relative kidney weights at 800 ppm and increased absolute and relative weights of the adrenals at 400 and 800 ppm. No gross pathological changes were observed that appeared to be related to treatment. Hepatic changes were seen histologically at dietary concentrations of 400 ppm and above at 1 month; these comprised hepatocyte swelling in males and large vacuoles in females. Adrenal changes (cortical cell swelling) were observed at 3 months in 2/10 females at 800 ppm and 1/10 females at 400 ppm; the study authors considered that this finding corresponded to the increase in adrenal weights observed. No histopathological differences between groups were seen in the liver at 3 months. No histopathological differences were observed in the thyroid at 1 or 3 months (Vermeir, Lavrijsen & Meuldermans, 1995).

Groups of 10 male and 10 female SPF Wistar Hannover rats were given diets containing technical-grade imazalil (purity not specified) at a concentration of 0, 800, 1600, 2400 or 3200 ppm (equal to 0, 64, 129, 181 and 252 mg/kg bw per day for males and 0, 79, 150, 236 and 333 mg/kg bw per day for females, respectively). The rats were observed daily, and body weights and feed consumption were measured weekly. Deaths were recorded. Haematological and clinical chemical measurements were performed on blood samples from all surviving animals before the terminal sacrifice, and the concentration of imazalil was determined by gas chromatography with mass spectrometry by Sterkins (1996). Urine was analysed before terminal sacrifice. TSH, T_3, T_4 and testosterone concentrations were measured in serum from blood taken at termination. At necropsy, the animals were examined and weighed, and any gross pathological changes were noted. The liver was examined histopathologically, as were other organs judged to be grossly abnormal. Electron microscopy was carried out on the livers of one male and one female control animal and one male and one female animal fed the highest dietary concentration of imazalil. Liver microsomes from four rats of each sex per group were assayed for protein and cytochrome P450 content, and the activities of UGT and several monooxygenases were determined by Vermeir & Lavrijsen (1996).

A statistically significantly lower body weight compared with concurrent controls was found from week 2 of the study in males at the two highest concentrations and from time to time in males at 1600 ppm. The females showed decreased body weight from week 1 at the two highest concentrations and from week 2 at 1600 ppm. The findings for body weight gain were similar, except that decreased body weight gain in comparison with controls was found in animals of both sexes at 2400 and 3200 ppm throughout the study. Sporadic decreases in feed consumption were seen in all groups of treated males, particularly frequently at 2400 ppm. Among females, decreased feed consumption was observed at concentrations of 1600 ppm and above from time to time.

Sporadic changes in haematological parameters were seen, most of which were not dose related and did not therefore appear to be related to treatment. However, a decrease in monocyte count was seen in males at dietary concentrations of 1600 ppm and above. Furthermore, increased erythrocyte volume fraction, haemoglobin and red cell count were seen, with decreases in mean cell volume and increases in mean cell haemoglobin concentration in females at most concentrations, including the lowest. Decreased AST activity was seen at all doses in both sexes, and decreased ALT activity and blood urea nitrogen concentration were seen at concentrations of 1600 ppm and above in males; such changes are not generally considered to be adverse. Decreased serum albumin was seen in females at the two highest dietary concentrations. A decrease in serum calcium and an increase in phosphate concentration were seen in females at 1600, 2400 and 3200 ppm. Decreased triglyceride and phospholipid concentrations were observed in males at all dietary concentrations, and a decreased triglyceride concentration was seen in females at the highest dietary concentration. No treatment-related intergroup differences in urinary end-points were seen. The TSH, T_3, T_4 and testosterone concentrations in serum showed no clear differences between groups.

At termination, the body weights of males were decreased at concentrations of 1600 ppm and above. Also in males, decreased absolute lung weights were seen at 2400 and 3200 ppm, and increases in relative liver weight were seen at all concentrations. Other changes in organ weights of males either were not dose related or reflected changes in total body weight. Similar findings were

made in females, which showed a decrease in body weight at dietary concentrations of 1600 ppm and above. A decrease in absolute lung weight was also seen in females at these concentrations. At postmortem examination, gross changes that appeared to be related to treatment were seen only in the liver. The livers of males at 2400 and 3200 ppm were dark, and more pronounced lobulation was seen at dietary concentrations of 1600 ppm and above. Dark livers were also found in females at 2400 and 3200 ppm, and more pronounced lobulation was seen only at 3200 ppm. Histological examination revealed hepatocellular hypertrophy in males at all dietary concentrations, which was particularly pronounced in centrilobular zones. Also in males, an increased frequency of hepatocytes with small vacuoles was seen at 2400 and 3200 ppm, and an increased frequency of fine oil red O–positive material was seen at all concentrations. Similar changes were seen in females: they had hepatocellular hypertrophy, which was particularly pronounced in centrilobular zones, at all dietary concentrations. Further, they showed an increased frequency of hepatocytes with small vacuoles at all concentrations and large vacuoles at and above 2400 ppm. An increased frequency of fine oil red O–positive material was seen in females at concentrations of 1600 ppm and higher. Histological examination of the thyroid gland revealed no significant intergroup differences. On electron microscopy, the livers of all animals at all four dietary concentrations, but not the controls, showed small and/or large lipid droplets in both centrilobular and perilobular areas. Additionally, whorls of smooth endoplasmic reticulum were seen, this change not being present in controls.

The protein content of microsomes was increased in males at concentrations of 1600 ppm and above and in females at and above 2400 ppm. The activities of EMD, EROD, PROD and ECOD were statistically significantly induced in animals of both sexes at nearly all dietary concentrations. An increase in lauric acid hydroxylase activity was seen more frequently in the livers of males than in those of females, but was statistically significant only in males at 2400 ppm. Aniline hydroxylase activity was induced in females at all dietary concentrations. UGT activity was induced at the two highest dietary concentrations in females. At 800 ppm, the plasma concentration of imazalil was at or below the limit of detection of the assay, which was 1 ng/mL, in males and slightly above the limit of detection in females, at 1.3 ng/mL. In males, the concentrations of imazalil were 3.4. 4.4 and 11 ng/mL at 1600, 2400 and 3200 ppm, respectively, and the equivalent figures for females were 9.4, 6.8 and 17 ng/mL. These values suggest that detoxification pathways may have been saturated at dietary concentrations of 2400 ppm and higher.

It was concluded that imazalil is a nonspecific inducer of hepatic enzymes and that it caused morphological changes in the livers of rats. No effect was seen at 3 months in either the concentration of thyroid hormones or the histological appearance of the thyroid (Sterkins, 1996; Van Deun et al., 1996a, 1999; Vermeir & Lavrijsen, 1996).

Male Wistar rats were dosed orally by gavage at a dose of 10 or 40 mg/kg bw per day for 7 days. Hepatic microsomes were prepared by differential centrifugation, about 24 hours after the last dose for half of the rats and after a drug-free period of 7 days for the remaining animals. The concentrations of microsomal protein, cytochrome P450, NADPH–cytochrome *c* reductase, as well as the magnitude of the activities of the phase I enzymes aniline hydroxylase, ECOD and EROD, were determined.

The results of the study demonstrate that imazalil only slightly increased some cytochrome P450–dependent enzyme activities at 40 mg/kg bw per day. Relative liver weights, NADPH–cytrochrome *c* reductase activity and phase I enzyme aniline hydroxylase were not affected by the treatment. There was only a weak and dose-dependent increase in ECOD and EROD activities, but this was fully reversible, as these enzyme activities returned to the control values after a drug-free period of 7 days (Lavrijsen et al., 1987).

Twenty-one paraffin blocks of rat liver from a previously conducted 24-month control carcinogenicity study in rats (Van Deun, 1999b) were analysed in this study. Treatment was for 7

days with imazalil and phenobarbital at unknown levels or routes of administration. Animals were dosed intraperitoneally with BrdU (100 mg/kg bw) 6 hours prior to necropsy.

Cell proliferation in the liver determined by BrdU labelling was not increased upon imazalil treatment or upon phenobarbital treatment. The results indicate that there is apparently no mitogenic response in the liver following 7 days of administration of imazalil to rats. However, the positive control also resulted in no increase of hepatic cell proliferation; thus, the results of this study are unconvincing (Elmore, 2004b). This study was not previously evaluated by JMPR.

In a more recently completed study not previously evaluated by JMPR, imazalil (purity 97.46%) was administered in the diet to groups of five male Crl:WI(Han) rats for 1, 2, 7, 14 or 28 consecutive days at a concentration of 0, 200, 1200 or 2400 ppm. A positive control group was fed 1200 ppm phenobarbital on a similar regimen. All animals were observed twice daily for mortality and moribundity. Clinical examinations were performed daily, and detailed physical examinations were performed at least weekly. Individual body weights and feed consumption were recorded at least weekly. Blood samples collected at necropsy were analysed for levels of ALT and sorbitol dehydrogenase. Osmotic minipumps with a 30 mg/mL solution of BrdU were implanted 4 days prior to necropsy. The liver was removed, and slices of liver and duodenum were fixed in buffered formalin. Weights of liver and thyroids with parathyroids were determined for all animals. Microscopic examination was performed on the liver, thyroid and duodenum from all animals at each scheduled necropsy. Liver sections (right portion of the median lobe, right and left lobes), thyroid and duodenum were stained for BrdU, caspase and 4-hydoxy-2-nonenal. For each dose group, the remaining section of the median lobe of five animals was collected, subjected to preparation of microsomes and assayed for CYP2B1/2 activity (PROD) and UGT (4-methylumbelliferone assay). In addition, portions (approximately 50–100 mg) of the median liver lobe collected at the end of the 28-day exposure were analysed for mRNA expression using reverse transcriptase polymerase chain reaction for the following: constitutive androstane receptor (CAR), *Cyp2b1/2*, *Cyp3a1*, *Cyp3a2* and *Gadd45β* (using cyclophilin as reference gene).

There were no test substance–related effects on clinical observations, serum chemistry parameters or macroscopic examinations. Test substance–related lower mean body weights and body weight changes compared with the control group were observed at 1200 and 2400 ppm during multiple intervals throughout the study; at necropsy days 14 and 28, mean body weights of animals treated at 2400 ppm were reduced by more than 10%. The 1200 and 2400 ppm groups consumed less feed overall than the control group throughout the study. There were test substance–related higher mean absolute and/or relative liver weights on study days 14 and 28 in the 1200 and 2400 ppm groups. In comparison, the phenobarbital-treated group had higher mean absolute and relative liver and thyroid/parathyroid weights on study days 2, 7, 14 and 28. Dietary administration of imazalil was associated with alterations in hepatocellular staining affinity (increased cytoplasmic homogeneity), which was equivocally detectable as early as study day 1, more readily apparent at study day 2 and clearly visible at study days 7–28. The cytoplasmic alteration was interpreted as a metabolic response to imazalil. There was no histopathological indication of morphological injury to the liver in response to imazalil, and there were no imazalil-related alterations in serum ALT or sorbitol dehydrogenase. Phenobarbital-related hepatocellular changes were more pronounced than imazalil-related changes at later time points of the study and included overt centrilobular hepatocellular hypertrophy at study days 14 and 28. In addition, higher serum ALT and sorbitol dehydrogenase levels in phenobarbital-treated rats at study days 2, 7 and 14 indicated functional as well as morphological hepatocellular alterations. Results of BrdU immunohistochemical staining of liver and thyroid gland revealed an increased level of BrdU incorporation in the liver of phenobarbital-treated rats at study day 7, indicating hepatocellular proliferative activity, with no similar effect noted in imazalil-treated rats. An increased rate of BrdU incorporation was noted in the thyroid gland at study day 14 in rats receiving 2400 ppm imazalil and in phenobarbital-treated rats at study days 7, 14 and 28. Additionally, neither phenobarbital nor imazalil showed any increases in apoptosis (as measured by caspase immunohistochemistry) or oxidative stress (as measured by 4-hydroxy-2-nonenal immunohistochemistry). Significant increases in CYP2B1/2 and UGT1A activities were observed

following treatment with 1200 or 2400 ppm imazalil, with these increases appearing to be both dose and time dependent. Although a plateau effect in CYP2B1/2 activity was present at study day 7 with the 1200 ppm imazalil treatment level, exposure to 2400 ppm caused the largest changes in both CYP2B1/2 and UGT1A activities following a 28-day dosing regimen. The enzyme induction in the phenobarbital group was statistically significantly higher than that of the 2400 ppm imazalil group. The induction in the latter was, on average, 9% (PROD) and 39% (4-methylumbelliferone glucuronidation) as effective as that in the 1200 ppm phenobarbital group. Similarly, mRNA levels of *Cyp2b1* showed an induction by imazalil and a larger induction by phenobarbital. A striking similarity between both treatments was also noted for the induction at the mRNA level of CAR (NR1I3), *Cyp3a1*, *Cyp3a2* and *Gadd45*. Levels of CAR (NR1I3) did not show induction at the mRNA level when compared with animals on the basal diet. Levels of mRNA for *Cyp3a1, Cyp3a2* and *Gadd45β* were all increased in both the imazalil-exposed rats and the phenobarbital-exposed rats.

The results indicated that both imazalil and phenobarbital showed parallel effects, with phenobarbital being the more potent agent (Mertens, 2011). This study was not previously evaluated by JMPR.

Liver enzyme induction and liver cell proliferation: humans

The aim of a study by Elcombe (2012c), which was not previously evaluated by JMPR, was to investigate whether imazalil (purity 97.46%) is able to induce CYP2B6, CYP3A4 and cell proliferation (as measured by replicative DNA synthesis during cell cycle S-phase and using epidermal growth factor as the positive control) in isolated primary human hepatocytes. Following a range-finding test, freshly isolated female human hepatocytes were cultivated as monolayer cultures in appropriate culture flasks and well plates. Hepatocytes were exposed to imazalil at a concentration of 1, 3, 10 or 30 μmol/L or to vehicle alone (0.5% v/v DMSO) for 96 hours. Three to six replicates, depending on the assay, were performed. Cytotoxicity was measured as the change in intracellular ATP concentration. Bioluminescent determination of released ATP was carried out using the assay kit Cell-Titre Glo Luminescent Cell Viability Assay (Promega) according to the manufacturer's instructions. Levels of human cytochrome P450 activities were determined spectrofluorometrically by formation of resorufin (according to Burke et al., 1985) or 7-hydroxyquinoline from pentoxyresorufin (PROD – CYP2B6) or benzyloxyquinoline (BQ – CYP3A4), respectively. The number of hepatocytes undergoing replicative DNA synthesis was measured by incorporation of BrdU in DNA and subsequent immunochemical detection.

Treatment with imazalil at 3, 10 or 30 μmol/L resulted in statistically significantly lower intracellular ATP, with levels being reduced to 94%, 77% and 12% of control values, respectively. The cytotoxicity observed following treatment with 30 μmol/L was considered excessive, and therefore replicative DNA synthesis and CYP2B6 (PROD) data either could not be measured at this concentration or were excluded from data analysis. In addition, the cytotoxicity observed following treatment with 10 μmol/L was considered biologically relevant and sufficient to demonstrate that imazalil had been tested to a suitably high concentration. No concentration of imazalil induced increases in replicative DNA synthesis (S-phase of the cell cycle), indicating that it did not induce cell proliferation. Treatment with imazalil at 10 μmol/L resulted in a statistically significant decrease in CYP2B6 activity (PROD), whereas data could not be measured at 30 μmol/L. Treatment with imazalil at 10 or 30 μmol/L resulted in statistically significant decreases in CYP3A4 activity (BQ). The decreased enzyme activities are probably due to inhibition by imazalil. The viability of the human hepatocytes and hence the validity of the test were verified by the marked stimulation of S-phase by epidermal growth factor.

Imazalil-induced liver enzyme activity was not seen in this cultured human hepatocyte study (Elcombe, 2012c). In contrast, enzyme induction was seen both in a companion mouse hepatocyte study (Elcombe, 2012b) and in a study in humanized mice in vivo (Elcombe, 2012a). The inhibition of cytochrome P450 by residual imazalil explains the poor induction of enzyme activities. However, increased expression of mRNA and protein for CYP2B and CYP3A was seen in imazalil-treated hPXR/hCAR mice and to a much greater degree in imazalil-treated wild-type C57Bl/6 mice in

previous tests. This suggests that although imazalil is a CAR activator, human CAR is less responsive than mouse CAR to imazalil. In the in vivo study in C57B/6 mice and hPAXR/hCAR mice (Elcombe, 2012a), imazalil stimulated S-phase. However, imazalil did not stimulate S-phase in human hepatocytes in vitro. It should be noted that hPXR/hCAR mice are humanized only for two genes (CAR and pregnane X receptor [PXR]), but all other genes involved in pathways of indirect activation and downstream signalling leading to cell proliferation remain murine. No increase in cell proliferation was observed in human hepatocytes exposed to imazalil at concentrations up to and beyond the toxicity threshold. Moreover, treatment with imazalil did not induce CYP2B6 or CYP3A4. These data demonstrate that human hepatocytes, unlike mouse hepatocytes, exhibit no cell proliferation in response to imazalil (Elcombe, 2012c). This study was not previously evaluated by JMPR.

CAR/PXR receptor agonism: mice

The capacity of imazalil to act as a CAR/PXR agonist in wild-type mice and mice humanized for CAR and PXR was investigated in a recently completed study not previously evaluated by JMPR. Phenobarbital, a well-studied CAR/PXR activator, was included as a positive control. The strains used in this study were wild-type C57Bl/6NTac mice and mice humanized for PXR and CAR (hPXR/hCAR). Each control or treatment group consisted of 20 male wild-type and 20 male hPXR/hCAR mice. Animals were exposed to imazalil (purity 97.46%) at a dietary concentration of 0, 50, 200 or 600 ppm or to phenobarbital at a dietary concentration of 1000 ppm for either 7 or 28 days. All diets were found to be homogeneous, and achieved concentrations of test items were between 89% and 102% of the target concentrations. The mean achieved intakes of imazalil at 0, 50, 200 and 600 ppm, calculated for the duration of the study, were 0, 6.7, 30 and 96 mg/kg bw per day, respectively, for wild-type mice and 0, 7.2, 26 and 78 mg/kg bw per day, respectively, for hPXR/hCAR mice. The mean achieved intakes of phenobarbital at 1000 ppm were 155 mg/kg bw per day for wild-type mice and 128 mg/kg bw per day for hPXR/hCAR mice.

Seven days before termination, animals were subcutaneously implanted with osmotic minipumps containing approximately 200 µL BrdU solution. The animals were terminated in a staggered fashion on consecutive days, such that five animals from each group (replicates I and III) were terminated on day 8 or day 29 and five animals from each group (replicates II and IV) were terminated on day 9 or day 30. Only animals treated for 7 days were analysed for the study-specific end-points, whereas samples from the animals treated for 28 days were stored for potential later analysis. Animals were checked for clinical signs and mortality once a day. Body weights were determined on day 3 and at termination (7-day exposure) or weekly (28-day exposure). Feed consumption was determined weekly. Terminal plasma was collected by cardiac puncture; ALT, AST, ALP, cholesterol and triglycerides were determined. Liver weight was determined, and liver and duodenum samples were fixed and evaluated histopathologically. Liver samples were processed, and subcellular fractions were assayed for caspase-3-activity, lipid peroxidation and cytochrome P450 activities; peroxisome β-oxidation (cyanide-insensitive palmitoyl-coenzyme A oxidation) was also assessed in liver fractions (heavy pellets). CYP3A and CYP3B were determined by western blot. A TaqMan® gene expression analysis assay was performed to determine *Cyp2b10*, *Cyp3a11* and *Gadd45β* (murine β-actin was used as the internal standard) in hepatic mRNA.

There were no clinical signs or mortalities, and body weights and liver weights were unaffected. Imazalil had no effect on ALT, AST or ALP in either strain of mouse. Administration of phenobarbital at 1000 ppm increased ALT and ALP in both wild-type and hPXR/hCAR mice, whereas AST was unaffected. Plasma cholesterol levels were significantly reduced following 7 days of administration of imazalil at 600 ppm and of phenobarbital at 1000 ppm in both wild-type and hPXR/hCAR mice. At histopathological examination, a few microscopic findings were recorded in the liver. Minimal to moderate diffuse, periportal or centrilobular hepatocellular vacuolation was observed in the animals of both mouse lines treated with both imazalil and phenobarbital. In addition, focal or multifocal inflammatory cell infiltration was noted in almost all mice of all groups. Portal mononuclear cell infiltrates also occurred in individual animals of all groups. Hepatocellular

diffuse/centrilobular hypertrophy was noted in phenobarbital-treated animals only. Minimal to slight hypertrophy was also noted in hPXR/hCAR mice administered 600 ppm imazalil. Hepatocellular hypertrophy was not observed in any wild-type mice administered imazalil. An increase in total cytochrome P450 was observed at 200 and 600 ppm imazalil and with phenobarbital in wild-type mice (1.5-, 2.5- and 3.7-fold compared with control values, respectively) and at 600 ppm and with phenobarbital in humanized mice (1.8- and 3.8-fold compared with control values, respectively). Imazalil induced *Cyp2b10* mRNA and protein expression and enzyme activity in a dose-dependent manner in both wild-type and hPXR/hCAR mice, but to a lesser extent compared with phenobarbital, and markedly less in hPXR/hCAR mice than in wild-type mice. Imazalil induced *Cyp3a11* mRNA expression in a dose-dependent manner in both wild-type and hPXR/hCAR mice. *Cyp3a* protein expression and enzyme activity were induced at 600 ppm and with phenobarbital. Imazalil and phenobarbital induced hepatic apoptosis markers *Gadd45β* mRNA expression and Caspase-3 cleavage products in both wild-type and hPXR/hCAR mice. Dietary administration of phenobarbital or imazalil for 7 days had no effect on lipid peroxidation in the livers of wild-type or humanized mice. Administration of imazalil at all concentrations to wild-type mice increased S-phase in liver by up to 6-fold relative to control values, whereas administration of phenobarbital at 1000 ppm increased S-phase by 31.2-fold. Administration of imazalil at all concentrations to hPXR/hCAR mice increased S-phase by up to 3.4-fold relative to control values, whereas phenobarbital at 1000 ppm increased S-phase by 24.5-fold. The imazalil-mediated induction of *Cyp2b10* and, to a lesser degree, *Cyp3a11* in the presence of increased hepatocellular S-phase strongly suggests that imazalil is an activator of the xenosensing nuclear receptors CAR and (probably) PXR.

Imazalil appeared to be a more potent activator of mouse CAR than human CAR, as noted by generally smaller responses in humanized CAR mice when compared with the wild-type mice at comparable doses. It should be noted that hPXR/hCAR mice are humanized only for two genes, but all other genes involved in pathways of indirect activation and downstream signalling leading to cell proliferation remain murine. Phenobarbital, a known non-genotoxic rodent liver carcinogen and CAR activator, was evaluated alongside imazalil and induced similar, albeit more marked, findings to those seen at high imazalil doses. Overall, it is considered that the liver effects seen with imazalil are CAR dependent.

The CAR/PXR activation with dose-responsive changes were seen at imazalil dietary concentrations of 200 ppm and above (Elcombe, 2012a). This study was not previously evaluated by JMPR.

Effects on the thyroid gland, liver enzyme induction and proliferation in thyroid, liver and jejunum: rats

In a study to investigate the mechanisms underlying the effects of imazalil on the thyroid gland, groups of 50 SPF male Wistar (Hannover) rats were given diets containing imazalil (purity 98.8%) at a concentration of 0, 400, 1200 or 3200 ppm (equal to 0, 41, 123 and 328 mg/kg bw per day, respectively) for 4 weeks; a further group received a diet containing phenobarbital at 1200 ppm (equal to 126 mg base equivalent/kg bw per day). Interim kills were carried out after 1, 2 and 4 weeks of treatment and after a 4-week (week 8) or 9-week (week 13) recovery period, so that the size of the group at each dietary concentration at the time of termination was 10. The rats were observed daily, and body weights and feed consumption were measured weekly. Blood was taken at termination for measurement of TSH, T_3, T_4 and other clinical chemical end-points, including bilirubin, total protein and ALP, ALT and AST activities. The animals were killed at the times described above, five animals from each group being injected 6 hours before death with BrdU. At termination, a full postmortem examination was carried out, and macroscopic abnormalities were noted. The thyroid (including parathyroids) and liver were weighed. The thyroid and liver were examined histologically after haematoxylin and eosin staining, and the thyroid, liver and sections of the jejunum from BrdU-treated rats were stained immunohistochemically for BrdU-labelled cells. Liver and thyroid microsomes were prepared from liver pieces and the left thyroid glands (Vermeir, Lavrijsen & Meuldermans, 2001). Induction or inhibition of the hepatic microsomal cytochrome P450 enzymes aniline hydroxylase,

EMD, PROD, 5′-monodeiodinase and T_4 glucuronosyltransferase was assessed in tissue from the five animals from each group not injected with BrdU; microsomal protein and thyroid peroxidase activity in the thyroid were also measured.

No treatment-related deaths were seen. There was significant feed wastage at the high dose of 3200 ppm. Sedation was noted in the phenobarbital-treated animals during week 1 and week 2. Mid- and high-dose male rats, respectively, weighed up to 8% ($P < 0.01$) and 19% ($P < 0.01$) less than vehicle controls, gained 15% ($P < 0.01$) and 36% ($P < 0.01$) less weight and consumed 7% ($P < 0.01$) and 15% ($P < 0.01$) less feed during the 4-week treatment period. The positive control group had mean body weights similar to those of controls during the 4-week treatment period, but weighed 6–7% ($P < 0.01$) less than controls from weeks 5 to 8 of the recovery period. Feed consumption was decreased sporadically in animals at 400 ppm, but this was considered not biologically relevant. At 1200 ppm, feed consumption was decreased during dosing and for the first 3 weeks of the recovery period, whereas at 3200 ppm, feed consumption was lower than in the controls during treatment and for 1 week afterwards. In the group given phenobarbital, feed consumption was increased during week 2 of dosing. At 400 ppm, no difference in clinical chemical end-points relative to controls was seen.

At 1200 ppm, ALP activity was decreased by 22% ($P < 0.05$) in mid-dose rats treated for 2 weeks. Serum AST activity showed dose-related decreases during weeks 1 and 2; the decreases were 17–30% ($P < 0.05$) for the mid-dose group and 41–45% ($P < 0.01$) for the high-dose group. Data for the positive control showed statistically significant increases for serum total protein at weeks 1, 2 and 4 (7–13%, $P < 0.01$ or < 0.05), an increase in serum albumin at week 8 (6%, $P < 0.05$), a decrease in ALP activity at week 2 (31%, $P < 0.05$) and a small increase in ALT activity at week 2 (28%, $P < 0.05$). TSH concentrations were increased in the phenobarbital-treated animals and in all imazalil-treated animals at 8 weeks. No statistically significant increases in TSH concentration were seen at other times; nevertheless, the increases at 1 and 2 weeks may have been biologically relevant. The T_4 concentrations were lower in all imazalil-treated groups after 1 week. After 2 weeks, no differences in T_4 concentration were seen, whereas at 4 weeks, the concentrations were increased in rats at 1200 and 3200 ppm and in those given phenobarbital. At week 8 (i.e. during the recovery period), an increased T_4 concentration was seen in the group at 1200 ppm and in the group given phenobarbital. An increase was also seen in the group given 3200 ppm at week 13. The T_3 concentration was decreased in the group given 3200 ppm at week 4 and in the group given phenobarbital at weeks 1, 2 and 4.

Increased hepatic activities of cytochrome P450, aniline hydroxylase, EMD and PROD were seen in both the imazalil-treated and the phenobarbital-treated groups at weeks 1, 2 and 4. Induction was often found at the lowest dietary concentration. Enzyme induction was largely absent during week 8, indicating that it was a reversible phenomenon. The activity of 5′-monodeiodinase was decreased in the group given 3200 ppm imazalil, and T_4 glucuronosyltransferase activity was induced in all imazalil-treated groups after 1 week of treatment and also at week 4 in the group given 3200 ppm. Thyroid peroxidase activity was increased in the group at 3200 ppm after weeks 1 and 4 and reduced after 2 weeks of treatment; however, the changes were neither statistically significant nor clearly dose related.

Increases in the relative weight of the thyroid of animals at 3200 ppm were seen at week 2 only, whereas phenobarbital increased both the relative and absolute thyroid weights at this time. At week 2, a positive trend in the relative thyroid weight with dose of imazalil was found. At week 1, the relative and absolute weights of the liver were increased at 400 and 3200 ppm, and only the relative weight was increased at 1200 ppm. At week 2, the relative liver weight was increased at 1200 and 3200 ppm, whereas at week 4, the relative and absolute liver weights were increased at 400 and 1200 ppm. The relative liver weight was increased at week 4 at the highest dietary concentration. At 8 weeks, a decrease in absolute but not relative liver weight was observed at the intermediate and high dietary concentrations of imazalil. Phenobarbital increased the absolute and relative weights of the liver at 1, 2 and 4 weeks, but this effect had disappeared by 8 weeks. At the terminal kill at 13 weeks, the highest concentration of imazalil had produced a decrease in the absolute weight but not in the relative weight of the liver, whereas phenobarbital had no effect.

The only gross findings of interest were in the liver and consisted of swelling in a few animals at 400 and 1200 ppm at weeks 2 and 4, respectively, and swelling and increased lobulation at 3200 ppm from week 1 onwards. Histologically, hepatic centrilobular hypertrophy was found at concentrations of 400 ppm and above, and the occurrence of this change appeared to be dose related. Periportal hypertrophy was found at 1200 and 3200 ppm, whereas hepatic vacuolation was seen at the highest dietary concentration. All three changes in the liver were also seen with phenobarbital. These changes were not observed 8 and 13 weeks after the start of the study (i.e. during the recovery period). Significant increases in the frequency of thyroid follicular cell hypertrophy were seen only with phenobarbital. This lesion disappeared during the recovery period. Immunohistochemistry for BrdU-labelled cells did not reveal intergroup differences. The authors concluded that imazalil alters thyroid status by affecting hepatic and thyroid enzymes involved in the synthesis, metabolism and excretion of T_4 (Piccirillo, 2000; Verbeek et al., 2000; Vermeir, 2000). These studies were not previously evaluated by JMPR.

Conclusion

Qualitatively, these studies show that imazalil causes hypertrophy of the liver and, at high doses, some interference with thyroid function. The quantitative differences between the studies may be related to their duration. The results for induction of specific hepatic enzymes were not easy to interpret, as they tended to be inconsistent, and dose–effect relationships were not always clear. However, the 2001 Meeting concluded that imazalil is a relatively nonspecific enzyme inducer in the liver and that its enzyme-inducing effects are reversible (Annex 1, reference *94*).

(c) Endocrine disruption

Imazalil was tested for its ability to activate the estrogen receptor in vitro in a proliferation assay with MCF7 cells and a screening test for yeast estrogen. Statistically significant agonism was not observed (Vinggaard, Breinholt & Larsen, 1999).

11β-Hydroxylation and 19-hydroxylation in the mitochondria of gerbils (*Meriones unguiculatus*) in vitro were suppressed in parallel by about 75% by imazalil (purity not reported) at 1 µmol/L (Drummond, Mason & McCarthy, 1988).

In a study of the effect of imazalil (purity 97–99.9%) on CYP19 aromatase activity in human placental microsomes, the median inhibitory concentration was 0.34 µmol/L (Vinggaard et al., 2000).

(d) Studies on metabolites

The acute oral toxicity and/or the genotoxic potential of the metabolites R043449 (FK284), R061000 (FK772) and R014821 were evaluated in recently completed studies that have not previously been evaluated by JMPR. In addition, a study designed for setting an ARfD, also not previously evaluated by JMPR, was carried out with R14821.

R043449 (FK284) (3-[2-(2,4-dichlorophenyl)-2-hydroxymethyl]-2,4-imidazolidinedione)

In an acute oral toxicity study, six female Sprague Dawley rats received a single gavage dose of R043449 (purity 101.5%) in 10 mL/kg bw 0.8% aqueous hydroxypropyl methylcellulose at 2000 mg/kg bw. Animals were observed for clinical signs and mortality before and immediately after dosing, at 5, 15, 30 and 60 minutes after dosing, as well as at 3, 6 and 24 hours after administration. Thereafter, all animals were observed daily for up to 14 days. Observations for changes to skin and fur, eyes and mucous membranes, respiratory and circulatory systems, autonomic and central nervous systems, somatomotor activity as well as behaviour patterns were made at least once a day. Attention was also paid to possible tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. Individual body weights were recorded before administration of the test item and thereafter in weekly

intervals up to the end of the study. Changes in weight were calculated and recorded. At the end of the experiments, all animals were terminated, dissected and inspected macroscopically. All gross pathological changes were recorded. Necropsy and macroscopic inspection of animals that died prematurely were carried out as soon as possible after death.

There were no clinical signs or mortalities during the study. All animals gained weight normally during the study. There were no findings upon necropsy.

The LD_{50} was greater than 2000 mg/kg bw (Haferkorn, 2010a).

The mutagenicity of R043449 (purity 101.5%) was examined in a bacterial reverse mutation assay using five *Salmonella typhimurium* strains (TA98, TA100, TA102, TA1535 and TA1537). Two independent experiments were carried out, each with and without metabolic activation (S9 liver fraction derived from Aroclor 1254–induced rats). Ten concentrations ranging from 0.316 to 5000 µg/plate were tested. Pronounced cytotoxicity (scarce background lawn and reduction of the number of revertants by more than 50%) was noted at the top concentration of 5000 µg/plate.

No mutagenic effect (no increase in revertant colony numbers compared with control counts) was observed for R043449 tested up to a cytotoxic concentration of 5000 µg/plate in any of the five test strains in two independent experiments (plate incorporation and preincubation test), each without and with metabolic activation (Flügge, 2010).

In an in vitro chromosomal aberration test, R043449 (purity 100%) was tested in Chinese hamster V79 lung cells up to cytotoxic doses for 3 hours with and without S9 mix and for 20 hours without S9 mix. DMSO was used as the vehicle. In assay 1, insolubility was detected at the end of treatment in the 500–1000 µg/mL concentration range with and without S9 mix. Cytotoxicity was observed at 1000 µg/mL. Without S9 mix (20-hour treatment, harvesting at 28 hours), cytotoxicity was observed at 300 µg/mL. No statistically significant increases in mean percentage of aberrant cells (excluding gaps) were observed at any of the concentrations used in any of the experiments. Polyploid metaphases (1–4) were found in some cases in the negative (vehicle) control, test item–treated or positive control samples in both assays, whereas no endoreduplicated metaphases were found in any samples.

In conclusion, no statistically significant increases in mean percentage of aberrant cells (excluding gaps) were noted with and without S9 mix. Therefore, R043449 is considered to be not clastogenic under the conditions of this study (Hargitai, 2017a).

In an in vitro mammalian cell gene mutation test, R043449 (purity 100%) was tested in Chinese hamster ovary (CHO K1) cells at the *Hprt* locus. Two assays were carried out; in assay 1, treatments were carried out for 5 hours with and without S9 mix, and in assay 2, treatments were carried out for 5 hours with S9 mix and for 24 hours without S9 mix. DMSO was used as the vehicle. In both assays, minimal to full insolubility was detected in the 600–800 µg/mL range at the end of treatment with or without S9 mix. Cytotoxicity was observed at the top doses in all assays.

No statistically significant or biologically relevant increase in mutant frequency was observed at any concentration tested, and no dose–response relationship was evident after trend analysis both with and without S9 mix. Therefore, it is concluded that R043449 (FK284) is not mutagenic in mammalian cells in either the presence or absence of S9 mix under the conditions of this assay (Hargitai, 2017b).

R061000 (FK772) ((±)-3-[2-(2,4-dichlorophenyl)-2-(2,3-dihydroxypropoxy)ethyl]-2,4-imidazolidinedione)

In an acute oral toxicity study, six female Sprague Dawley rats received a single gavage dose of R061000 (purity ~97.3%) at 2000 mg/kg bw in 10 mL/kg bw 0.8% aqueous hydroxypropyl methylcellulose. Animals were observed for clinical signs and mortality before and immediately after dosing, at 5, 15, 30 and 60 minutes after dosing, as well as at 3, 6 and 24 hours after administration. Thereafter, all animals were observed daily for up to 14 days. Observations for changes to skin and fur, eyes and mucous membranes, respiratory and circulatory systems, autonomic and central nervous systems, somatomotor activity as well as behaviour patterns were made at least once a day. Attention was also paid to possible tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. Individual body weights were recorded before administration of the test item and thereafter in weekly intervals up to the end of the study. Changes in weight were calculated and recorded. At the end of the experiments, all animals were terminated, dissected and inspected macroscopically. All gross pathological changes were recorded. Necropsy and macroscopic inspection of animals that died prematurely were carried out as soon as possible after death.

There were no mortalities during the study. Piloerection was observed in all animals 15 minutes to 3 hours after administration. There were no other clinical signs. All animals gained weight normally during the study. There were no findings upon necropsy.

The LD_{50} was greater than 2000 mg/kg bw (Haferkorn, 2010b).

The mutagenicity of R061000 (purity ~97.3%) was examined in a bacterial reverse mutation assay using five *S. typhimurium* strains (TA98, TA100, TA102, TA1535 and TA1537) in the presence and absence of metabolic activation (S9 liver fraction derived from Aroclor 1254–induced rats). Six concentrations of R061000 ranging from 31.6 to 5000 μg/plate were employed in the plate incorporation test and in the preincubation test, each carried out without and with metabolic activation.

No signs of cytotoxicity were noted at the top concentration of 5000 μg/plate. No mutagenic effect (no increase in revertant colony numbers compared with control counts) was observed for R061000 tested up to 5000 μg/plate in any of the five test strains in two independent experiments (plate incorporation and preincubation test), each without and with metabolic activation.

R061000 (FK772) was not mutagenic up to the limit concentration of 5000 μg/plate in this bacterial reverse mutagenicity assay (Flügge, 2012).

In an in vitro chromosomal aberration test, R061000 (purity 97.7%) was tested in Chinese hamster V79 lung cells up to cytotoxic doses for 3 hours with and without S9 mix and for 20 hours without S9 mix. DMSO was used as the vehicle. In both assays, no insolubility and no large changes in pH or osmolality were detected at the end of treatment at concentrations up to 2000 µg/mL with and without S9 mix. In assay 1, no statistically significant increases in mean percentage of aberrant cells (excluding gaps) were observed at any of the concentrations used both with and without S9 mix. In assay 2, with S9 mix, no statistically significant increases in mean percentage of aberrant cells (excluding gaps) were observed at any of the concentrations used. Without S9 mix, statistically significant increases in mean percentage of aberrant cells (excluding gaps) were observed at 750 and 1000 µg/mL. The mean percentage of aberrant cells (excluding gaps) of 4.0% was at the upper limit of the historical control range of the laboratory (0–4%), and the response at 1000 µg/mL of 5.3% was obtained at a cytotoxic level (relative increase in cell count of 35%). However, based on the very weak response (20% aberration level is 4320 µg/mL, which is above the maximum concentration that is appropriate for testing), lack of repeatability and lack of biological relevance, the observed results were not considered indicative of clear and significant clastogenic activity under the conditions of this test system. The occurrence of polyploid and endoreduplicated metaphases was recorded. Polyploid metaphases (1–6) were found in some cases in the negative (vehicle) control, test item–treated or

positive control samples in both assays. No endoreduplicated metaphases were found in assay 1, whereas an endoreduplicated metaphase was recorded in assay 2 for the negative (vehicle) control sample without S9 mix and one test item–treated sample with S9 mix. The negative (vehicle) control data were within the acceptable range for the spontaneous chromosomal aberration frequency, and the positive control substances demonstrated the sensitivity of the test system.

Therefore, R061000 is considered not clastogenic under the conditions of this study (Hargitai, 2017c).

In an in vitro mammalian cell gene mutation test, R061000 (purity 97.7%) was tested in Chinese hamster ovary (CHO K1) cells at the *Hprt* locus in the presence and absence of metabolic activation. DMSO was used as the vehicle. Minimal to full insolubility was detected at the end of the treatment in assay 1 at 1000–1750 µg/mL with S9 mix and in assay 2 at 1900–2000 µg/mL without S9 mix and at 1000–2000 µg/mL with S9 mix.

No statistically significant increase in the mutant frequency value was observed in this experiment at any examined concentration. No dose–response relationship was observed (a trend analysis showed no effect of treatment). Therefore, it is concluded that R061000 (FK772) is not mutagenic in mammalian cells in either the presence or absence of S9 mix under the conditions of this assay (Hargitai, 2017d).

R014821 ((RS)-1-(2,4-dichlorophenyl)-2-imidazol-1-yl-ethanol)

In a single-dose oral toxicity study, two parallel sets of Wistar rats (five of each sex per dose per set) were gavaged with a single dose of R014821 (batch no. M12GB4078; purity 98.0%), at 0, 125, 500 or 2000 mg/kg bw. The vehicle was PEG 400. One set of animals (control group and three treated dose groups) was terminated at 24 hours for examination of acute effects, whereas the parallel set was subjected to termination at 7 days post-administration for delayed effects or recovery. Mortality was assessed at least twice daily. Clinical signs were assessed on all animals daily up to and including the day of necropsy. Necropsy was performed on day 2 for main group animals and on day 8 for recovery groups. Body weights were recorded on days 1, 2, 4, 7 and 8. Feed consumption was recorded for days 1–2, 2–5 and 5–7. Neurobehavioural examinations were made on all recovery group males prior to treatment and at 4 and 24 hours and 7 days after treatment. Clinical laboratory investigations were made on day 2 for main group animals and on day 8 for recovery group animals. Haematological and clinical chemistry parameters were evaluated. Prothrombin clotting time was determined. At necropsy, all animals were investigated for macroscopic abnormalities, and samples of all organs (according to guidelines) were subjected to histopathological examination. Organ weights were recorded for selected organs. Formulation analyses confirmed that formulations of test substance in PEG 400 were prepared accurately and homogeneously distributed and were stable over at least 6 hours.

No preterminal mortalities were observed. Lethargy was noted in one male at the top dose. Body weight generally remained static at 2000 mg/kg bw in males over the entire study; cumulative total feed consumption up to the end of week 1 was reduced by 20% in this group. In females, body weight gain at 500 and 2000 mg/kg bw was about half those of the controls and the 125 mg/kg bw group; cumulative total feed consumption up to the end of week 1 was likewise 12% lower in these groups. Neurobehavioural observations at 2000 mg/kg bw comprised lethargy in two animals at 4 hours post-dosing, abnormal posture in one animal at 4 hours post-dosing, both being reversible within 24 hours, hunched posture after 7 days in one animal and loss of righting reflex in three animals after 24 hours, fully reversible within 7 days. Owing to the transient and incidental nature of these findings, they were not considered to be toxicologically relevant. Rectal temperature was not affected. No toxicologically relevant effects on haematological parameters were observed that could be attributed to treatment. No microscopic correlate for the significant prolongation of prothrombin clotting was seen in males at 2000 mg/kg bw. Various changes in clinical biochemistry parameters were noted that were essentially reversible within 7 days, except for decreased bilirubin in males and

decreased urea levels in females, which were considered to be within the normal range for rats of this age and strain. At 125 and 500 mg/kg bw, these findings were not accompanied by clear histopathological lesions. These findings comprised lower ALP, lower total protein in males and higher total protein in females. Albumin, bilirubin, urea, phospholipids, potassium and calcium were reduced, and sodium levels were increased. At the top dose (2000 mg/kg bw), liver, adrenal and spleen weights (absolute and relative to body weight) were increased, without macroscopic pathological findings. At the end of recovery, absolute and relative liver weights remained increased in both sexes at the top dose. Increased adrenal weights in all male dose groups did not show any dose–effect relationship and were considered to be caused by relatively low control values and hence not of toxicological relevance. Microscopic examination revealed a minimal increase in myelopoiesis of the sternal bone marrow in males treated with 2000 mg/kg bw and terminated at 24 hours. After recovery of 7 days, hepatocellular vacuolation of liver in both sexes at the middle and top doses was found. Hypertrophy of the follicular epithelium of the thyroid gland in males and cortical hypertrophy of adrenal glands in females were seen at 2000 mg/kg bw. All other findings were considered to be within the normal background ranges encountered for Wistar-Han strain rats.

The NOAEL was 125 mg/kg bw, based on reduced feed consumption and body weight gain at 500 mg/kg bw (Beerens-Heijnen, 2013).

In an in vitro chromosomal aberration test, R014821 (purity 99.4%) was tested in Chinese hamster V79 lung cells up to cytotoxic doses for 3 hours with and without S9 mix and for 20 hours without S9 mix. DMSO was used as the vehicle. Cytotoxicity was observed at 400 µg/mL. No statistically significant increases in the mean percentage of aberrant cells (excluding gaps) were observed at any of the concentrations used both with and without S9 mix.

Therefore, R014821 is considered to be not clastogenic under the conditions of this study (Hargitai, 2017f).

In an in vitro mammalian cell gene mutation test, R014821 (purity 99.4%) was tested in Chinese hamster ovary (CHO K1) cells at the *Hprt* locus in the presence and absence of metabolic activation. DMSO was used as the vehicle. Excessive cytotoxicity was observed at 400–500 µg/mL. The mutation frequency of the vehicle control was within the historical control range of the laboratory in all assays. The positive controls gave the anticipated increases in mutation frequency over the controls and were in good concordance with historical data in all assays.

It can be concluded that R14821 is not mutagenic in mammalian cells in either the presence or absence of S9 mix under the conditions of this assay (Hargitai, 2017e).

3. Observations in humans

3.1 Medical surveillance on manufacturing plant personnel

A statement was received from the occupational medical advisor for one of the imazalil manufacturing plants. Up to 2016, there was no evidence or indication of negative effects in workers as a result of active participation in imazalil processing (Wiener, 2016).

3.2 Case reports

A 43-year-old female veterinary technician developed contact dermatitis following exposure to the veterinary antimycotic Imaverol, which contains imazalil (enilconazole). Patch testing showed sensitivity to several imidazoles, including imazalil (Van Hecke & de Vos, 1983).

A case of palatal and nasal infection with *Alternaria* was treated with imazalil at doses progressing from 50 to 1200 mg administered topically and orally. The drug was tolerated, but it was

found to be bitter and unpleasant, with an aftertaste lasting for hours, and nausea occurred at high doses (≥800 mg). Very limited studies of pharmacokinetics were carried out: at a dose of 400 mg/day, the maximum serum concentration was about 2 µg/mL, whereas at a dose of 1200 mg/day, the maximum serum concentration was about 4 µg/mL. The half-life was about 2 hours, and administration of 1200 mg daily for 1 month did not result in accumulation. No changes in clinical chemical parameters were seen (Stiller & Stevens, 1986).

3.3 Experimental studies

A study in volunteers (19 males and one female) was undertaken to examine the local effects of imazalil on the skin. Imazalil base (0.2% w/w), imazalil base (0.2% w/w) ointment, imazalil sulfate (526 mg base/L), Fungaflor (68% w/w imazalil) and two control formulations not containing imazalil were applied under occlusive adhesive strips for 48 hours. The skin was then examined for redness, oedema, irritation or inflammation. There were no complaints of burning, itching or pain, and the skin at the sites of application appeared normal in all cases (Desplenter & Verhamme, 1979).

Comments

Biochemical aspects

After administration of [^{14}C]imazalil to rats by gavage at a single dose of 1.25 or 20 mg/kg bw or a repeated dose of 1.25 mg/kg bw of non-radiolabelled material for 14 days followed by a single oral dose of radiolabelled test compound, [^{14}C]imazalil was rapidly and nearly completely absorbed. Most of the label was excreted (approximately 90%) within 24 hours. More imazalil appeared in the urine (49–60% of the administered dose) than in the faeces (36–48% of the administered dose) with all dosing regimens. At 96 hours after oral administration of [^{14}C]imazalil, tissue concentrations (including carcass) of radioactivity were about 1% of the administered dose. Nearly 50% of the radiolabel retained in the body was found in the liver (Mannens, Van Leemput & Heykants, 1993).

Very little imazalil was excreted unchanged, and the compound was metabolized to at least 25 metabolites. The metabolic profiles in the urine and faeces were largely comparable. Moreover, identical metabolites were recovered in the excreta regardless of sex, dose or dosing regimen. The main routes of metabolism were epoxidation, epoxide hydration, oxidative *O*-dealkylation, imidazole oxidation and scission, and oxidative *N*-dealkylation. The metabolic pattern was similar for both sexes after both oral and intravenous administration (Mannens, Van Leemput & Heykants, 1993).

Toxicological data

In rats, the lowest acute oral LD_{50} for imazalil was 227 mg/kg bw (Niemegeers, 1979), the acute dermal LD_{50} was greater than 2000 mg/kg bw (Dreher, 1990b) and the acute inhalation LC_{50} was 1.84 mg/L (Blagden, 1990). Imazalil was mildly irritating to the skin of rabbits (Dreher, 1990c) and severely irritating to their eyes (Teuns et al., 1990b). It was not sensitizing to the skin of guinea-pigs, as determined by the Magnusson and Kligman test (Teuns et al., 1990c) and the Buehler test (Wnorowski, 1997).

In a single-dose oral toxicity study, rats were administered imazalil via gavage at a dose of 0, 25, 100 or 400 mg/kg bw. The NOAEL was 100 mg/kg bw, based on an increased incidence of slight hepatocellular vacuolation in both sexes and increased liver weights in females at 400 mg/kg bw (Teunissen, 2013).

Short- and long-term studies in mice, rats and dogs showed that the main target organ of toxicity was the liver.

In a 3-month toxicity study in mice, imazalil was administered in the diet at a concentration of 0, 200, 400 or 800 ppm (equivalent to 0, 30, 60 and 120 mg/kg bw per day, respectively). A NOAEL

could not be identified in this study, as hepatocytic vacuolation was seen at all doses in males (Verstraeten et al., 1993a).

In a 4-week range-finding toxicity study in rats, imazalil was administered at a dietary concentration of 0, 100, 1000, 2000 or 3000 ppm (equal to 0, 11.5, 116.7, 232.1 and 351.3 mg/kg bw per day for males and 0, 11.7, 124.0, 229.0 and 351.6 mg/kg bw per day for females, respectively). The NOAEL was 100 ppm (equal to 11.5 mg/kg bw per day), based on decreased body weight, feed consumption and heart, spleen, kidney and testis weights in males and increased liver weights in females at 1000 ppm (equivalent to 116.7 mg/kg bw per day) (Gur, Nyska & Crown, 1990).

In a 13-week study in rats, imazalil was administered at a dietary concentration of 0, 40, 200 or 1000 ppm (equal to 0, 4.02, 18.78 and 94.58 mg/kg bw per day for males and 0, 4.05, 20.28 and 99.34 mg/kg bw per day for females, respectively). The NOAEL was 40 ppm (equal to 4.02 mg/kg bw per day), on the basis of liver histopathology and associated clinical chemistry changes at 200 ppm (equal to 18.78 mg/kg bw per day) (Gur, Nyska & Waner, 1991).

Imazalil was administered to rats for 6 months at a dietary concentration of 0, 25, 100 or 400 ppm (equivalent to 0, 1.25, 5.0 and 20 mg/kg bw per day, respectively). The NOAEL was 100 ppm (equivalent to 5.0 mg/kg bw per day), on the basis of changes in liver, kidney, lung and thymus weights in females and increased relative kidney weights in males at 400 ppm (equivalent to 20 mg/kg bw per day) (Lina et al., 1983).

In a 1-year study, dogs received imazalil at a dose of 0, 1.25, 2.5 or 20 mg/kg bw per day orally in gelatine capsules. The NOAEL was 2.5 mg/kg bw per day, on the basis of clinical signs, decreased body weight gain and feed consumption, decreased serum calcium concentration, increased serum ALP activity and increased liver weights at 20 mg/kg bw per day (Verstraeten et al., 1989).

In a 2-year study, dogs received imazalil at a dose of 0, 1.25, 5 or 20 mg/kg bw per day by capsule. The NOAEL was 1.25 mg/kg bw per day, based on decreased body weight gain and slight ground glass aspect of the cytoplasm in centrilobular hepatocytes at 5 mg/kg bw per day (Marsboom et al., 1977).

The overall NOAEL for imazalil in dogs was 2.5 mg/kg bw per day, on the basis of decreased body weight, decreased body weight gain and liver toxicity seen at 5 mg/kg bw per day.

In a study of carcinogenicity, mice received imazalil sulfate via the drinking-water at a concentration of 0, 6.25, 25 or 100 ppm (equivalent to 0, 2.5, 10 and 40 mg/kg bw per day, respectively) for 18 months (Marsboom & Herin, 1979a). This study was not considered to be acceptable for the evaluation because of several shortcomings in its design and conduct.

In a 23-month study of carcinogenicity, mice received imazalil at a dietary concentration of 0, 50, 200 or 600 ppm (equal to 0, 8.1, 33.4 and 105 mg/kg bw per day for males and 0, 9.9, 41.6 and 131 mg/kg bw per day for females, respectively). The NOAEL for toxicity was 50 ppm (equal to 8.1 mg/kg bw per day), on the basis of morphological changes (foci and nodules) in the livers of males at 200 ppm (equal to 33.4 mg/kg bw per day). Liver adenomas were observed in males at 200 and 600 ppm and in females at 600 ppm. The NOAEL for carcinogenicity was 50 ppm (equal to 8.1 mg/kg bw per day), based on an increased incidence of adenomas seen in males at 200 ppm (equal to 33.4 mg/kg bw per day) (Verstraeten et al., 1993b).

In an 18-month study of toxicity in rats, imazalil was administered at a dietary concentration of 0, 25, 100 or 400 ppm (equivalent to 0, 1.2, 5 and 20 mg/kg bw per day, respectively). The NOAEL was 100 ppm (equivalent to 5 mg/kg bw per day), on the basis of decreased body weight gain in females, decreased plasma albumin concentration in males and pathological changes in the livers of males at 400 ppm (equivalent to 20 mg/kg bw per day) (Lina et al., 1984). Although there was no evidence that imazalil was carcinogenic, the duration of the study and the number of animals used were insufficient to exclude that possibility.

In a 24-month study of toxicity and carcinogenicity, rats received diets containing imazalil at a concentration of 0, 50, 200, 1200 or 2400 ppm (equal to 0, 2.4, 9.7, 58 and 120 mg/kg bw per day for males and 0, 3.4, 13.5, 79 and 157 mg/kg bw per day for females, respectively). The NOAEL for

toxicity was 50 ppm (equal to 2.4 mg/kg bw per day), on the basis of minor haematological changes in both sexes and increased blood glucose concentrations and hepatic changes (increased relative liver weight and an increased frequency of pigment-laden hepatocytes) in females at 200 ppm (equal to 9.7 mg/kg bw per day). The NOAEL for carcinogenicity was 200 ppm (equal to 9.7 mg/kg bw per day), based on an increased incidence of follicular cell neoplasia (adenoma and carcinoma combined) of the thyroid in males at 1200 ppm (equal to 58 mg/kg bw per day) (Van Deun, 1999a).

In a 30-month study of carcinogenicity, imazalil was administered to rats at a dietary concentration of 0, 25, 100 or 400 ppm (equal to 0, 1.0, 3.6 and 15.0 mg/kg bw per day for males and 0, 1.2, 4.7 and 19.7 mg/kg bw per day for females, respectively). The NOAEL for toxicity was 100 ppm (equal to 3.6 mg/kg bw per day), on the basis of decreased body weight gain in males at 400 ppm (equal to 15.0 mg/kg bw per day). No treatment-related histopathological effects were observed in the liver, and there was no treatment-related increase in the incidence of tumours (Til et al., 1985). The Meeting noted that the highest dose tested was lower than the LOAEL for carcinogenicity in the 24-month study.

The Meeting concluded that imazalil is carcinogenic in mice and rats.

Imazalil was tested for genotoxicity in an adequate range of in vitro and in vivo assays. No evidence of genotoxicity was found (Steelman & Schreiner, 1977; Vanparys & Marsboom, 1979, 1982, 1987a,b, 1988a,b; Fautz, 1990; Jenkinson, 1990a,b; Vanparys et al., 1990; Watabe, 1992; Van Gompel, Vanparys & Van Cauteren, 1995; Clare, 1996).

The Meeting concluded that imazalil is unlikely to be genotoxic.

The results of several mechanistic studies of the liver effects of imazalil in mice and rats and thyroid effects in rats indicate that imazalil has a phenobarbital-like mode of action in the induction of liver and thyroid tumours in rodents. The modes of action for these tumours were assessed using the International Programme on Chemical Safety (IPCS) human relevance framework (see Appendix 1). It was concluded that these carcinogenic responses are not relevant to humans.

In view of the lack of genotoxicity and the lack of human relevance of the tumours observed in mice and rats, the Meeting concluded that imazalil is unlikely to pose a carcinogenic risk to humans.

A two-generation study of reproductive toxicity was conducted in rats, in which imazalil was administered in the diet at a nominal dose of 0, 5, 20 or 80 mg/kg bw per day. The NOAEL for parental toxicity was 20 mg/kg bw per day, on the basis of reduced maternal weight gain and hepatotoxicity (vacuoles) in males at 80 mg/kg bw per day. The NOAEL for offspring toxicity was 20 mg/kg bw per day, on the basis of decreased numbers of live pups, decreased survival rate of pups and increased numbers of stillbirths at 80 mg/kg bw per day. The NOAEL for reproductive toxicity was 20 mg/kg bw per day, on the basis of increased duration of gestation for the F_0 and F_1 females and decreased gestation rate in F_1 females at 80 mg/kg bw per day (Dirkx et al., 1992b).

In a developmental toxicity study in mice, imazalil was administered by gavage during gestation days 6 through 16 at a dose of 0, 40, 80 or 120 mg/kg bw per day. The NOAEL for maternal toxicity was 40 mg/kg bw per day, on the basis of reduced body weight gain and feed consumption at 80 mg/kg bw per day. No NOAEL was identified for embryo and fetal toxicity, as litter size and the number of live pups were decreased in all dose groups (Gillardin, Sanz & Marsboom, 1987).

In a second study in mice, imazalil was administered by gavage on gestation days 6 through 16 at a dose of 0, 10, 40, 80 or 120 mg/kg bw per day. The NOAEL for maternal toxicity was 10 mg/kg bw per day, on the basis of decreased body weight gain and reduced feed consumption at 40 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 80 mg/kg bw per day, on the basis of reduced number of live fetuses, increased number of resorptions, and decreased pup body weights at 120 mg/kg bw per day. There was no evidence of teratogenicity (Levron et al., 1991).

In a study of developmental toxicity in rats, imazalil was administered by gavage on gestation days 6 through 16 at a dose of 0, 40, 80 or 120 mg/kg bw per day. No teratogenic effects were seen. The NOAEL for embryo and fetal toxicity was 40 mg/kg bw per day, on the basis of reduced pup

body weight at 80 mg/kg bw per day. A NOAEL for maternal toxicity could not be identified because of lower maternal body weight in all the groups when compared with controls (Gillardin et al., 1988).

The developmental toxicity of imazalil in rabbits was studied at gavage doses of 0, 1.25, 2.5 and 5 mg/kg bw per day administered on gestation days 6–18. The NOAEL for both maternal and embryo/fetal toxicity was 5 mg/kg bw per day, the highest dose tested (Dirkx & Marsboom, 1985).

In another study of developmental toxicity in rabbits administered imazalil sulfate by gavage at a dose of 0, 5, 10 or 20 mg/kg bw per day on gestation days 6–18, the NOAEL for maternal toxicity was 5 mg/kg bw per day, on the basis of reduced feed consumption at 10 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 5 mg/kg bw per day, on the basis of an increased incidence of resorptions and a decrease in the number of live pups at 10 mg/kg bw per day (Dirkx et al., 1992a).

The Meeting concluded that imazalil is not teratogenic.

In an acute neurotoxicity study, rats were given a single oral dose of imazalil at 0, 60, 180 or 600 mg/kg bw and observed for 14 days. The NOAEL for systemic toxicity was 180 mg/kg bw, on the basis of decreased body weight gains in males, deaths in females, and FOB and motor activity alterations at the time of peak effect on day 0 in females at 600 mg/kg bw. The NOAEL for neurotoxicity was 600 mg/kg bw, the highest dose tested (Beck, 2006b). The Meeting noted that the highest dose used was above the LD_{50}, and therefore the FOB results are due to general toxicity.

In a published study of reproductive toxicity in which neurobehavioural end-points were measured, mice were fed imazalil in the diet at a concentration of 0, 120, 240 or 480 ppm (equal to 0, 19, 39 and 79 mg/kg bw per day for F_0 males and 0, 26, 45 and 102 mg/kg bw per day for F_0 females before conception). The NOAEL for developmental neurotoxicity was 120 ppm (equal to 19 mg/kg bw per day), based on effects on surface righting in males at 240 ppm (equal to 39 mg/kg bw per day) (Tanaka, 1995). The Meeting noted that parameters other than body weight were not measured in the other reproductive toxicity studies and that the lowest concentration used was higher than the NOAEL in other studies.

In a second published study of reproductive toxicity in which neurobehavioural end-points were measured, mice were fed imazalil in the diet at a concentration of 0, 6, 18 or 54 ppm (equal to 0, 0.85, 2.49 and 7.87 mg/kg bw per day, respectively, during gestation). Some neurobehavioural parameters were inconsistently affected at all doses (Tanaka et al., 2013).

The Meeting concluded that imazalil is not neurotoxic.

Toxicological data on metabolites and/or degradates

R061000 (rat and ruminant metabolite)

For metabolite R061000 (also known as rat metabolite 8), the acute oral LD_{50} in rats was greater than 2000 mg/kg bw (Haferkorn, 2010b). R061000 was negative for mutagenicity in a bacterial reverse mutation assay (Flügge, 2012), an in vitro chromosomal aberration test (Hargitai, 2017c) and an in vitro mammalian cell gene mutation test (Hargitai, 2017d).

The Meeting concluded that, based on the structure of R061000 and its low acute toxicity, this metabolite would be covered by the health-based guidance values for the parent compound.

R043449 (ruminant metabolite)

For metabolite R043449, the acute oral LD_{50} in rats was greater than 2000 mg/kg bw (Haferkorn, 2010a). R043449 was negative for mutagenicity in a bacterial reverse mutation assay (Flügge, 2010), an in vitro chromosomal aberration test (Hargitai, 2017a) and an in vitro mammalian cell gene mutation test (Hargitai, 2017b).

For R043449, the Meeting noted that the threshold of toxicological concern (TTC) approach (Cramer class III) could be applied for chronic toxicity.

R014821 (plant and rat metabolite)

A single-dose study was conducted for the metabolite R014821 (also known as rat metabolite 11). Rats were gavaged with R014821 at a single dose of 0, 125, 500 or 2000 mg/kg bw. The NOAEL was 125 mg/kg bw, based on reduced feed consumption and body weight gain at 500 mg/kg bw (Beerens-Heijnen, 2013). R014821 was negative for mutagenicity in an in vitro chromosomal aberration test (Hargitai, 2017f) and an in vitro mammalian cell gene mutation test (Hargitai, 2017e).

The Meeting concluded that, based on the structure of R014821 and its acute toxicity profile, this metabolite would be covered by the health-based guidance values for the parent compound.

Human data

A published case-study involving one woman indicated that imazalil used to treat a fungal infection was well tolerated after oral ingestion at doses of 50 mg/day progressing to 1200 mg/day over 6 months. The only adverse effect noted was nausea.

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

Toxicological evaluation

The Meeting reaffirmed the ADI of 0–0.03 mg/kg bw established by the 2001 Meeting. The present Meeting used the overall NOAEL of 2.5 mg/kg bw per day from 1-year and 2-year studies in dogs as the basis for this ADI and a safety factor of 100. The ADI is supported by a NOAEL of 2.4 mg/kg bw per day identified in a combined long-term toxicity and carcinogenicity study in rats. The Meeting noted that the LOAEL in the 2-year rat study was higher than the overall LOAEL in the two dog studies, and therefore the overall NOAEL from the dog studies was used as the basis of the ADI.

The Meeting reaffirmed the ARfD of 0.05 mg/kg bw established by the 2005 Meeting on the basis of a NOAEL of 5 mg/kg bw per day for both maternal (decreased feed consumption) and embryo/fetal toxicity (resorptions and decrease in number of live pups) in a developmental toxicity study in rabbits and a safety factor of 100. The Meeting was not able to determine whether the effects could occur following a single dose.

The ADI and ARfD for imazalil would also apply to the metabolites R061000 and R014821.

Levels relevant to risk assessment of imazalil

Species	Study	Effect	NOAEL	LOAEL
Mouse	Twenty-three-month study of toxicity and carcinogenicity[a]	Toxicity	50 ppm, equal to 8.1 mg/kg bw per day	200 ppm, equal to 33.4 mg/kg bw per day
		Carcinogenicity	50 ppm, equal to 8.1 mg/kg bw per day	200 ppm, equal to 33.4 mg/kg bw per day
	Developmental toxicity study[b]	Maternal toxicity	40 mg/kg bw per day	80 mg/kg bw per day
		Embryo and fetal toxicity	–	40 mg/kg bw per day[c]
	Developmental toxicity study[b]	Maternal toxicity	10 mg/kg bw per day	40 mg/kg bw per day

Species	Study	Effect	NOAEL	LOAEL
		Embryo and fetal toxicity	80 mg/kg bw per day	120 mg/kg bw per day
Rat	Two-year study of toxicity and carcinogenicity[a]	Toxicity	50 ppm, equal to 2.4 mg/kg bw per day	200 ppm, equal to 9.7 mg/kg bw per day
		Carcinogenicity	200 ppm, equal to 9.7 mg/kg bw per day	1 200 ppm, equal to 58 mg/kg bw per day
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	20 mg/kg bw per day	80 mg/kg bw per day
		Parental toxicity	20 mg/kg bw per day	80 mg/kg bw per day
		Offspring toxicity	20 mg/kg bw per day	80 mg/kg bw per day
	Developmental toxicity study[b]	Maternal toxicity	–	40 mg/kg bw per day[c]
		Embryo and fetal toxicity	40 mg/kg bw per day	80 mg/kg bw per day
Rabbit	Developmental toxicity study[b]	Maternal toxicity	5 mg/kg bw per day	10 mg/kg bw per day
		Embryo and fetal toxicity	5 mg/kg bw per day	10 mg/kg bw per day
Dog	One- and 2-year studies of toxicity[d,e]	Toxicity	2.5 mg/kg bw per day	5 mg/kg bw per day

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

Acceptable daily intake (ADI) (applies to imazalil and metabolites R061000 and R014821, expressed as imazalil)

0–0.03 mg/kg bw

Acute reference dose (ARfD) (applies to imazalil and metabolites R061000 and R014821, expressed as imazalil)

0.05 mg/kg bw

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

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

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

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid and nearly complete

Dermal absorption	No data
Distribution	Extensive; highest concentrations in liver followed by kidney
Potential for accumulation	No evidence of accumulation
Rate and extent of excretion	Rapid: >90% (about 50% in urine and about 40% in faeces) within 24 hours
Metabolism in animals	Extensive metabolism by epoxidation, epoxide hydration, oxidative *O*-dealkylation, imidazole oxidation and scission, oxidative *N*-dealkylation
Toxicologically significant compounds in animals and plants	Imazalil
Acute toxicity	
Rat, LD_{50}, oral	227 mg/kg bw
Rat, LD_{50}, dermal	>2 000 mg/kg bw
Rat, LC_{50}, inhalation	1.84 mg/L
Rabbit, dermal irritation	Mildly irritating
Rabbit, ocular irritation	Severely irritating
Guinea-pig, dermal sensitization	Not sensitizing (Magnusson and Kligman; Buehler)
Short-term studies of toxicity	
Target/critical effect	Decreased body weight gain and liver toxicity
Lowest relevant oral NOAEL	2.5 mg/kg bw per day (dog)
Lowest relevant dermal NOAEL	40 mg/kg bw per day (rabbit)
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Haematological changes, blood glucose, hepatic changes, follicular cell neoplasia
Lowest relevant NOAEL	2.4 mg/kg bw per day (rat)
Carcinogenicity	Carcinogenic in rats and mice; liver and thyroid tumours not relevant for humans based on mechanistic data[a]
Genotoxicity	
	No evidence of genotoxicity[a]
Reproductive toxicity	
Target/critical effect	Reduced pup viability
Lowest relevant parental NOAEL	20 mg/kg bw per day
Lowest relevant offspring NOAEL	20 mg/kg bw per day
Lowest relevant reproductive NOAEL	20 mg/kg bw per day
Developmental toxicity	
Target/critical effect	Increased number of resorptions, reduced number of live pups
Lowest relevant maternal NOAEL	5 mg/kg bw per day (rabbit)
Lowest relevant embryo/fetal NOAEL	5 mg/kg bw per day (rabbit)

Neurotoxicity	
Acute neurotoxicity NOAEL	600 mg/kg bw, highest dose tested (rat)
Subchronic neurotoxicity NOAEL	No data
Developmental neurotoxicity NOAEL	19 mg/kg bw per day (mouse)
Other toxicological studies	
Immunotoxicity	No data
Studies on toxicologically relevant metabolites	
R061000	
Acute oral LD_{50}	>2 000 mg/kg bw
Genotoxicity	No evidence of genotoxicity in vitro
R014821	
Single-dose study NOAEL	125 mg/kg bw
Genotoxicity	No evidence of genotoxicity in vitro
R043449	
Acute oral LD_{50}	> 2 000 mg/kg bw
Genotoxicity	No evidence of genotoxicity in vitro
Human data	
	High doses (up to 1 200 mg/day over 6 months) were well tolerated

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

Summary

	Value	Study	Safety factor
ADI[a]	0–0.03 mg/kg bw	One- and 2-year studies of toxicity in dogs	100
ARfD[a]	0.05 mg/kg bw	Developmental toxicity study in rabbits	100

[a] Applies to imazalil and metabolites R061000 and R014281, expressed as imazalil.

References

Appelman L, Woutersen R (1983). Acute inhalation toxicity study of an imazalil containing smoke, developed by a smoke-generator in rats. Unpublished report no. V83.308/230831. Civo Institutes TNO, Zeist, the Netherlands. Submitted to WHO by Janssen Pharmaceutica NV.

Beck MJ (2006a). An oral (gavage) dose range–finding acute neurotoxicity study of imazalil technical in rats. Unpublished report no. WIL-436007. WIL Research Laboratories, LLC, Ashland, OH, USA. Submitted to WHO by Janssen Pharmaceutica NV.

Beck MJ (2006b). An oral (gavage) acute neurotoxicity study of imazalil technical in rats. Unpublished report no. WIL-436006. WIL Research Laboratories, LLC, Ashland, OH, USA. Submitted to WHO by Janssen Pharmaceutica NV.

Beerens-Heijnen CGM (2013). Single dose oral gavage toxicity study with R14821 in Wistar rats. Unpublished report no. 502619. WIL Research Europe B.V., ’s-Hertogenbosch, the Netherlands. Submitted to WHO by Janssen Pharmaceutica NV.

Blagden SM (1990). Imazalil technical: acute inhalation toxicity study four-hour exposure (nose only) in the rat. Unpublished report no. 306/3 (MCW report no. R-5740). Safepharm Laboratories Ltd, Derby, United Kingdom. Submitted to WHO by ADAMA.

Burke DM, Thompson S, Elcombe CR, Halpert J, Haaparanta T, Mager RT (1985). Ethoxy-, pentoxy- and benzyloxyoxazones and homologues: a series of substrates to distinguish between different induced cytochrome P-450. Biochem Pharmacol. 34:3337–45.

Butler WH (1995). Pathology working group imazalil. Unpublished report. BIBRA International, Carshalton, Surrey, United Kingdom. Submitted to WHO by Janssen Pharmaceutica NV.

Clare C (1996). Imazalil: measurement of unscheduled DNA synthesis and replicative DNA synthesis in mouse liver using an in vivo/in vitro procedure. Unpublished report no. 1073/3-1052. Corning Hazleton, Harrogate, United Kingdom. Submitted to WHO by Janssen Pharmaceutica NV.

Desplenter L, Verhamme G (1979). Local effects of imazalil after application to the human skin. Unpublished preclinical research report. Department of Veterinary Clinical Research, Janssen Pharmaceutica Laboratories, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Đikić D, Mojsovic-Cuic A, Cupor I, Benkovic V, Horvat-Knezevic A, Lisi D et al. (2012). Carbendazim combined with imazalil or cypermethrin potentiate DNA damage in hepatocytes of mice. Hum Exp Toxicol. 31(5):492–502.

Dirkx P, Marsboom R (1985). R 18531 oral embryotoxicity and teratogenicity study in New Zealand white rabbits (segment II) – administration: oral. Unpublished report no. 1482 (85.18.04). Department of Toxicology, Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Dirkx P, Lampo A, Coussement W, Van Cauteren H (1992a). R 23979 – imazalil sulphate. Embryotoxicity and teratogenicity study in albino rabbits. Administration: orally by gavage. Unpublished report no. 2615. Department of Toxicology, Janssen Research Foundation, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Dirkx P, Lampo A, Vandenberghe J, Coussement W, Van Cauteren H (1992b). R 23979 – imazalil 2-generation reproduction study with 1 litter per generation in Wistar rats. Administration orally through the diet. Unpublished report no. 2337. Department of Toxicology, Janssen Research Foundation, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Dreher DM (1990a). Imazalil technical: acute oral toxicity test in the rat. Unpublished report no. 306/1 (MCW report no. R-5738). Safepharm Laboratories Ltd, Derby, United Kingdom. Submitted to WHO by ADAMA.

Dreher DM (1990b). Imazalil technical: acute dermal toxicity (limit test) in the rat. Unpublished report no. 306/2 (MCW report no. R-5739). Safepharm Laboratories Ltd, Derby, United Kingdom. Submitted to WHO by ADAMA.

Dreher DM (1990c). Imazalil technical: acute dermal irritation test in the rabbit. Unpublished report no. 306/4 (MCW report no. R-5741). Safepharm Laboratories Ltd, Derby, United Kingdom. Submitted to WHO by ADAMA.

Dreher DM (1990d). Imazalil technical: acute eye irritation test in the rabbit. Unpublished report no. 306/5 (MCW report no. R-5742). Safepharm Laboratories Ltd, Derby, United Kingdom. Submitted to WHO by ADAMA.

Dreher DM (1990e). Imazalil technical: Magnusson & Kligman maximisation study in the guinea pig. Unpublished report no. 306/6 (MCW report no. R-5743). Safepharm Laboratories Ltd, Derby, United Kingdom. Submitted to WHO by ADAMA.

Drummond TD, Mason JI, McCarthy JL (1988). Gerbil adrenal 11β- and 19-hydroxylating activities respond similarly to inhibitory or stimulatory agents: two activities of a single enzyme. J Steroid Biochem. 29:641–8.

Durando J (2006). Fungaflor technical (code ZR023979) primary skin irritation study in rabbits. Unpublished report no. 19035. Product Safety Laboratories, Dayton, NJ, USA. Submitted to WHO by Janssen Pharmaceutica NV.

Elcombe B (2012a). Imazalil: dietary 7-/28-day investigative toxicity study in wild type C57BL/6J, and PXR/CAR humanised mice. Final report 7 day exposure. Unpublished report no. CXR1085. CXR Biosciences, Dundee, United Kingdom. Submitted to WHO by Janssen Pharmaceutica NV.

Elcombe B (2012b). Imazalil – induction of cytochrome P450 2b, 3a and DNA-synthesis in cultured female mouse hepatocytes. Unpublished report no. CXR1227. CXR Biosciences, Dundee, United Kingdom. Submitted to WHO by Janssen Pharmaceutica NV.

Elcombe B (2012c). Imazalil – cytochrome P450 2B, 3A and DNA-synthesis induction in cultured female human hepatocytes. Unpublished report no. CXR1226. CXR Biosciences, Dundee, United Kingdom. Submitted to WHO by Janssen Pharmaceutica NV.

Elmore AR (2004a). Cell proliferation study in mice following dietary imazalil administration. Unpublished report no. C131-001. Integrated Laboratory Systems (ILS), Durham, NC, USA. Submitted to WHO by Janssen Pharmaceutica NV.

Elmore AR (2004b). BrdU assessment of rat livers following imazalil exposure. Unpublished report no. C131-002. Integrated Laboratory Systems (ILS), Durham, NC, USA. Submitted to WHO by Janssen Pharmaceutica NV.

Fautz R (1990). Unscheduled DNA synthesis in primary hepatocytes of male rats in vitro with imazalil. Unpublished report no. 192600. Cytotest Cell Research GmbH and Co. KG, Rossdorf, Germany, and Laboratory for Mutagenicity Testing, Darmstadt, Germany. Submitted to WHO by Janssen Pharmaceutica NV.

Flügge C (2010). Mutagenicity study of FK284 in the *Salmonella typhimurium* reverse mutation assay (in vitro). Unpublished report no. R-27265 (LPT report no. 25984). LPT Laboratory of Pharmacology and Toxicology GmbH & Co., Hamburg, Germany. Submitted to WHO by Janssen Pharmaceutica NV.

Flügge C (2012). Mutagenicity study of FK772 in the *Salmonella typhimurium* reverse mutation assay (in vitro). Unpublished report no. R-27261 (LPT report no. 25982). LPT Laboratory of Pharmacology and Toxicology GmbH & Co., Hamburg, Germany. Submitted to WHO by Janssen Pharmaceutica NV.

Gillardin JM, Sanz G, Marsboom R (1987). R 27180 embryotoxicity and teratogenicity study in COBS CD1 mice (segment II): oral by gavage. Unpublished report no. 86-07 (86.09.18). Research Department, Laboratoires Janssen, Aubervilliers, France. Submitted to WHO by Janssen Pharmaceutica NV.

Gillardin JM, Van Cauteren H, Sanz G, Marsboom R (1988). R 27180 embryotoxicity and teratogenicity study in Sprague-Dawley rats (segment II): oral by gavage. Unpublished report no. 2003/88-05. Research Department, Laboratoires Janssen, Aubervilliers, France. Submitted to WHO by Janssen Pharmaceutica NV.

Glaza S (1996). Primary eye irritation study of Magnate (imazalil) technical in rabbits. Lab project no. CHW 6413-138. Corning Hazelton. Submitted to WHO by Janssen Pharmaceutica NV.

Goodwine WR (1990). Comparative acute oral toxicity studies of the different salts of imazalil in rats. Unpublished report no. R23979/15, 81-1, REC # 21. Department of Pharmacology, Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Gur E, Nyska A, Crown S (1990). R 5767 imazalil: oral toxicity in dietary administration to rats; a 4-week preliminary range finding study. Unpublished preclinical research report no. LSRI MAK/208/IMA (MCW report no. R-5767). Life Science Research Israel Ltd, Ness Ziona, Israel. Submitted to WHO by ADAMA.

Gur E, Nyska A, Waner T (1991). R 5766 imazalil: toxicity in dietary administration to rats for 13 weeks. Unpublished preclinical research report no. LSRI MAK/209/IMA (MCW report no. R-5766). Life Science Research Israel Ltd, Ness Ziona, Israel. Submitted to WHO by ADAMA.

Haferkorn J (2010a). Acute oral toxicity study of FK284 in rats. Unpublished report no. R-27266 (LPT report no. 25985). LPT Laboratory of Pharmacology and Toxicology GmbH & Co., Hamburg, Germany. Submitted to WHO by Janssen Pharmaceutica NV.

Haferkorn J (2010b). Acute oral toxicity study of FK772 in rats. Unpublished report no. R-27262 (LPT report no. 25983). LPT Laboratory of Pharmacology and Toxicology GmbH & Co., Hamburg, Germany. Submitted to WHO by Janssen Pharmaceutica NV.

Hargitai J (2017a). FK284: in vitro mammalian chromosome aberration test. Unpublished report no. 15/508-020C (Janssen study no. AGR5570). CiToxLAB Hungary Ltd, Veszprém, Szabadságpuszta, Hungary. Submitted to WHO by Janssen Pharmaceutica NV.

Hargitai J (2017b). FK284: in vitro mammalian cell gene mutation test (HPRT assay). Unpublished report no. 15/508-015C (Janssen study no. AGR5545). CiToxLAB Hungary Ltd, Veszprém, Szabadságpuszta, Hungary. Submitted to WHO by Janssen Pharmaceutica NV.

Hargitai J (2017c). FK772: in vitro mammalian chromosome aberration test. Unpublished report no. 15/507-020C (Janssen study no. AGR5571). CiToxLAB Hungary Ltd, Veszprém, Szabadságpuszta, Hungary. Submitted to WHO by Janssen Pharmaceutica NV.

Hargitai J (2017d). FK772: in vitro mammalian cell gene mutation test (HPRT assay). Unpublished report no. 15/507-015C (Janssen study no. AGR5568). CiToxLAB Hungary Ltd, Veszprém, Szabadságpuszta, Hungary. Submitted to WHO by Janssen Pharmaceutica NV.

Hargitai J (2017e). R014821: in vitro mammalian chromosome aberration test. Unpublished report no. 15/506-020C (Janssen study no. AGR5569). CiToxLAB Hungary Ltd, Veszprém, Szabadságpuszta, Hungary. Submitted to WHO by Janssen Pharmaceutica NV.

Hargitai J (2017f). R014821: in vitro mammalian cell gene mutation test (HPRT assay). Unpublished report no. 15/506-015C (Janssen study no. AGR5546). CiToxLAB Hungary Ltd, Veszprém, Szabadságpuszta, Hungary. Submitted to WHO by Janssen Pharmaceutica NV.

Hess R (1996). Imazalil (enilconazole): relevance of liver tumor formation in mice. Unpublished report to Janssen Research Foundation, Beerse, Belgium, by University of Basle, Basel, Switzerland. Submitted to WHO by Janssen Pharmaceutica NV.

Heykants J, Meuldermans W, Hurkmans R (1975). On the excretion, metabolism and tissue distribution of imazalil-^{3}H in the male and female Wistar rat. Unpublished report no. 7512. Department of Drug Metabolism, Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Jenkinson PC (1990a). Imazalil: reverse mutation assay “Ames test” using *Salmonella typhimurium*. Unpublished report no. 306/31 (MCW report no. R-5793). Safepharm Laboratories Ltd, Derby, United Kingdom. Submitted to WHO by ADAMA.

Jenkinson PC (1990b). Imazalil: micronucleus test in the mouse. Unpublished report no. 306/33 (MCW report no. R-5792). Safepharm Laboratories Ltd, Derby, United Kingdom. Submitted to WHO by ADAMA.

Lavrijsen K, Van Houdt J, Van Dijck D, Meuldermans W, Heykants J (1987). Study on the induction and/or inhibition potential of imazalil towards drug metabolizing enzymes in rat liver. Unpublished report no. R23979/56. Department of Pharmacokinetics, Janssen Research Foundation, Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Lawrence AC (2001). Three month oral mechanistic toxicity study with one month interim sacrifices in albino Swiss mice (PCNA quantitative analysis of livers). Unpublished report no. JPA 076/012132. Huntingdon Life Sciences, Huntingdon, United Kingdom. Submitted to WHO by Janssen Pharmaceutica NV.

Levron JC, Van Cauteren H, Sanz G, Marsboom R (1991). Imazalil sulfate R 27180S embryotoxicity and teratogenicity study in COBS CD1 mice. Unpublished report no. 2374/90-06. Department de Recherche, Laboratoires Janssen, Aubervilliers, France. Submitted to WHO by Janssen Pharmaceutica NV.

Lina B, Til H, Van Nesselrooij JHJ, Kupe C, Falke H (1983). Six-month oral toxicity study with imazalil technical grade in rats. Unpublished report no. V 83.186/220555. Division for Nutrition and Food Research, Netherlands Organization for Applied Scientific Research, TNO, Zeist, the Netherlands. Submitted to WHO by Janssen Pharmaceutica NV.

Lina B, Til HP, Van Nesselrooij JHJ, Beems RB, Falke HE (1984). Eighteen-month oral toxicity study with imazalil base-R 23979 in rats. Unpublished report no. V 84.140/220555. Division for Nutrition and Food Research, Netherlands Organization for Applied Scientific Research, TNO, Zeist, the Netherlands. Submitted to WHO by Janssen Pharmaceutica NV.

Mannens G, Van Leemput L, Heykants J (1993). General metabolism of imazalil. Unpublished report no. R 23979/FK1116. Department of Drug Metabolism and Pharmacokinetics, Janssen Research Foundation, Turnhoutseweg, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Marsboom R (1970). Potential of R18531 for embryotoxicity and teratogenic effects in rats receiving R18531 orally. Unpublished report. Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Pennwalt Corporation.

Marsboom R (1974). Potential of oral R 18531 for embryotoxicity and teratogenic effects in rabbits. Unpublished report no. 535. Janssen Research Laboratories, Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Marsboom R (1975a). Oral three generation study in Wistar rats. Unpublished report. Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Pennwalt Corporation.

Marsboom R (1975b). Oral embryotoxicity and teratogenicity study in Wistar rats during the peri- and postnatal study (segment III). Unpublished report. Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Pennwalt Corporation.

Marsboom R (1977). Oral male and female fertility study in Wistar rats (segment I). Unpublished report. Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Pennwalt Corporation.

Marsboom H (1978). Oral three-generation study in Wistar rats. Unpublished report. Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Marsboom R, Dirkx P (1981). Imazalil: R 18531. Oral embryotoxicity and teratogenicity study in New Zealand white rabbits (segment II). Unpublished report no. 10816. Janssen Research Laboratories, Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Marsboom R, Herin V (1979a). Imazalil-sulfate R 27180 – oral carcinogenicity study in albino Swiss mice (repeated dosage for 18 months). Unpublished report no. 666 (Experiment No. 666 (79.04.04)). Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Marsboom R, Herin V (1979b). Oral carcinogenicity study in Wistar rats. Unpublished report no. 667. Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Marsboom R, Van Ravestyn C (1975). R 23979 Experiment No. 583 (75.04.23) – acute dermal toxicity in rats (single dosage). Janssen Research Laboratories, Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Marsboom R, Gillardin JM, Sanz G (1985). R 27180 imazalil. Embryotoxicity and teratogenicity study in COBS mice. Unpublished report no. 85-02. Janssen Research Laboratories, Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Marsboom R, Herin V, Vandesteene R (1972). A study of the safety of R 18531 in Wistar rats. Study report no. 342. Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Marsboom R, Herin V, Vandesteene R, Pardoell L (1975). Oral toxicity study in Wistar rats (repeated dosage for 6, 12 or 24 months). Unpublished report. Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Pennwalt Corporation.

Marsboom R, Herin V, Vandesteene R, Van Belle H (1977). Oral toxicity study in beagle dogs (repeated dosage for 24 months). Unpublished report. Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Pennwalt Corporation.

Martens M (2015). Addendum to the assessment of the ARFD of imazalil – 6 February 2015. Submitted to WHO by Certis Europe, Janssen PMP and ADAMA.

Mertens JJWM (2011). Cell proliferation study in male Wistar rats after administration of imazalil in the diet for 1, 2, 7, 14, or 28 days. Unpublished report no. WIL-436011. WIL Research Laboratories, Inc., Ashland, OH, USA. Submitted to WHO by Janssen Pharmaceutica NV.

Meuldermans W, Swysen E, Lauwers W, Heykants J (1977a). An in-vitro study of imazalil-^{3}H in the rat. Unpublished report. Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Pennwalt Corporation.

Meuldermans W, Hurkmans J, Lauwers W, Heykants J (1977b). The excretion and biotransformation of imazalil and one of its metabolites (R14821) in the Wistar rat. Unpublished report. Department of Drug Metabolism and Analytical Department, Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Pennwalt Corporation.

Moore GE (2005a). Fungaflor technical: acute oral toxicity up and down procedure in rats. Unpublished report no. AGR 1162. Product Safety Laboratories, East Brunswick, NJ, USA. Submitted to WHO by Janssen Pharmaceutica NV.

Moore GE (2005b). Acute inhalation toxicity study in rats – limit test. Unpublished report (Study no. 18503). Product Safety Laboratories, Dayton, NJ, USA. Submitted to WHO by Janssen Pharmaceutica NV.

Muto N, Hirai H, Tanaka T, Itoh N, Tanaka K (1997). Induction and inhibition of cytochrome P450 isoforms by imazalil, a food contaminant, in mouse small intestine and liver. Xenobiotica. 27:1215–23.

Nakagawa Y, Moore GA (1995). Cytotoxic effects of postharvest fungicides, *ortho*-phenylphenol, thiabendazole and imazalil, on isolated rat hepatocytes. Life Sci. 57:1433–40.

Nakagawa Y, Tayama K (1997). Acute hepatotoxic potential of imazalil fungicide in rats. Bull Environ Contam Toxicol. 58:402–7.

Niemegeers C (1979). Comparative acute oral toxicity studies of the different salts of imazalil in rats. Unpublished report no. N17417. Department of Pharmacology, Janssen Research Foundation, Turnhoutseweg, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

O'Neill TP (2002). Cell proliferation study in male CD-1 mice after administered imazalil in the diet for 2 or 13 weeks. Unpublished report no. WIL-436001. WIL Research Laboratories, Inc., Ashland, OH, USA. Submitted to WHO by Janssen Pharmaceutica NV.

Piccirillo VJ (2000). Imazalil: one-month repeated dose oral toxicity study with 1 and 2 week interim sacrifices and 4 and 9 week recovery periods to evaluate thyroid effects. Unpublished report no. VJP 5452-00-1. VJP Consulting Inc., Sterling, VA, USA. Submitted to WHO by Janssen Pharmaceutica NV.

Sparrow S (1993). Peer review contract histopathology report. Unpublished report no. 93/JED003/0793. Pharmaco-LSR Ltd, Suffolk, United Kingdom. Submitted to WHO by Janssen Pharmaceutica NV.

Steelman JA, Schreiner CA (1977). In vitro mutagenicity screening of imazalil by microsomal activation bacterial assays. Unpublished report no. McN-JR-23979 (Toxicological Research Report No. 515 (770914)). McNeil Laboratories Inc. Submitted to WHO by Janssen Pharmaceutica NV.

Sterkins P (1996). The toxicokinetics of imazalil (R023979) in SPF Wistar rats at the end of a three-month dose-range-finding and mechanistic toxicity study. Unpublished addendum to study no. 3672. Department of Pharmacokinetics, Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Stiller RL, Stevens DA (1986). Studies with a plant fungicide, imazalil, with vapor phase activity, in the therapy of human alternariosis. Mycopathologia. 93:169–72.

Tanaka T (1995). Reproductive and neurobehavioral effects of imazalil administered to mice. Reprod Toxicol. 9:281–8.

Tanaka T, Ogata A, Inomata A, Nakae D (2013). Effects of maternal exposure to imazalil on behavioral development in F_1-generation mice. Birth Defects Res B Dev Reprod Toxicol. 98(4):334–42.

Teunissen MS (2013). Single dose oral gavage toxicity study with imazalil technical in Wistar rats. Unpublished report no. 502620 (AGR 5108). WIL Research Europe B.V., 's-Hertogenbosch, the Netherlands. Submitted to WHO by Janssen Pharmaceutica NV.

Teuns G, Marsboom R (1987). Imazalil (R 23979) primary dermal irritation study in rabbits. Unpublished report no. 1864. Department of Toxicology, Janssen Research Foundation, Turnhoutseweg, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Teuns G, Ligtvoet T, Coussement W, Lampo A, Van Cauteren H, Marsboom R (1990a). Study of the acute dermal toxicity of imazalil: R 23979 (technical grade) in New Zealand white rabbits. Unpublished report no. 2344. Department of Toxicology, Janssen Research Foundation, Turnhoutseweg, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Teuns G, Peeters V, Coussement W, Lampo A, Van Cauteren H, Marsboom R (1990b). Imazalil: R 23979 technical grade primary eye irritation study in New Zealand white rabbits. Unpublished report no. 2253. Department of Toxicology, Janssen Research Foundation, Turnhoutseweg, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Teuns G, Coussement W, Van Cauteren H, Marsboom R (1990c). Imazalil: R 23979 (technical grade) dermal sensitization study according to the Magnusson guinea-pig maximization test. Unpublished report no. 2417. Department of Toxicology, Janssen Research Foundation, Turnhoutseweg, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Teuns G, Vandenberghe J, Lampo A, Coussement W, Van Cauteren H (1991). Imazalil: R 23979. Repeated dose dermal toxicity study in New Zealand white rabbits (21-days). Unpublished report no. 2418. Department of Toxicology, Janssen Pharmaceutica NV, Turnhoutseweg, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Til HP, Lina BAR, Van Nesselrooij JHJ, Beems RB, Falke HE (1985). Lifespan oral carcinogenicity study with imazalil base-R 23979 in rats. Unpublished report no. V 85.153/220555. Division for Nutrition and Food Research, Netherland Organization for Applied Scientific Research, TNO, Zeist, the Netherlands. Submitted to WHO by Janssen Pharmaceutica NV.

Van Deun K (1999a). Combined oral chronic toxicity/carcinogenicity study in the SPF Wistar rat. Unpublished report no. 3817 (study no. R023979). Department of Toxicology, Janssen Pharmaceutica NV, Turnhoutseweg, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Van Deun K (1999b). 24-month control carcinogenicity study in the SPF Wistar rat. Unpublished report no. 4032. Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Van Deun K, Vandenbergh J, Lammens L, Lampo A, Coussement W, Van Cauteren H (1994). Three-month oral mechanistic toxicity study with one month interim sacrifice in SPF albino Swiss mice. Unpublished report no. 3140. Department of Toxicology, Janssen Research Foundation, Turnhoutseweg, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Van Deun K, Lammens L, Benze J, Coussement W, Vandenberghe J, Lampo A et al. (1996a). 3-month dose range finding and mechanistic toxicity study in SPF Wistar rats. Unpublished report no. 3672. Department of Pharmacokinetics, Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Van Deun K, Lammens L, Benze J, Coussement W, Vandenberghe J, Lampo A et al. (1996b). 3-month dose range finding and mechanistic toxicity study with one month interim sacrifice in SPF Wistar rats. Unpublished study no. 3514. Department of Pharmacokinetics, Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Van Deun K, Lammens L, Vandenberghe J, Lampo A, Jansen T, Coussement W (1999). 3-month dose range finding and mechanistic toxicity study in SPF Wistar rats. Unpublished amendment no. N117794 to the final report (report no. 3672). Department of Pharmacokinetics, Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Van Gompel J, Vanparys P, Van Cauteren H (1995). R023979 – imazalil in vitro mammalian gene mutation assay administration: incubation with or without a rat liver metabolic activation system. Unpublished report no. 3470. Department of Toxicology, Janssen Research Foundation, Turnhoutseweg, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Van Hecke E, de Vos L (1983). Contact sensitivity to enilconazole. Contact Dermatitis. 9:144–73.

Vanparys P, Marsboom R (1979). Imazalil R 23979 Experiment No. 916 (79.12.18) – micronucleus test in rats. Department of Toxicology, Janssen Research Laboratories, Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Vanparys P, Marsboom R (1982). Imazalil R 23979 Experiment No. 1146 (82.07.9) – sex-linked recessive lethal test in *Drosophila melanogaster*. Department of Toxicology, Janssen Research Laboratories, Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Vanparys P, Marsboom R (1987a). R 23979 Experiment No. 649 (revised 87.02.27) – dominant lethal test in male mice. Administration: single oral dose. Department of Toxicology, Janssen Research Laboratories, Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Vanparys P, Marsboom R (1987b). R 23979 Experiment No. 650 (revised 87.05.14) – dominant lethal test in female mice. Administration: single oral dose. Department of Toxicology, Janssen Research Laboratories, Janssen Pharmaceutica, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Vanparys P, Marsboom R (1988a). Ames reverse mutation test of imazalil technical. Unpublished report no. 1999. Department of Toxicology, Janssen Research Foundation, Turnhoutseweg, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Vanparys P, Marsboom R (1988b). Micronucleus test in mice at a single oral dose of imazalil technical. Unpublished report no. 1911. Department of Toxicology, Janssen Research Foundation, Turnhoutseweg, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Vanparys P, Marsboom R, Lenaerts P, Deknudt G (1990). R 23979 – in vitro chromosome aberration study on human lymphocytes incubated with or without a metabolic activation system. Unpublished report no. SCK 86/O2D/R23979. Mammalian Genetics Laboratory, Department of Biology, SCK·CEN, Mol, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Verbeek J, Verstyenen B, Vandenberghe J, Vinckier A, de Coster R, Lampo A et al. (2000). Imazalil: one-month repeated dose oral toxicity study in the Wistar rat with 1 and 2 week interim sacrifice and 4 and 9 week recovery periods to evaluate thyroid effects. Unpublished report no. R023979/5009. Department of

Toxicology, Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Vermeir M (2000). Study on the possible induction and/or inhibition of effects of hepatic drug metabolising enzymes and of hepatic 5′-monodeiodinase and thyroid peroxidase activities in male SPF Wistar rats, after oral adminstration through the diet for one, two and four weeks at levels of 400, 1200 and 3200 ppm. Unpublished study no. FK 3378. Department of Pharmacokinetics, Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Vermeir M, Lavrijsen K (1996). Study on the possible induction and/or inhibition of hepatic drug metabolizing enzymes by imazalil in male and female SPF Wistar rats, after oral administration through the diet for three months at levels of 800, 1,600 and 2,400 and 3,200 ppm. Unpublished study no. R023979/FK 2060. Department of Pharmacokinetics, Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Vermeir M, Lavrijsen K, Meuldermans W (1995). Study on the possible induction and/or inhibition of effects of hepatic drug metabolizing enzymes by imazalil in male and female SPF Wistar rats, after oral administration through the diet for one or three consecutive months at levels of 200, 400 and 800, 1600 ppm. Unpublished study no. R023979/FK 1960. Department of Pharmacokinetics, Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Vermeir M, Lavrijsen K, Meuldermans W (2001). Study on the possible induction and/or inhibition of effects of hepatic drug metabolizing enzymes and of the hepatic 5′-monodeiodinase and thyroid peroxidase activities by imazalil in male SPF Wistar rats, after oral administration through the diet for one, two and four weeks at levels of 400, 1200 and 3200 ppm. Unpublished study no. FK 3378. Department of Pharmacokinetics, Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Vermeir M, Lavrijsen K, Van Leemput L (1994). Study on the possible induction and/or inhibition of hepatic drug metabolizing enzymes by imazalil in male and female SPF albino Swiss mice, after oral administration through the diet for one and three consecutive months. Unpublished study no. FK 1600. Department of Pharmacokinetics, Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Verstraeten A, Teuns G, Vandenberghe J, Coussement W, Van Cauteren H, Marsboom R (1989). Imazalil base: R 23979 technical. Chronic toxicity study in beagle dogs subjected to repeated dosage for 12 months by oral administration. Unpublished report no. 1899. Department of Toxicology, Janssen Research Foundation, Turnhoutsweg, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Verstraeten A, Vandenberghe J, Lampo A, Van Deun K, Coussement W, Van Cauteren H (1993a). Imazalil: R 23979 – three-month oral toxicity study in albino Swiss mice. Unpublished report no. 2020. Department of Toxicology, Janssen Research Foundation, Turnhoutsweg, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Verstraeten A, Vandenberghe J, Lampo A, Van Deun K, Coussement W, Van Cauteren H (1993b). Imazalil base: carcinogenicity study in Swiss mice – administration through the diet for 2 years. Unpublished report no. 2194. Department of Toxicology, Janssen Research Foundation, Turnhoutsweg, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Vinggaard AM, Breinholt V, Larsen JC (1999). Screening of selected pesticides for oestrogen receptor activation in vitro. Food Addit Contam. 16:533–42.

Vinggaard AM, Hnida C, Breinholt V, Larsen JC (2000). Screening of selected pesticides for inhibition of CYP19 aromatase activity in vitro. Toxicol In Vitro. 14:227–34.

Watabe H (1992). A reverse mutation assay of imazalil. Unpublished report no. 92-001. Mitsubishi Yuka Bio-Clinical Laboratories, Inc., Ibaraki-ken, Japan. Submitted to WHO by Janssen Pharmaceutica NV.

Wiener A (2016). Letter dated 19 September 2016. Institute of Occupational and Environmental Medicine, Rambam Medical Center, State of Israel Ministry of Health, Haifa, Israel.

Wnorowski G (1997). Dermal sensitization test – Buehler method. Unpublished report (Project no. 5337). Product Safety Laboratories, Dayton, NJ, USA. Submitted to WHO by Janssen Pharmaceutica NV.

Appendix 1. IPCS framework for the preliminary analysis of the relevance of a cancer mode of action for humans: liver and thyroid tumours

Introduction

Hepatic tumours are seen in mouse and rat studies with imazalil. In mice, liver adenomas were reported to be increased over controls at 200 and 600 ppm (equal to 33 and 100 mg/kg bw per day for male mice, respectively), with a NOAEL for carcinogenic effects of 50 ppm (equal to 8.1 mg/kg bw per day) (Verstraeten et al., 1993a). There was also a trend for the increase in the incidence of combined hepatocellular adenomas and carcinomas in female mice at 600 ppm (equal to 131 mg/kg bw per day), with a NOAEL of 200 ppm (equal to 41.6 mg/kg bw per day) (Verstraeten et al., 1993a). Increased incidences of liver adenomas in male rats over controls were reported at 2400 ppm (equal to 120 mg/kg bw per day), with a NOAEL for liver adenomas of 1200 ppm (equal to 58 mg/kg bw per day) (Van Deun, 1999). Thyroid follicular cell adenomas were reported in male rats at 1200 and 2400 ppm (equal to 58 and 120 mg/kg bw per day, respectively), with a NOAEL of 200 ppm (equal to 9.7 mg/kg bw per day) (Van Deun, 1999).

The postulated mode of action (MOA) for imazalil-induced rodent hepatocellular tumours is non-genotoxic activation of CAR, followed by the key events of altered gene expression specific to CAR activation, increased cell proliferation, formation of altered hepatic foci and, ultimately, the development of liver tumours. Elcombe et al. (2014) provided a review of the evidence that mouse or rat liver tumours that occur via a CAR MOA are not relevant to humans, because, although activation of CAR, the induction of CYP2B and other cytochrome P450 forms and liver hypertrophy can be observed in humans, the cell proliferation and subsequent altered hepatic foci and liver tumours do not occur in humans. Extensive data exist with known CAR-activating compounds that show a fundamental qualitative difference between the species and indicate that the key events leading to tumours in mice or rats are not operative in humans (Peffer et al., 2018). Imazalil did not demonstrate any increase in cell proliferation in human hepatocytes.

In addition, chronic exposure of male rats to imazalil resulted in neoplastic changes in thyroid follicular cells. The underlying cause is the increase in circulating TSH secondary to CAR activation with consequent hepatic enzyme induction, especially phase II enzymes responsible for clearance of thyroid hormones. The postulated MOA for the imazalil-induced thyroid follicular cell tumours involves the perturbation of homeostasis of the hypothalamic–pituitary–thyroid axis by an extra-thyroidal mechanism to which rats are particularly sensitive. Specifically, the MOA includes induction of conjugation of thyroid hormones with glucuronic acid through CAR activation and subsequent excretion in the bile, leading to a decrease in thyroid hormone plasma levels and activation of the hypothalamic–pituitary–thyroid axis. Rodents are much more sensitive to changes in thyroid hormone levels because of the lack of T_4 binding globulin (TBG), which acts as a buffer. This mechanism is not considered to be quantitatively relevant to humans, as the thyroid of humans is much less sensitive to this pathogenic phenomenon than that of rodents.

Postulated mode of action (theory of the case): hepatocellular tumours

The postulated MOA for imazalil-induced rodent hepatocellular tumours is non-genotoxic activation of CAR, followed by the key events of altered gene expression specific to CAR activation, increased cell proliferation, formation of altered hepatic foci and, ultimately, the development of liver tumours. Associative events in the MOA include altered epigenetic changes, induction of hepatic CYP2B enzymes, liver hypertrophy and decreased apoptosis, with inhibition of gap junctional intercellular communication being an associative event or modulating factor. The key events (i.e. CAR activation, altered gene expression, cell proliferation, altered foci and increased adenomas/carcinomas) can be demonstrated by measuring a combination of the key events themselves or associative events that are known markers for the key events (Peffer et al., 2018).

The MOA for imazalil-induced liver tumours is depicted in Fig. A1-1.

Fig. A1-1. MOA for imazalil-induced rodent liver tumour formation

Key Events
CAR Activation
Associative Events
Hypertrophy
CYP2B induction
Decreased apoptosis
Altered gene expression specific to CAR activation
Associative Events
Altered epigenetic changes specific to CAR activation
Cell proliferation
Associative Event or Modulating Factor
Inhibition of gap junction communication
Clonal expansion leading to altered foci
Liver adenomas/carcinomas

Source: Elcombe et al. (2014)

The sequence of key events for the proposed MOA for rat and mouse liver tumours (designated LKE1 to LKE5) is:

LKE1	Activation of CAR (molecular initiating event)
LKE2	Upregulation and downregulation of a set of genes secondary to CAR activation (*Cyp2b* up and *Cyp3a* down)
LKE3	Increased cell proliferation
LKE4	Increase in foci of altered hepatocytes
LKE5	Increase in hepatocellular adenomas/carcinomas (adverse outcome).

Postulated mode of action (theory of the case): thyroid follicular cell tumours

The postulated MOA for the imazalil-induced thyroid follicular cell tumours involves the perturbation of homeostasis of the hypothalamic–pituitary–thyroid axis by an extra-thyroidal mechanism to which rats are particularly sensitive. The typical MOA for extra-hepatic induction of thyroid tumours in rats includes induction of hepatic uridine diphosphate–glucuronosyltransferase (UGT, or UDPGT, as in Fig. A1-2 below) activity (secondary to CAR activation), leading to enhanced metabolism of the thyroid hormones T_4 and T_3 by conjugation and increased biliary excretion of the conjugated hormones. The result of this enhanced liver metabolism and excretion via UGT is a decrease in serum T_4 and T_3 levels. The reduced concentrations of circulating T_3 and T_4 are detected in the hypothalamus, which maintains homeostasis of thyroid hormones via the secretion of thyrotropin-releasing hormone (TRH). Increased TRH levels stimulate the pituitary to secrete more TSH. Prolonged elevation of circulating TSH levels stimulates the thyroid gland to deplete its stores of thyroid hormone (T_4) and continues to induce hormone production. Thus, the thyroid follicular cells enlarge (hypertrophy) and are induced to proliferate at an increased rate, increasing in number (hyperplasia). With chronic exposure, thyroid hyperplasia eventually progresses to thyroid neoplasia (USEPA, 1998; Dellarco et al., 2006). This mechanism is not considered to be quantitatively relevant to humans, as the thyroid of humans is much less sensitive to this pathogenic phenomenon than that of rodents due to the presence of TBG and longer T_4 and T_3 half-lives in humans.

The MOA for imazalil-induced thyroid tumours is depicted in Fig. A1-2.

Fig. A1-2. MOA for imazalil-induced thyroid tumours

KE1: Induction of Hepatic UDPGTs (CAR-induced)

KE2: Enhanced Biliary Excretion of T3/T4

KE3: Decreased Serum Levels of T3/T4

KE4: Stimulation Release of TSH from pituitary

KE5: TSH stimulation of thyroid follicular cells to release T3/T4

KE6: Thyroid follicular cell hypertrophy, hyperplasia, neoplasia

KE: key event
Source: Elcombe et al. (2014)

The sequence of key events for the proposed MOA for rat thyroid tumours (designated TKE1 to TKE6) is:

TKE1	Induction of hepatic enzymes, including UGT activity
TKE2	Increased hepatic metabolism and biliary excretion of T_4/T_3
TKE3	Decrease in serum T_4/T_3 half-life and/or concentration
TKE4	Feedback to pituitary gland and increase in circulating TSH concentrations
TKE5	Thyroid follicular cell hypertrophy and/or hyperplasia
TKE6	Increased thyroid follicular cell adenomas/carcinomas.

Interplay between the two proposed MOAs

The toxicological database for imazalil is reviewed in this appendix in the context of the hepatic enzyme induction rodent-specific MOA for liver tumours and for the thyroid secondary specific responses detailed above. Because of the interplay between the two proposed MOAs for liver and thyroid tumours, the mechanistic studies that support similar key events are addressed together. For instance, the studies applicable to key events LKE1, LKE2 and TKE1 all evaluate liver gene expression and enzyme activity. Additionally, key events LKE3 and TKE2 are addressed together because the same set of data addresses increased hepatic cell proliferation and increased hepatic metabolism and supports the increased biliary excretion of T_4/T_3 (TKE2).

Liver key events 1 and 2 and thyroid key events 1 and 2: CAR activation (LKE1), induction of liver enzymes (LKE2) and hepatic UGT activity (TKE1) and increased hepatic metabolism and biliary excretion of T_4/T_3 (TKE2)

For imazalil, the adverse outcome for the liver (hepatocellular adenomas and carcinomas) was reported at 200 ppm (equal to 33 mg/kg bw per day) in male mice, with a NOAEL of 50 ppm (equal to 8.1 mg/kg bw per day), and at 600 ppm (equal to 130 mg/kg bw per day) in female mice, with a NOAEL of 200 ppm (equal to 42 mg/kg bw per day). In male rats, hepatocellular adenomas were reported at 2400 ppm (equal to 120 mg/kg bw per day), with a NOAEL of 1200 ppm (equal to 58 mg/kg bw per day). Evidence of LKE1 and LKE2 events include altered gene expression secondary to CAR activation (Elcombe, 2012c) and associative events supporting LKE1 and LKE2, including increased CYP2B and CYP3A enzyme activities, increased liver weight and hepatocellular hypertrophy.

The adverse outcome in the thyroid (thyroid follicular cell adenomas) was reported in male rats at 1200 ppm (equal to 58 mg/kg bw per day) and 2400 ppm (equal to 120 mg/kg bw per day), with a NOAEL of 200 ppm (equal to 9.7 mg/kg bw per day). Evidence of TKE1, induced hepatic UGT activity, was demonstrated in female mice and male and female rats (at 600 ppm, equal to 170 mg/kg bw per day, with a NOAEL of 200 ppm, equal to 55 mg/kg bw per day, in female mice; and at 400 ppm, equal to 42.0 mg/kg bw per day, with a NOAEL of 200 ppm, equal to 21.0 mg/kg bw per day, in male and female rats). Associative events supporting TKE2 include increased liver weight relative to body weight and increased incidence of hepatocellular hypertrophy, reported in male and female mice and male and female rats.

Mouse liver

Increased expression of Cyp2b10 and Cyp3a11 mRNA and protein and Gadd45β mRNA was demonstrated in male mice

In the study by Elcombe (2012c), male mice were administered imazalil in the diet for 7 days at 0, 50, 200 or 600 ppm. This study evaluated levels of hepatic murine *Cyp2b10*, *Cyp3a11* and *Gadd45β* mRNA.[1] Imazalil induced *Cyp2b10*, *Cyp3a11* and *Gadd45β* mRNA expression in a dose-dependent manner at all doses (50, 200 and 600 ppm), with maximal levels of induction at 600 ppm, at which target genes were induced 38.9-fold, 6.2-fold and 4-fold, respectively, in male mice when compared with controls. Elcombe (2102c) also evaluated the induction of murine CYP2B10 and CYP3A11 protein in liver microsomes. Marked dose-dependent increases in the microsomal expression of CYP2B10 protein were observed with imazalil at all doses (50, 200 and 600 ppm). The expression of CYP3A protein was induced by 600 ppm imazalil. Increased expression of *Cyp2b10* mRNA and CYP2B10 protein was reported at 50 ppm (the lowest dose tested; no NOAEL identified). Increased expression of *Cyp3a11* and *Gadd45β* mRNA was also reported at 50 ppm (the lowest dose tested), and increases in microsomal expression of CYP3A11 protein were reported at 600 ppm (NOAEL of 200 ppm). The response to CAR activation is apparent at lower doses in male mice than in female mice. In female mice, a trend for an increased incidence of combined hepatocellular adenomas and carcinomas was noted at 600 ppm (compared with 200 and 600 ppm in male mice), with a NOAEL for hepatocellular neoplasms in females of 200 ppm (compared with 50 ppm in male mice). Therefore, data available in male mice are also applicable to females.

---

[1] Mechanistically, GADD45β is an anti-apoptotic factor, a negative regulator of the p53 tumour suppressor. GADD45β has been implicated in pathways activated by CAR and contributory to the enhanced tumorigenic response in CAR wild-type animals (NIEHS, 2010).

An increase in CYP2B activation greater than the increase in CYP3A activation was demonstrated in both male and female mice

Microsomal PROD was used as a marker for CYP2B activity in all studies. CYP2B and CYP3A enzyme induction was evaluated in male mice after 7 days of dietary exposure in Elcombe (2012c) and after 1 month and 3 months of dietary exposure in Vermeir, Lavrijsen & Van Leemput (1994). CYP2B enzyme induction was highest at 7 days, but was also present at 1 month. In the study by Elcombe (2012c), after 7 days of dietary exposure, CYP2B activity was increased in male mice at 200 and 600 ppm by 3.2- and 6.7-fold over control values, respectively, with a NOAEL of 50 ppm. In the Vermeir, Lavrijsen & Van Leemput (1994) study, after 1 month, a statistically significant increase in PROD activity was reported at 600 ppm in male mice (92% increase over controls) and female mice (63% increase over controls[2]). After 3 months, PROD was not induced in male or female mice up to 600 ppm. Elcombe (2012a) evaluated CYP2B and CYP3A activity in freshly isolated female CD-1 mouse hepatocytes cultivated as monolayer cultures for 96 hours and treated with imazalil at 3, 10, 30 or 100 µmol/L or with vehicle alone (0.5% v/v DMSO). Treatment with imazalil at 3 µmol/L resulted in a statistically significant increase in CYP2B10 (PROD) activity (17% increase over controls). Treatment at 10 µmol/L did not result in an increase in CYP2B10 (PROD) activity in vitro, and excess toxicity prohibited evaluation of the results at 30 and 100 µmol/L.

Microsomal BQ was used as a marker for CYP3A11 activity by Elcombe (2012a,c). In Elcombe (2012c), administration of imazalil to male mice for 7 days at 600 ppm increased the BQ activity by 1.9- and 2.7-fold, respectively (NOAEL of 200 ppm). In Elcombe (2012a), BQ activity was increased dose-wise at 3, 10 and 30 µmol/L (34%, 100% and 105.3% increases over controls, respectively). Excess toxicity prohibited evaluation at 100 µmol/L. In Vermeir, Lavrijsen & Van Leemput (1994), EMD was used as a marker for CYP3A1 and CYP3A2 activities. Induction (expressed in nanomoles of product formed per milligram of protein per minute) by treatment with imazalil was observed for both male (41% increase over controls at 600 ppm) and female (71% increase over controls at 600 ppm) mice. However, the inductive effect in male mice was not revealed when the activity was expressed as nanomoles of product formed per nanomole of cytochrome P450 per minute. Dosing for 3 months with imazalil up to 600 ppm had hardly any effect on the EMD activity in male or female mice.

Significant increases in liver weight (relative to body weight) in male and female mice were reported in the toxicological database for imazalil

Elmore (2004a) reported a statistically significant increase in relative liver weight of male mice at 1200 ppm after 4 days of treatment (133% over controls). O'Neill (2002) reported statistically significant increases in relative liver weight in male mice after 2 weeks of dietary administration of imazalil at 600 and 1200 ppm (NOAEL of 400 ppm). Van Deun et al. (1994) reported statistically significant increases in relative liver weight in male and female mice at 600 ppm after 1 and 3 months (no effects reported at 50 or 200 ppm). Verstraeten et al. (1993b) reported a statistically significant increase in relative liver weight in male mice at 400 ppm and in male and female mice at 800 ppm after 3 months (NOAEL of 200 ppm for males and 400 ppm for females). O'Neill (2002) reported statistically significant increases in relative liver weight in male mice after 3 months of dietary administration of imazalil at 1200 ppm (NOAEL of 600 ppm). Verstraeten et al. (1993a) reported statistically significant increases in relative liver weight of male mice at 800 ppm (NOAEL of 400 ppm) and no statistically significant increases in relative liver weight in females (NOAEL of 800 ppm) in the mouse carcinogenicity study (23 months).

[2] PROD activity in female mice at 1 month was not increased, as reported in nanomoles per nanomole cytochrome P450 per minute.

Evidence of hepatocellular hypertrophy was reported in both male and female mice

Elmore (2004a) reported a statistically significant increase in centrilobular hepatocellular hypertrophy (minimal) in male mice at 1200 ppm after 4 days of treatment (6/6, compared with 0/6 in controls). O'Neill (2002) reported an increased incidence of hepatocellular hypertrophy in male mice after 2 weeks of dietary administration of imazalil at 200, 400, 600 or 1200 ppm (NOAEL of 100 ppm). A non-statistically significant increase in diffuse hepatocellular swelling was reported after 3 months in male mice at 600 ppm (Van Deun et al., 1994). Verstraeten et al. (1993b) reported a statistically significant increase in the incidence of centrilobular swelling in male mice at 400 ppm and in male and female mice at 800 ppm after 3 months (NOAEL of 200 ppm for males and 400 ppm for females). O'Neill (2002) reported an increased incidence of hepatocellular hypertrophy in male mice after 3 months of dietary administration of imazalil at 200, 400, 600 or 1200 ppm (NOAEL of 100 ppm).

Rat liver

Increased expression of Cyp2b10 and Cyp3a11 and Gadd45β mRNA was demonstrated in male rats

Cyp2b1, *CAR (Nr113)*, *Cyp3a1*, *Cyp3a2* and *Gadd45β* mRNA levels were evaluated after 14 and 28 days of dietary administration of 0, 200, 1200 or 2400 ppm imazalil to male rats by Mertens (2011). In rats exposed to imazalil for either 14 or 28 days, analysis of mRNA levels of *Cyp2b1* showed statistically significant and dose-related increases when compared with the mRNA levels of *Cyp2b1* in rats fed the basal diet; the LOAEL was 200 ppm (no NOAEL identified). Imazalil administered for 14 or 28 days induced the mRNA levels of *Cyp2b1*, *Cyp3a1*, *Cyp3a2* and *Gadd45β* in the liver of rats in a dose-dependent manner, with statistically significant changes reported at 1200 and 2400 ppm (NOAEL of 200 ppm).

An increase in CYP2B activation greater than the increase in CYP3A activation was demonstrated in both male and female rats

Microsomal PROD was used as a marker for CYP2B activity in all studies. Mertens (2011) evaluated CYP2B1/2 induction in male rats at 0, 200, 1200 and 2400 ppm via dietary exposure after 1, 2, 7, 14 and 28 days. In Mertens (2011), administration of imazalil to male rats at 0, 200, 1200 or 2400 ppm and phenobarbital (1200 ppm) across all treatment periods (1, 2, 7, 14 and 28 days) resulted in increases in CYP2B1/2 activity (as measured by PROD). Statistically significant increases were observed for all intervals at 1200 and 2400 ppm imazalil (with the exception of 14-day 1200 ppm imazalil) and the 1200 ppm phenobarbital treatment groups. The NOAEL for CYP2B induction in Mertens (2011) was 200 ppm. CYP2B enzyme induction was reported in male rats 1, 2 and 4 weeks after dietary administration of imazalil at 400, 1200 or 3200 ppm (Vermeir, Lavrijsen & Meuldermans, 2001). CYP2B enzyme induction was reported in male and female rats after 1 and 3 months of dietary administration of imazalil at 200, 400 or 800 ppm (Vermeir, Lavrijsen & Meuldermans, 1995). CYP2B enzyme induction was reported in male and female rats after 3 months of dietary administration of imazalil at 800, 1600, 2400 or 3200 ppm (Vermeir & Lavrijsen, 1996). The LOAEL was 200 ppm, the lowest dose tested.

EMD was used as a marker for CYP3A1 and CYP3A2 enzyme induction activity in Vermeir, Lavrijsen & Meuldermans (1995, 2001) and Vermeir & Lavrijsen (1996). In Vermeir, Lavrijsen & Meuldermans (2001), CYP3A enzyme induction (statistically significant) was reported in male rats after 2 weeks of dietary administration of imazalil at 400, 1200 or 3200 ppm. At 4 weeks, no statistically significant or biologically relevant increases were reported after 1 week of treatment. In Vermeir, Lavrijsen & Meuldermans (1995),[3] CYP3A enzyme induction was reported in females at 200, 400 and 800 ppm at 1 month and 3 months. Non-statistically significant increases were reported

[3] Livers taken from Van Deun et al. (1996b).

in males at 1 month at all doses and at 3 months at 400 and 800 ppm. CYP3A enzyme induction was reported in male and female rats after a 3-month dietary administration of imazalil at 800, 1600, 2400 or 3200 ppm (Vermeir & Lavrijsen 1996). The LOAEL was 200 ppm, the lowest dose tested; no NOAEL was identified.

Significant increases in liver weight (relative to body weight) in male and female rats were reported in the toxicological database for imazalil

Mertens (2011) reported a statistically significant increase in liver weight relative to body weight at 28 days in male rats (24.1% increase over controls) at 2400 ppm, with no increases recorded at 1, 2, 7 or 14 days at doses up to 2400 ppm. Statistically significant, dose-related increases in relative liver weight were reported in male rats at 200, 400 and 800 ppm and in females at 400 and 800 ppm at 1 month. Gur, Nyska & Crown (1990) reported statistically significant increases in relative liver weight in female rats at 1000, 2000 and 3000 ppm (NOAEL of 100 ppm). At 3 months, relative liver weight was increased only at 800 ppm in male rats (Van Deun et al., 1996b[3]). Van Deun et al. (1996a) reported increased relative liver weight in male rats at 800, 1600, 2400 and 3200 ppm after 3 months; no increase in relative liver weight was reported in female rats at any dose. Gur, Nyska & Crown (1990) reported statistically significant increases in relative liver weight in male and female rats at 1000 ppm (NOAEL of 200 ppm). Van Deun (1999) reported statistically significant increases in relative liver weight in male rats at 1200 and 2400 ppm (NOAEL of 200 ppm) and in female rats at 200, 1200 and 2400 ppm (NOAEL of 50 ppm).

Evidence of hepatocellular hypertrophy was reported in both male and female rats in the toxicological database for imazalil

Van Deun et al. (1996b) reported an increase in centrilobular swollen hepatocytes at 400 and 800 ppm in male rats (5/10 in controls, 5/10 at 200 ppm, 8/10 at 400 ppm and 8/10 at 800 ppm) after 1 month of treatment. No increases in female rats were reported at 1 or 3 months, and no changes were reported in males at 3 months. Van Deun et al. (1996a) reported an increase in the incidence of hepatocellular hypertrophy at 800, 1600, 2400 and 3200 ppm after 3 months in both male and female rats. Gur, Nyska & Waner (1991) reported an increase in the incidence of hepatocellular hypertrophy at 1000 ppm in male rats (15/19 compared with 0/20 in controls) and female rats (18/18 compared with 0/20 in controls) (NOAEL of 200 ppm). Van Deun (1999) reported an increased incidence (statistically significant) of hepatocellular hypertrophy (centriacinar and periacinar) after 24 months in male and female rats at 1200 and 2400 ppm (NOAEL of 200 ppm).

Rat thyroid

Evidence of UGT4 induction in male rats has been experimentally demonstrated

Vermeir, Lavrijsen & Meuldermans (2001) reported a statistically significant increase in T_4-UGT activity (T_4 substrate) in male rats after 1 week of treatment at 400, 1200 or 3200 ppm (LOAEL of 400 ppm; no NOAEL identified). Mertens (2011) reported increased UGT activity (UGT1A activity as measured by 4-methylumbelliferone glucuronidation) in male rats after 14 days at 2400 ppm and after 28 days at 1200 ppm and 2400 ppm (NOAEL of 200 ppm). After 2 and 4 weeks, UGT activity was statistically significantly increased in male rats at 3200 ppm (NOAEL of 1200 ppm). Vermeir, Lavrijsen & Meuldermans (1995) reported increased UGT activity (β-naphthoflavone) in male and female rats after 1 and 3 months at 800 ppm (NOAEL of 400 ppm). Van Deun et al. (1996a) reported non-statistically significant increases in UGT activity (β-naphthoflavone-induced) in male rats at 800, 1600, 2400 and 3200 ppm (LOAEL of 800 ppm; no NOAEL identified) and in female rats at 2400 and 3200 ppm.

Liver key events 3, 4 and 5: Cell proliferation (LKE3), foci (LKE4) and neoplasia (LKE5)

For liver key events 3, 4 and 5, the available toxicological database includes studies evaluating cell proliferation responses in vitro and in vivo and increased incidences of liver foci and tumours in chronic mouse and rat studies. An increase of murine hepatocyte cell proliferation in response to imazalil was demonstrated in vitro by Elcombe (2012a) and in vivo by Elmore (2004a) in male mice. An increase in eosinophilic foci is most commonly observed after long-term administration of CAR activators (Peffer et al., 2018), but mixed foci (i.e. including basophilic or clear cells) can at times be observed (Peffer et al., 2018).

Mouse and human liver: cell proliferation studies

Elcombe (2012a) evaluated the ability of imazalil to induce hepatocyte cell proliferation (measured as the change in replicative DNA synthesis [S-phase of the cell cycle]) in isolated male mouse hepatocyte cultures. The number of hepatocytes undergoing replicative DNA synthesis was measured by incorporation of BrdU into DNA and subsequent immunohistochemical detection. Treatment with imazalil at 10 or 30 μmol/L resulted in statistically significant increases in replicative DNA synthesis at 1.8- and 1.5-fold relative to controls, respectively. Elmore (2004a) evaluated the ability of imazalil to induce cell proliferation in male mice administered 1200 ppm imazalil in the diet for 4 days. Each mouse was implanted with mini-osmotic pumps (day −1) that delivered BrdU for the assessment of hepatic cell proliferation. There was a statistically significant increase in the BrdU labelling index of the imazalil-treated mice compared with controls.

Elcombe (2012b) evaluated the ability of imazalil to induce cell proliferation (measured as the change in replicative DNA synthesis [S-phase of the cell cycle]) in isolated female human hepatocyte cultures. No increase in cell proliferation was observed in human hepatocytes exposed to imazalil at concentrations up to and beyond the toxicity threshold.

Elcombe (2012c) demonstrated hepatocyte cell proliferation in wild-type mice at doses of 50 ppm and higher. Although cell proliferation was also observed in humanized mice, the response was lower than that observed in wild-type mice. This lack of cell proliferation differences between wild-type and hCAR/hPXR mice (Elcombe, 2012c) is further discussed in the "Uncertainties, inconsistencies and data gaps" section below.

Rat liver: cell proliferation studies

Mertens (2011) evaluated the ability of imazalil to induce hepatocyte proliferation in male rats. Osmotic pumps loaded with BrdU were implanted subcutaneously in all animals 4 days prior to the scheduled necropsy (study day −3, −2, 3, 10 or 24). Results of BrdU immunohistochemical staining of the liver revealed an increased level of BrdU incorporation in the liver of phenobarbital-treated rats on study day 7, indicating hepatocellular proliferative activity, with no similar effect noted in imazalil-treated rats. Only a single hepatocellular nucleus in a 200 ppm animal was found to be positive for BrdU at the study week 2 interim necropsy. Elmore (2004b)[4] reported an increase in hepatocyte cell proliferation in male rats on day 7 at 3200 ppm with intraperitoneal administration of BrdU 6 hours prior to necropsy.

Mouse liver: liver foci, hepatocellular adenoma and carcinoma

In addition to hepatocellular adenomas and carcinomas, liver foci, described as focal cellular changes by Verstraeten et al. (1993b) or as basophilic or eosinophilic focus of alteration by Sparrow (1993), were reported in male mice (NOAEL of 50 ppm) after 18 months of dietary administration of imazalil (Table A1-1).

[4] Samples obtained from Verbeek et al. (2000).

Table A1-1. Incidence of liver foci, hepatocellular adenoma and carcinoma in mice (18 months)

Parameter	Males				Females			
	0 ppm	**50 ppm**	**200 ppm**	**600 ppm**	**0 ppm**	**50 ppm**	**200 ppm**	**600 ppm**
Dose (mg/kg bw per day)	0	8.1	33	100	0	9.9	42	130
Focal cellular changes (Verstraeten et al., 1993b)	2/50	4/49	10/50*	8/50	0/50	0/50	0/50	0/50
Basophilic focus of alteration (Sparrow, 1993)	1/50	3/50	2/50	5/50	0/50	0/50	0/50	0/50
Eosinophilic focus of alteration (Sparrow, 1993)	0/50	0/50	1/50	0/50	0/50	0/50	0/50	0/50
Hepatocellular adenoma (Verstraeten et al., 1993b)	8/50	5/49	23/50**	17/50**	4/50	6/50	0/50	10/50
Hepatocellular carcinoma (Verstraeten et al., 1993b)	5/50	7/49	6/50	11/50	0/50	1/50	2/50	2/50

bw: body weight; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$
Source: Verstraeten et al. (1993b)

Rat liver: liver foci, hepatocellular adenoma and carcinoma

Van Deun (1999) reported a statistically significant increase in eosinophilic foci at 2400 ppm in male rats (Table A1-2).

Table A1-2. Incidence of liver foci and hepatocellular adenoma and carcinoma in rats (24 months)

	Males					Females				
	0 ppm	**50 ppm**	**200 ppm**	**1 200 ppm**	**2 400 ppm**	**0 ppm**	**50 ppm**	**200 ppm**	**1 200 ppm**	**2 400 ppm**
Dose (mg/kg bw per day)	0	2.5	10	60	120	0	3.5	14	80	160
Clear cell focus/ foci	24/50	14/50	21/50	20/50	16/50	10/50	5/50	14/50	4/50	1/50
Basophilic focus/ foci of hepatocellular alteration	8/50	10/50	15/50	7/50	12/50	25/50	20/50	18/50	20/50	6/50
Eosinophilic focus/foci of hepatocellular alteration	20/50	19/50	22/50	28/50	37/50**	6/50	8/50	5/50	7/50	6/50
Hepatocellular adenomas	4/50 (8%)	2/50 (4%)	3/50 (6%)	4/50 (8%)	13/50* (26%)	2/50 (4%)	1/50 (2%)	2/50 (4%)	1/50 (2%)	2/50 (4%)
Hepatocellular carcinomas	1/50 (2%)	0/50 (0%)	0/50 (0%)	0/50 (0%)	1/50 (2%)	0/50 (0%)	0/50 (0%)	0/50 (0%)	0/50 (0%)	0/50 (0%)

bw: body weight; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$
Source: Van Deun (1999)

Thyroid key events 3 and 4: Effects on serum T_3/T_4 (TKE3) and TSH (TKE4)

Verbeek et al. (2000) evaluated T_3, T_4 and TSH levels in male rats after 1, 2 and 4 weeks of dietary treatment followed by a recovery period of 4 or 9 weeks. The study demonstrated biologically relevant and statistically significant reductions in T_4 levels after 1 week at 1200 and 3200 ppm (12% and 17%, respectively) compared with the controls and non-statistically significant increases in TSH levels at 1200 and 3200 ppm (15% and 19%, respectively) compared with the controls. Similar non-statistically significant increases in TSH levels at 1200 and 3200 ppm were reported after 2 weeks of treatment (22% and 18%, respectively) compared with the controls. At 4 weeks of treatment, following the 1- and 2-week increases in TSH, serum levels of T_4 were increased compared with the controls, and TSH levels were decreased compared with the controls, indicating the homeostatic negative feedback relationship between T_4 and TSH.

Van Deun (1999) evaluated changes in thyroid hormone concentrations in the 30-month carcinogenicity study in rats. Changes in thyroid hormone concentrations in serum were inconsistent. In males, the concentrations of TSH tended to be higher and those of T_4 definitely lower at the two highest dietary concentrations (1200 and 2400 ppm); in females, the level of T_3 was decreased at the two highest dietary concentrations (1200 and 2400 ppm), and that of T_4 was decreased only at 1200 ppm. Piccirillo (2000) concluded that dose-related decreases in T_4 were reported at 1200 and 2400 ppm at the terminal interval, with a no-effect level of 200 ppm.

Thyroid key events 5 and 6: Thyroid hypertrophy, hyperplasia and tumours (TKE5 and TKE6)

Mertens (2011) evaluated BrdU incorporation in the thyroid of male rats exposed to 2400 ppm imazalil for 1–28 days. BrdU incorporation in the thyroid of imazalil-treated animals on study day 14 was higher, but not statistically significantly higher, than that in the control group. Phenobarbital-treated animals had statistically significantly higher thyroid BrdU incorporation on study days 7, 14 and 28.

In the study by Verbeek et al. (2000), male rats (10 per dose per time point) were administered imazalil in the diet at a concentration of 0, 400, 1200 or 3200 ppm or phenobarbital at 1200 ppm for 4 weeks with 1- and 2-week interim evaluations and 4- and 9-week recovery groups. Relative thyroid weight was increased at 3200 ppm after 2 weeks (119% compared with controls; statistically significant) and after 4 weeks (119% compared with controls; not statistically significant). A non-statistically significant increase in the incidence of follicular cell hypertrophy was reported after 2 weeks at 3200 ppm.

A statistically significant increase in relative thyroid weight was reported in male rats by Van Deun et al. (1996b) at 800 ppm after 1 month of dietary exposure to imazalil (118% compared with control value). At 3 months, relative thyroid weight was increased (110%), but not statistically significantly, compared with controls. A statistically significant increase in relative thyroid weight was reported by Van Deun et al. (1996a) at 3200 ppm after 3 months of dietary exposure to imazalil (126% compared with control value); increases over control were reported at 800, 1200 and 2400 ppm, but these values were not statistically significant and/or did not demonstrate a dose–response relationship.

In the 24-month rat carcinogenicity study (Van Deun, 1999), statistically significant increases in relative thyroid weight were reported only for male rats at all doses (50, 200, 1200 and 2400 ppm), and statistically significant increases in the incidence of follicular cell hyperplasia of the thyroid were observed at 1200 and 2400 ppm (Table A1-3).

Summary of dose concordance for the key events

Table A1-4 summarizes the dose concordance of the key events for imazalil-induced mouse liver tumours. Table A1-5 summarizes the dose concordance of the key events in the MOA for imazalil-induced rat thyroid tumours. For imazalil, the key events necessary to demonstrate the CAR-

induced liver tumour MOA and UGT-induced thyroid tumour MOA are available, and the key events demonstrate dose concordance with the adverse outcome.

Table A1-3. Thyroid effects in male rats (24 months)

Parameter	**0 ppm**	**50 ppm**	**200 ppm**	**1 200 ppm**	**2 400 ppm**
Dose (mg/kg bw per day)	0	2.5	10	60	120
Number of individuals	50	50	50	50	50
Absolute thyroid weight (mg)	44	51	101	76*	67*
Relative thyroid weight (mg/kg bw)	84	103*	202*	154*	145***
Follicular cell hyperplasia	4	8	6	11	12
Follicular cell adenoma	4	8	5	9	10
Follicular cell carcinoma	0	0	2	2	2

bw: body weight; ppm: parts per million; *: $P < 0.05$; ***: $P < 0.001$
Source: Van Deun (1999)

Table A1-4. Summary of the key event dose concordance for imazalil-induced mouse liver tumours

End-point	**NOAEL (ppm)**	**LOAEL (ppm)**	**Reference**
Key event 1: Activation of CAR (molecular initiating event)			
Not directly measured			
Key event 2: Upregulation and downregulation of a set of genes secondary to CAR activation			
Increased CYP2B and CYP3A mRNA	–	50 m[a]	Elcombe (2012c)
Associative event – Increased CYP2B enzyme activity	– 200 f	50 m[a] 600 f	Vermeir, Lavrijsen & Van Leemput (1994); Elcombe (2012c)
Associative event – Increased CYP3A enzyme activity	200 mf	600 mf	Vermeir, Lavrijsen & Van Leemput (1994); Elcombe (2012c)
Associative event – Increased liver weight	200 m 200 f	400 m 600 f	Verstraeten et al. (1993b) Verstraeten et al. (1993a)
Associative event – Hepatocellular hypertrophy	100 m 400 f	200 m 800 f	Verstraeten et al. (1993b); Van Deun et al. (1994); Vermeir, Lavrijsen & Van Leemput (1994); O'Neill (2002)
Key event 3: Increased cell proliferation			
BrdU in vitro	10 µmol/L	30 µmol/L	Elcombe (2012a)
BrdU in vivo (mouse)	<50 m	50 m	Elmore (2004a); Elcombe (2012c)
Key event 4: Increase in foci of altered hepatocytes			
Focal cellular changes (mouse)	50 m 600 f[b]	200 m –	Verstraeten et al. (1993b)
Eosinophilic focus/foci of hepatocellular alteration (rat)	1 200 m 2 400 f[b]	2 400 m –	Van Deun (1999)
Key event 5: Increase in hepatocellular adenomas/carcinomas (adverse outcome)			
Increase in hepatocellular adenomas	50 m	200 m	Verstraeten et al. (1993a)

End-point	NOAEL (ppm)	LOAEL (ppm)	Reference
	200 f	600 f (trend)	
Increase in hepatocellular carcinomas	200 m	600 m	Verstraeten et al. (1993a)
	600 f[b]	–	

BrdU: 5-bromo-2′-deoxyuridine; f: female; LOAEL: lowest-observed-adverse-effect level; m: male; NOAEL: no-oberved-adverse-effect level; ppm: parts per million

[a] Lowest dose tested.

[b] Highest dose tested.

Table A1-5. Summary of the key event dose concordance for imazalil-induced male rat thyroid tumours

End-point	NOAEL (ppm)	LOAEL (ppm)	Reference
Key event 1: CAR-induced increased in hepatic UGT activity (molecular initiating event)			
CAR-induced increase in T_4 UGT activity	200	400	Vermeir, Lavrijsen & Meuldermans (1995); Vermeir & Lavrijsen (1996); Vermeir, Lavrijsen & Meuldermans (2001); Mertens (2011)
Key event 2: Increased hepatic metabolism and biliary excretion of T_4/T_3			
Associative event – Increase in relative liver weight	50	100	Gur (1990); Van Deun et al. (1996a,b); Van Deun (1999); Mertens (2011)
Associative event – Hepatocellular hypertrophy	200	400	Gur, Nyska & Crown (1990); Van Deun et al. (1996a,b); Van Deun (1999); Mertens (2011)
Key event 3: Decrease in serum T_4/T_3 half-life and concentration			
T_3 decrease	3 200[a]	–	Verbeek et al. (2000)
T_4 decrease	400	1 200	Verbeek et al. (2000)
Key event 4: Increase in circulating TSH concentrations			
TSH increase	400	1 200	Verbeek et al. (2000)
Key event 5/6: Thyroid follicular cell hypertrophy, hyperplasia, adenomas			
Increase in relative thyroid weight	400	800	Van Deun et al. (1996a,b); Van Deun (1999)
Follicular cell hypertrophy	400	1 200	Verbeek et al. (2000)
Follicular cell hyperplasia	200	1 200	Van Deun (1999)
Follicular cell adenomas	200	1 200	Van Deun (1999)

CAR: constitutive androstane receptor; LOAEL: lowest-observed-adverse-effect level; NOAEL: no-oberved-adverse-effect level; ppm: parts per million; T_3: triiodothyronine; T_4: thyroxine; TSH: thyroid stimulating hormone; UGT: uridine diphosphate–glucuronosyltransferase

[a] Highest dose tested.

Temporal association

If an event (or events) is an essential element of tumorigenesis, it must precede tumour appearance.

For imazalil, the key events necessary to demonstrate the CAR-induced liver tumour MOA are available, and a temporal association of progression is demonstrated. Multiple exposure time data are available at 1, 2 and 4 days, 1 and 2 weeks, and 1, 3, 18 and 24 months in studies in which mice and rats were offered diets containing imazalil. Increased CAR-activated *Cyp2b* and *Cyp3a* mRNA levels and enzyme activity were demonstrated in mice in vitro at 96 hours (Elcombe, 2012a) and in vivo at 7 days (Elcombe, 2012c) and 1 month (Vermeir, Lavrijsen & Van Leemput, 1994); and in rats at 1 and 2 days, 1 and 2 weeks, and 1 and 3 months (Vermeir, Lavrijsen & Meuldermans, 1995, 2001; Vermeir & Lavrijsen, 1996; Mertens, 2011). Increased relative liver weights were recorded in mice at 4 days (Elmore, 2004a) and 2 weeks (O'Neill, 2002), 1 and 3 months (Verstraeten et al., 1993b; Van Deun et al., 1994; Vermeir, Lavrijsen & Van Leemput, 1994; O'Neill, 2002) and 18 months (Verstraeten et al., 1993a); and in rats at 1 and 3 months (Gur, Nyska & Crown, 1990; Gur, Nyska & Waner, 1991; Vermeir, Lavrijsen & Meuldermans, 1995, 2001; Vermeir & Lavrijsen, 1996; Mertens, 2011) and 24 months (Van Deun, 1999). Increased hepatocellular hypertrophy was reported in mice at 4 days (Elmore, 2004a), 2 weeks (O'Neill, 2002) and 3 months (Verstraeten et al., 1993b; Van Deun et al., 1994; Vermeir, Lavrijsen & Van Leemput, 1994; O'Neill, 2002); and in rats at 1 month (Van Deun et al., 1996a,b; Mertens, 2011), 3 months (Gur, Nyska & Waner, 1991) and 24 months (Van Deun, 1999). Increased cell proliferation was demonstrated in mouse hepatocytes at 96 hours (Elcombe, 2012a), in male mice at 4 days (Elmore, 2004a) and in male rats at 7 days (Elmore, 2004b). Increased incidences of liver foci were reported in male mice at 18 months (Verstraeten et al., 1993b) and in male rats at 24 months (Van Deun, 1999).

For imazalil, the key events necessary to demonstrate the MOA for UGT-induced thyroid tumours are available, and a temporal association of progression is demonstrated. Multiple exposure time data are available at 1, 2 and 4 days, 1 and 2 weeks and 1, 3 and 24 months in studies in which male rats were offered diets containing imazalil. Increased hepatic UGT activity in male rats was reported at 1 and 2 weeks and 1 and 3 months (Vermeir, Lavrijsen & Meuldermans, 1995, 2001; Vermeir & Lavrijsen, 1996; Mertens, 2011). Relative liver weights were increased at 1 and 3 months (Gur, Nyska & Crown, 1990; Gur, Nyska & Waner, 1991; Vermeir, Lavrijsen & Meuldermans, 1995, 2001; Vermeir & Lavrijsen, 1996; Mertens, 2011) and 24 months (Van Deun, 1999). Increased hepatocellular hypertrophy was noted at 1 month (Van Deun et al., 1996a,b; Mertens, 2011), 3 months (Gur, Nyska & Waner, 1991) and 24 months (Van Deun, 1999). Biliary excretion of T_4/T_3 was not measured in any study; however, serum T_4 levels were reduced at 1 week (Verbeek et al., 2000). Increased TSH levels in the serum were demonstrated at 1 and 2 weeks (Verbeek et al., 2000). Increased thyroid weight was demonstrated at 1 and 3 months (Van Deun et al., 1996b) and at 24 months (Van Deun, 1999). Microscopic examination of the thyroid demonstrated an increase in thyroid hypertrophy at 2 weeks (Verbeek et al., 2000), and thyroid hyperplasia was reported after 2 years of treatment (Van Deun, 1999).

Strength, consistency and specificity of association of tumour response with key events

The strength, consistency and specificity of the association can be established from the studies detailed above. The quantifiable precursor events, fundamental to the proposed MOAs, are consistent with the emergence of hepatocellular and thyroid follicular cell tumours in rodents.

Induction of CAR-induced CYP2B and CYP3A enzymes, associated with increased liver weight, hepatocellular hypertrophy and hepatocyte cell proliferation, is consistent with a CAR-mediated MOA. The rat appears to be more resistant than the mouse to imazalil-induced tumour formation, which is consistent with the findings for phenobarbital (Elcombe et al., 2014).

Observation of induction of hepatic UGT activity, associated with increased liver weight and hepatocellular hypertrophy, decreased T_4 and increased TSH levels in rats receiving imazalil in the

diet, is consistent with the perturbation of homeostasis of the hypothalamic–pituitary–thyroid axis by an extra-thyroidal mechanism.

Biological plausibility and coherence

Liver tumours

The MOA demonstrated for imazalil is consistent with the well-known MOA for CAR activation in rodents and is consistent with current understanding of cancer biology and nuclear receptor–mediated carcinogenesis. Phenobarbital is a standard example of a CAR-mediated cytochrome inducer (LeBaron et al., 2013; Elcombe et al., 2014). Treatment with phenobarbital induces a range of phase I (e.g. CYP2B, CYP2C and CYP3A subfamily enzymes) and phase II enzymes (e.g. microsomal epoxide hydrolase, some microsomal UGT enzymes), as well as phase III enzymes (Elcombe et al., 2014). The MOA includes activation of a nuclear receptor, selective cytochrome P450 induction, an increase in cell proliferation and suppression of apoptosis. This creates an environment in the liver where spontaneously initiated cells have a greater chance to survive and divide, eventually leading to tumour formation (Elcombe et al., 2014).

An important step in demonstrating the importance of CAR in liver tumour induction was the finding that phenobarbital does not increase liver weight or DNA synthesis or result in enzyme induction, and consequently there is no increased incidence of liver tumours in human hepatocytes or genetically modified mice lacking CAR. Similarly, studies in rats have demonstrated that CAR is needed for induction of enzymes and replicative DNA synthesis. Based on results in the knockout rodent systems and in human hepatocytes and a chimeric mouse/human model (in which the mouse liver is replaced with human hepatocytes), the lack of replicative DNA synthesis is identified as the key species difference in response to CAR activators, leading to the difference in tumour response (Peffer et al., 2018). Thus, there is a qualitative interspecies difference in tumour response between rats and humans.

The proposed MOA for imazalil-induced rodent liver tumour formation is biologically plausible and consistent with our current understanding of liver tumour formation by non-genotoxic mitogenic agents that can activate nuclear receptors. Imazalil did not induce cell proliferation in human hepatocytes.

Thyroid tumours

There exist considerable data from laboratory studies in rats that demonstrate the relationship between sustained perturbation of the hypothalamic–pituitary–thyroid axis, prolonged stimulation of the thyroid gland by TSH and the progression of thyroid follicular cells from hypertrophy to hyperplasia and eventually to neoplasia (McClain, 1995; Hard, 1998; Hurley, Hill & Whiting, 1998; Capen et al., 1999; IARC, 2001; Dellarco et al., 2006). Increased secretion of TSH may result via several mechanisms, including increased hepatic clearance of T_4, as is the case with imazalil.

Circulating levels of thyroid hormones (T_3/T_4) in the blood are checked and regulated by the thyrotropic cells of the hypothalamus (releasing TRH) and the pituitary gland, which is responsible for the synthesis of TSH. In the pituitary gland, T_4 is metabolized by 5′-deiodinase type II to T_3, which then binds to specific receptors in the cell nucleus. A decrease in T_3 receptor occupancy results in stimulation of TRH from the hypothalamus and TSH synthesis and secretion in the pituitary. Studies in vivo have shown that injection of rats with TSH leads to reductions in thyroid follicular cell nuclear statin, a non-proliferation-specific nuclear protein, indicating that these cells were leaving the non-dividing state to resume the cell cycle (Bayer et al., 1992). This study demonstrated that low, repeated doses of TSH (0.25 IU per rat twice daily) produced a cumulative response in nuclear statin levels over 10 days, which returned to normal resting levels within 5 days of cessation of TSH injections. Reduction in nuclear statin is also an early event that parallels the earliest known pinocytotic response to TSH. These data are consistent with increased TSH concentrations alone causing thyroid follicular cells of rats to enter a state of pre-proliferation. Therefore, the proposal that

imazalil causes thyroid follicular cell neoplasms in rats by initially inducing hepatic UGT is consistent with the known physiology of the hypothalamus–pituitary–thyroid dynamic control system, at least to the stage of hypertrophy and hyperplasia (Dellarco et al., 2006).

The tumour response elicited from chronic treatment with imazalil is typical of a rat thyroid carcinogen. For imazalil, the thyroid follicular cell tumours were found in male rats, but not in female rats or mice. Rats tend to be more sensitive to thyroid carcinogenesis than mice, and male rats are frequently found to be more sensitive than female rats with respect to the finding of thyroid follicular cell tumours in carcinogenicity studies (Hurley, Hill & Whiting, 1998; USEPA, 1998). The TSH levels are typically higher in male rats than in female rats (Hill et al., 1989). In addition, male rats are more prone to hepatic enzyme induction than females of the same strain, but this is dependent on the specific inducible enzyme, the dose of the inducing compound and the age of the animals (Sundseth & Waxman, 1992; Agrawal & Shapiro, 1996; Oropeza-Hernandez, Lopez-Romero & Albores, 2003).

Other modes of action

Liver tumours

Other potential modes of action for liver tumours include genotoxicity, cytotoxicity, hormonal perturbation, oxidative stress, peroxisome proliferator–activated receptor alpha (PPARα) activation, aryl hydrocarbon receptor (AhR)–mediated enzyme induction, infection and iron/copper overload (Elcombe et al., 2014).

Genotoxicity

The genetic toxicology studies with imazalil demonstrate that there is no concern for genotoxicity.

Hepatic cytotoxicity

Cytotoxicity is unlikely to be a relevant MOA for imazalil, as relevant toxicity data from numerous repeated-dose toxicity studies indicated a lack of treatment-related necrosis and necrosis-related end-points in rats and mice (e.g. increases in ALT, AST, ALP; data not shown). Taken together, the weight of evidence does not support a consistent association of cytotoxicity/necrosis in imazalil-treated animals with eventual hepatocellular tumours.

Elcombe (2012c) evaluated the effect of dietary administration of imazalil on the number of hepatocytes undergoing replicative DNA synthesis (S-phase of the cell cycle). Increased S-phase in liver was reported at all concentrations by up to 6-fold relative to control values.

Elcombe (2012a) evaluated cytotoxicity in response to imazalil in vitro in mouse hepatocytes as changes in intracellular ATP concentration and the number of hepatocytes undergoing replicative DNA synthesis (S-phase of the cell cycle). No effect on hepatocyte cytotoxicity (ATP) was reported at 10 or 30 µmol/L, whereas statistically significant increases in S-phase were demonstrated at 10 and 30 µmol/L.

As hepatic cytotoxicity was not evident, several specific regenerative proliferative MOAs can be excluded (e.g. iron/copper overload, infection).

Apoptosis

Imazalil experimentally did not demonstrate any increase in hepatocellular apoptosis (as measured by caspase immunohistochemistry) in male rats at doses up to 2400 ppm for up to 28 days (Mertens, 2011). Elcombe (2012c) evaluated the imazalil-induced increase in the concentration of cleavage products and reported that imazalil induction was less than that of the phenobarbital positive control.

Imazalil-induced *Gadd45β* mRNA[5] was reported in male rats at doses up to 2400 ppm for up to 28 days (Mertens, 2011) and in both wild-type and hPXR/hCAR mice (Elcombe, 2012c).

Oxidative stress

Imazalil experimentally did not demonstrate any increase in hepatocellular oxidative stress (as measured by 4-hydroxy-2-nonenal immunohistochemistry) in male rats at doses up to 2400 ppm for up to 28 daya (Mertens, 2011).

Elcombe (2012c) evaluated lipid peroxidation as thiobarbituric acid reactive substances (largely malondialdehyde) in liver homogenates. Dietary administration of phenobarbital or imazalil for 7 days had no effect on lipid peroxidation in the livers of wild-type or humanized mice.

PPARα

Although there can be considerable crosstalk between CAR and PXR receptors, the key and associative events for imazalil-induced liver tumour formation appear to be predominantly CAR dependent, as such effects are absent in mice lacking CAR. The imazalil-mediated induction of *Cyp2b10* and, to a lesser degree, *Cyp3a11* in the presence of increased hepatocellular S-phase strongly suggests that imazalil is an activator of the xenosensing nuclear receptors CAR and (possibly) PXR. Importantly, imazalil appeared to be a more potent activator of mouse CAR than human CAR, as noted by generally smaller responses in humanized CAR mice when compared with wild-type mice at comparable doses (Elcombe, 2012c).

Elcombe (2012c) evaluated the effect of dietary administration of imazalil on the number of hepatocytes undergoing replicative DNA synthesis (S-phase of the cell cycle) in wild-type and humanized (hPXR/hCAR) mice. Increased S-phase in liver was reported at all concentrations in wild-type mice by up to 6-fold relative to control values; administration of phenobarbital at 1000 ppm increased S-phase by 31.2-fold. Administration of imazalil at all concentrations to hPXR/hCAR mice increased S-phase by up to 3.4-fold relative to control values; administration of phenobarbital at 1000 ppm increased S-phase by 24.5-fold. S-phase was increased in all groups administered imazalil, but no dose–response relationship was observed, and, furthermore, the maximum response was less than 20% and 14% in wild-type and humanized mice, respectively, following the administration of phenobarbital. This lack of cell proliferation differences between wild-type and hCAR/hPXR mice (Elcombe, 2012c) has no effect on the validity of the proposed CAR MOA and is further discussed in the “Uncertainties, inconsistencies and data gaps” section below. It is important to note that imazalil-induced increases in hepatocellular cell proliferation were demonstrated in vitro in mouse hepatocytes (Elcombe, 2012a), but not in female human hepatocytes (Elcombe, 2012b). No concentration of imazalil induced any increases in replicative DNA synthesis (S-phase of the cell cycle) in human hepatocytes, indicating that, opposite to what occurs in mice, imazalil does not induce cell proliferation in human hepatocytes.

Thyroid tumours

Relevant alternative MOAs for rodent thyroid follicular cell tumours include genotoxicity, disruption of thyroid–pituitary functioning, chemicals inhibiting the uptake of iodide into the thyroid, chemicals inhibiting thyroid peroxidase, chemicals inhibiting release of thyroid hormone from the

---

[5] GADD45β is an anti-apoptotic factor, a negative regulator of the p53 tumour suppressor. GADD45β has been implicated in pathways activated by CAR and contributory to the enhanced tumorigenic response in CAR wild-type animals (NIEHS, 2010).

thyroid gland, chemicals damaging thyroid follicular cells and chemicals inhibiting the conversion of T_4 to T_3 (USEPA, 1998).

The genetic toxicology studies with imazalil demonstrate that there is no concern for genotoxicity.

Vermeir, Lavrijsen & Meuldermans (2001) evaluated the possible induction and/or inhibition of hepatic 5′-monodeiodinase (the enzyme that converts T_4 to T_3) and thyroid peroxidase activities by imazalil in male SPF Wistar rats after oral administration through the diet for 1, 2 and 4 weeks at concentrations of 0, 400, 1200 and 3200 ppm; phenobarbital (1200 ppm) was used as the positive control. Thyroid sections were obtained at 1, 2 and 4 weeks as well as after recovery periods of 4 and 9 weeks. 5′-Monodeiodinase activity was decreased with 3200 ppm imazalil and 1200 ppm phenobarbital at week 1, but not in the dose groups at weeks 2 and 4 or in the recovery groups. Thyroid peroxidase activities tended to be increased in the thyroids of the imazalil dose groups at weeks 1 and 4 and in the phenobarbital group at week 4.

Imazalil experimentally did not demonstrate any increase in thyroid follicular cell apoptosis (as measured by caspase immunohistochemistry) (Mertens, 2011).

Imazalil was included in the Hornung et al. (2018) screen for inhibition of deiodinase type 1 activity and did not demonstrate inhibition over 20%.

Imazalil experimentally did not demonstrate any increase in oxidative stress in thyroid follicular epithelial cells (as measured by 4-hydroxy-2-nonenal immunohistochemistry) in male rats at doses up to 2400 ppm for up to 28 days (Mertens, 2011).

Thyroid receptor effects of imazalil have been evaluated in the United States Environmental Protection Agency's Endocrine Disruption Screening Program for the 21st Century[6] and the Toxicity Forecaster[7] programmes. Imazalil was inactive in all assays above the limit of cytotoxicity.

Additional effects on the hypothalamic–pituitary–thyroid axis and disruption of other pathways of thyroid hormone metabolism are other possibilities for altering thyroid homeostasis. These variations would not differ in any fundamental way from the MOA that has been proposed for imazalil, in that all would lead to prolonged TSH stimulation with continuous exposure, and quantitatively humans are less sensitive than rodents to developing cancer from perturbations in the thyroid–pituitary axis (USEPA, 1998).

Uncertainties, inconsistencies and data gaps

Limited uncertainties, inconsistencies and data gaps exist for the proposed liver and thyroid tumour MOAs for imazalil. The identified inconsistencies and data gaps have no effect on the validity of the proposed liver or thyroid MOAs.

Liver tumours

The biological relevance of the liver tumours in female mice at 2400 ppm is uncertain. The incidence of hepatocellular adenomas was not statistically significant, but was increased over the available control and historical controls. There was no increase in the incidence of hepatocellular carcinomas in female mice. A trend for the increase in the incidence of hepatocellular adenomas and carcinomas combined was noted by Vandenberghe (2014). However, increases in CYP2B and CYP3A enzyme activity, liver weight and hepatocellular hypertrophy were reported in female mice. This does not affect the validity of the proposed imazalil-induced CAR MOA. The liver tumours in female mice may not be biologically relevant. If the liver tumours in female mice were considered to

[6] https://actor.epa.gov/edsp21/

[7] https://actor.epa.gov/dashboard/

be biologically relevant, the data generated in male mice in support of the CAR MOA are applicable to the female mice. The response to CAR activation in male mice and male rats is apparent at doses lower than those in female mice, as evidenced by the derived NOAEL values for hepatocellular neoplasms; therefore, data available in males are applicable to females. In addition, Peffer et al. (2018) stated that "in cases where both sexes were affected, in the interest of animal ethics, information gathered using just one sex that is representative of the responses in both males and females should be sufficient".

Increased *Cyp2b* and *Cyp3a* mRNA expression and LKE3 (increased cell proliferation) and LKE4 (increased in foci of altered hepatocytes) were reported only in male mice. As noted above, Peffer et al. (2018) stated that "in cases where both sexes were affected, in the interest of animal ethics, information gathered using just one sex that is representative of the responses in both males and females should be sufficient". This data gap has no effect on the validity of the proposed CAR MOA.

No direct measurement of CAR activation was made in mice or rats. Mertens (2011) did evaluate the levels of CAR (Nr113) and did not demonstrate induction at the mRNA level when compared with animals on the basal diet for any dose (0, 200, 1200 and 2400 ppm) of imazalil or the phenobarbital (1200 ppm) positive control at 24 or 28 days. Because phenobarbital, the positive control, did not elicit a response, the interpretation of this result is that the study was unable to detect changes in CAR mRNA levels. This data gap has no effect on the validity of the proposed CAR MOA, as the other key events and associative events were experimentally demonstrated in the toxicological database for imazalil.

Hepatocellular proliferation was reported in the in vitro mouse hepatocyte study (Elcombe, 2012a) and in male mice (Elmore, 2004a; Elcombe, 2012c) and male rats (Elmore, 2004b). Some studies, such as O'Neill (2002) and Mertens (2011), failed to demonstrate cell proliferation. This inconsistency has no effect on the validity of the proposed CAR MOA, as cell proliferation in both male mice and male rats was experimentally demonstrated within the toxicological database for imazalil. In addition, no cell proliferation was reported in the in vitro human hepatocyte study (Elcombe, 2012b).

Elcombe (2012c) did not report any differences in cell proliferation between wild-type and humanized (hCAR/hPXR) mice when dosed with imazalil or phenobarbital. This is also an identified inconsistency in the literature on phenobarbital (Elcombe et al., 2014). Elcombe et al. (2014) summarized two studies conducted with phenobarbital in wild-type and hCAR or hCAR/hPXR mice. One study demonstrated no cell proliferation in hCAR/hPXR mice, and the other demonstrated cell proliferation in hCAR mice. Elcombe et al. (2014) concluded that "the present data for effects on cell proliferation and apoptosis obtained with transgenic mice expressing either hCAR/hPXR or just hCAR is equivocal". Both phenobarbital and imazalil have been shown not to increase cell proliferation in cultured human hepatocytes (Elcombe, 2012b), whereas cell proliferation was demonstrated in cultured mouse hepatocytes (Elcombe, 2012a). Peffer et al. (2018) listed primary hepatocellular cultures in humans and rodents as specific tests to establish the non-human relevance of the CAR MOA. Therefore, this lack of cell proliferation differences between wild-type and hCAR/hPXR mice in vivo (Elcombe, 2012c) has no effect on the validity of the proposed CAR MOA. Furthermore, the hCAR/hPXR transgenic mouse model is an invalid model, because although the mice are humanized for CAR/PXR, all downstream events are still murine. As stated in describing the MOA for CAR-induced rodent tumours, the species differences are specific to the stimulation of cell proliferation, not to the activation of CAR. Elcombe et al. (2014) provided a review of the evidence that mouse or rat liver tumours that occur via a CAR MOA are not relevant to humans, because although activation of CAR, the induction of CYP2B and other cytochrome P450 forms, and liver hypertrophy can be observed in humans, the cell proliferation and subsequent altered hepatic foci and liver tumours do not occur in humans.

Thyroid tumours

No direct measurement of TKE2 (i.e. increased biliary excretion of T_3/T_4) was conducted in the data package. This is consistent with Dellarco et al. (2006), in that biliary excretion of T_3/T_4 was not explicitly measured for pyraziflumid. The associative events of increased hepatic metabolism and biliary excretion of T_4 are supported by the associative events that demonstrated increased phase I and phase II (T_4-UGT) enzymes as well as increases in liver weight and corresponding liver histopathology demonstrated in the toxicological database for pyraziflumid. T_4 is conjugated with glucuronic acid catalysed by T_4-UGT and excreted in the bile, which is generally accepted by the scientific community without the need for further animal testing (Japundžić, Bastomsky & Japundžić, 1976; McClain, 1989; Capen, 1997; Lecureux et al., 2009). This data gap has therefore no effect on the validity of the proposed thyroid MOA for imazalil.

Assessment of postulated mode of action: human applicability of the proposed MOA

Is the weight of evidence sufficient to establish an MOA in animals?

Liver tumours

As described in detail in this document, the rodent MOA consists of key events that are dose concordant, occur in a logical temporal sequence and are reproducible and consistent across multiple studies. Other potential MOAs have been considered and have been excluded. Thus, the weight of evidence is sufficient to establish an MOA for liver tumours in animals.

Thyroid tumours

As described in detail in this document, there is clear evidence that imazalil alters thyroid homeostasis by UGT induction, by reducing serum T_4 levels and consequently elevating serum TSH. Thus, the weight of evidence is sufficient to establish an MOA for thyroid tumours in animals.

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

Liver tumours

A number of effects of imazalil that are produced in the rodent liver can also be observed in humans. Such effects include activation of CAR, the induction of CYP2B and other cytochrome P450 forms, and liver hypertrophy. However, some clear species differences have been reported.

For example, although imazalil stimulates replicative DNA synthesis in cultured rodent (mouse) hepatocytes, as well as after in vivo administration, imazalil does not increase replicative DNA synthesis in cultured human hepatocytes (Elcombe, 2012a,b). Furthermore, imazalil inhibited apoptosis in mice and rats, which is not expected to occur in human hepatocytes. As described previously, the present literature data on phenobarbital for effects on cell proliferation and apoptosis obtained with transgenic mice expressing either hCAR/hPXR or just hCAR are equivocal. As discussed in the "Uncertainties, inconsistencies, and data gaps" section, the hCAR/hPXR transgenic mouse model is an invalid model, because although mice are humanized for CAR/PXR, all downstream events are still murine. The differences between humans and rodents are specific to the stimulation of cell proliferation, not to the activation of CAR. Elcombe et al. (2014) provided a review of the evidence that mouse or rat liver tumours that occur via a CAR MOA are not relevant to humans, because although activation of CAR, the induction of CYP2B and other cytochrome P450 forms, and liver hypertrophy can be observed in humans, the cell proliferation and subsequent altered hepatic foci and liver tumours do not occur in humans. The results of Elcombe (2012a) demonstrate the cell proliferation in mouse hepatocytes, and the results of Elcombe (2012b) demonstrate the lack of cell proliferation in human hepatocytes. Peffer et al. (2018) listed primary hepatocyte cultures (rodent and human) as a key study to evaluate the qualitative differences in hepatocyte response

between humans and rodents. Based on the known species differences data, it can be concluded that the animal CAR MOA for imazalil-induced rodent liver tumour formation is not qualitatively plausible for humans (Elcombe et al., 2014).

Thyroid tumours

The current understanding of the regulation of thyroid hormone homeostasis in humans and the resulting role of increased TSH levels as a risk factor for thyroid cancer was considered in order to assess the relevance of the key events in the MOA for thyroid follicular cell tumours in rats resulting from chronic exposure to imazalil. The general mechanisms involved in the function and regulation of the hypothalamic–pituitary–thyroid axis in humans and rats are qualitatively similar (Bianco et al., 2002). Therefore, an agent that decreases the serum levels of T_4 in rats could similarly reduce T_4 levels in humans and thereby could potentially lead to an increase in TSH levels.

However, there are quantitative differences between the rat and the human in the function and regulation of the hypothalamic–pituitary–thyroid axis. In contrast to rats, increases in TSH levels have not been demonstrated to result from agents that induce hepatic microsomal enzymes and reduce circulating T_4 levels (Lehman-McKeeman & Hill in Meek et al., 2003). Pharmaceuticals (rifampin, phenytoin and carbamazepine) induce hepatic microsomal enzymes in humans, including UGT, and reduce circulating T_4 levels, but do not result in increased TSH levels (Curran & DeGroot, 1991). Agents that produce thyroid tumours in rats by increasing glucuronidation and biliary excretion of T_4 at high experimental doses produce no changes to thyroid hormone levels at clinically relevant doses (Masubuchi, Hakusui & Okazaki, 1997). Differences in the basic physiological processes between humans and rats account for the dose–response differences and differences in the response of the hypothalamic–pituitary–thyroid axis in triggering increases in TSH levels.

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

Liver tumours

As the data suggest that there are sufficient qualitative differences between the species that develop liver tumours after imazalil treatment (rats and mice) and humans, it is not necessary to answer the third question regarding quantitative differences (Elcombe et al., 2014). The key events of CAR activation, cell proliferation, production of altered foci and production of tumours are well characterized in animals that develop liver tumours. However, although CAR activation does occur in humans, certain other key events do not appear to occur in humans. Both imazalil and phenobarbital have been shown not to increase cell proliferation in cultured human hepatocytes, and the development of altered hepatic foci has not been reported in the literature for phenobarbital (Elcombe et al., 2014). It is therefore not likely that liver tumours would occur through this MOA as a consequence of imazalil or phenobarbital exposure in humans.

As a further weight of evidence, although phenobarbital can act as a non-genotoxic carcinogen and tumour promoter in the rat and mouse, it does not appear to produce liver tumours in humans. Because of the therapeutic uses of phenobarbital, a number of epidemiological and other human studies are available. Moreover, in these studies showing no evidence of increased liver tumour risk, the subjects received phenobarbital for many years at doses producing plasma concentrations similar to those that are carcinogenic in rodents. For example, plasma levels of phenobarbital in three strains of mice given 500 ppm phenobarbital in the drinking-water ranged from 5 to 29 µg/mL, whereas plasma concentrations of phenobarbital in human subjects given therapeutic doses of 3–6 mg/kg bw ranged from 10 to 25 µg/mL (Munro, 1993). Overall, the human epidemiological studies support the conclusion that the MOA for phenobarbital-induced rodent liver tumours is not relevant to humans (Elcombe et al., 2014).

Thyroid tumours

The thyroid is not a direct or the initial target organ of imazalil. The primary target organ of imazalil is the liver and its hepatic metabolizing enzymes, and the increased metabolic activity indirectly increases the systemic clearance of T_4, leading to the hypothyroid state and compensatory increases in TSH in rats. Although there are no chemical-specific data on the possibility for imazalil to disrupt thyroid homeostasis in humans, a number of other microsomal enzyme inducers have been extensively studied, such as phenobarbital, primidone, phenytoin and carbamazepine (Benedetti et al., 2005). As discussed above, agents that produce hypothyroidism by altering the hepatic clearance of T_4 do not appear to result in elevated TSH levels in humans.

Conclusion: statement of confidence, analysis and implications

Liver tumours

There is sufficient experimental evidence to establish an imazalil-induced CAR MOA for rodent liver tumours. The data on species differences are sufficient to determine that this MOA would be qualitatively not relevant for humans (Table A1-6).

Table A1-6. A comparison of key events for CAR-induced liver tumours in rodents and humans

Key event	Evidence in rodents	Evidence in humans
CAR activation	Yes	Yes
Altered gene expression	Yes	Yes
CYP2B induction	Yes	Yes
Hypertrophy	Yes	Yes
Increased cell proliferation	Yes	No
Clonal expansion (foci)	Yes	No
Tumours	Yes	No

CAR: constitutive androstane receptor; CYP: cytochrome P450
Source: Adapted from Elcombe et al. (2014)

Thyroid tumours

There is sufficient experimental evidence to establish an extra-thyroidal disruption MOA for imazalil-induced thyroid follicular cell tumours in male rats. Although imazalil may potentially result in hypothyroidism in humans, there is sufficient quantitative evidence on the basic physiological processes in the general literature to conclude that thyroid tumours induced by a process involving increased hepatic clearance of thyroid hormone and altered homeostasis of the pituitary–thyroid axis in rodents is not likely to lead to an increase in susceptibility to tumour development in humans. Although there are no human data on imazalil, clinical data on other hepatic microsomal enzyme inducers were critical to this human relevance analysis. The general literature provided sufficient evidence to show that, unlike in the rat, decreased T_4 levels typically show no evidence of compensatory increases in TSH levels in humans. There is also cellular and biochemical evidence that the rat pituitary–thyroid axis is much more sensitive than that of humans to such perturbations. This sensitivity is likely the result of the rapid turnover of T_4 in rats coupled with the higher demand for TSH to maintain thyroid activity (Table A1-7). Table A1-8 summarizes the thyroid-specific species differences between rats and humans.

Table A1-7. A comparison of key events for thyroid tumours in rats and humans

Key event	Evidence in rats	Evidence in humans
Increased hepatic clearance of T_4	Yes	Plausible.
Decreased serum T_4	Yes	Plausible, but less likely due to buffering capacity of TBG.
Increased TSH levels	Yes	No. Microsomal enzyme inducers have not been shown to increase TSH levels even when T_4 is decreased.
Increased TSH increases thyroid cell proliferation and tumour formation	Yes	No. Induction of thyroid follicular cell tumours secondary to hypothyroidism is remote in humans, given the quantitative differences in thyroid function/homeostasis. Occurrence of thyroid cancer is rare even in severely hypothyroid individuals.

T_4: thyroxine; TBG: thyroxine binding globulin; TSH: thyroid stimulating hormone
Source: Adapted from Dellarco et al. (2006)

Table A1-8. Thyroid-specific species differences between rats and humans

Parameter	Rat	Human	Consequence
Protein binding of T_3 and T_4	Absent	Extensive binding to TBG and weak binding to albumin	Humans are able to replenish plasma T_3 and T_4 levels without the need for increased production by the thyroid gland.
Plasma TSH levels	60 times higher than in humans	Reference range 0.3–3.0 mIU/L	The rat thyroid is considerably more active than the human thyroid.
T_4 half-life	0.5–1 day	5–9 days	Much higher baseline rate of clearance from plasma in rats than in humans.
T_3 half-life	0.25 day	1 day	Much higher baseline rate of clearance from plasma in rats than in humans.
T_4 production rate/kg bw	10-fold higher than in humans	10-fold lower than in rats	Basal rate of thyroid hormone synthesis is much higher in rats than in humans.
Follicular cell morphology	Cuboidal	Low cuboidal	The rat thyroid produces more thyroid hormones than the human thyroid.

IU: international units; T_3: triiodothyronine; T_4: thyroxine; TBG: thyroxine binding globulin; TSH: thyroid stimulating hormone
Source: Adapted from Dellarco et al. (2006)

Implications of the IPCS human relevance framework

Imazalil is an illustration of an induced tumour response consistent with MOAs that have previously been defined and established as not likely to lead to an increase in susceptibility to tumour development in humans. The first question in the framework analysis, "Is the weight of evidence sufficient to establish an MOA in animals?", has been convincingly met by the data set on this chemical, which conforms to the same key events defined for the pathway of interest. The case for imazalil further demonstrates how data on the basic understanding of the biological processes involved in the MOA provide an important means to compare the rodent and human key events. Thus, the relevant human information on other chemicals was essential to evaluating the qualitative and quantitative differences between experimental animals and humans in addressing the plausibility of the cancer MOAs for humans (i.e. "Can human relevance of the MOA be reasonably excluded on the basis of fundamental, qualitative differences in key events between animals and humans?" and "Can human relevance of the MOA be reasonably excluded on the basis of quantitative differences in either kinetic or dynamic factors between animals and humans?" in the human relevance framework).

In conclusion, for liver tumours, the CAR MOA is qualitatively not relevant to humans. For thyroid tumours, whereas the thyroid MOA is qualitatively relevant to humans, quantitatively, humans are less sensitive than rodents in regard to developing cancer from perturbations in the thyroid–pituitary axis (USEPA, 1998).

References for Appendix 1

Agrawal AK, Shapiro BH (1996). Phenobarbital induction of hepatic CYP2B1 and CYP2B2: pretranscriptional and post-transcriptional effects of gender, adult age, and phenobarbital dose. Mol Pharmacol. 49(3):523–31 [cited in IPCS, 2007].

Bayer I, Mitmaker B, Gordon PH, Wang E (1992). Modulation of nuclear statin expression in rat thyroid follicle cell following administration of thyroid stimulating hormone. J Cell Physiol. 150:276–82 [cited in IPCS, 2007].

Benedetti MS, Whomsley R, Baltes E, Tonner F (2005). Alteration of thyroid hormone homeostasis by antiepileptic drugs in humans: involvement of glucuronosyltransferase induction. Eur J Clin Pharmacol. 61(12):863–72.

Bianco AC, Salvatore D, Gereben B, Berry MJ, Larsen PR (2002). Biochemistry, cellular and molecular biology, and physiological roles of the iodothyronine selenodeiodinases. Endocr Rev. 23(1):38–89 [cited in IPCS, 2007].

Capen CC (1997). Mechanistic data and risk assessment of selected toxic end points of the thyroid gland. Toxicol Pathol. 25(1):39–48.

Capen CC, Dybing E, Rice JM, Wilbourn JD, editors (1999). Species differences in thyroid, kidney and urinary bladder carcinogenesis. Lyon: International Agency for Research on Cancer (IARC Scientific Publications No. 147) [cited in IPCS, 2007].

Curran PG, DeGroot LJ (1991). The effect of hepatic enzyme–inducing drugs on thyroid hormones and the thyroid gland. Endocr Rev. 12:135–50 [cited in IPCS, 2007].

Dellarco VL, McGregor D, Berry SC, Cohen SM, Boobis AR (2006). Thiazopyr and thyroid disruption: case study within the context of the 2006 IPCS human relevance framework for analysis of a cancer mode of action. Crit Rev Toxicol. 36:793–801.

Elcombe B (2012a). Imazalil – induction of cytochrome P450 2b, 3a and DNA-synthesis in cultured female mouse hepatocytes. Unpublished report no. CXR1227. CXR Biosciences, Dundee, United Kingdom. Submitted to WHO by Janssen Pharmaceutica NV.

Elcombe B (2012b). Imazalil – cytochrome P450 2B, 3A and DNA-synthesis induction in cultured female human hepatocytes. Unpublished report no. CXR1226. CXR Biosciences, Dundee, United Kingdom. Submitted to WHO by Janssen Pharmaceutica NV.

Elcombe B (2012c). Imazalil: dietary 7-/28-day investigative toxicity study in wild type C57BI/6J, and PXR/CAR humanised mice. Final report 7 day exposure. Unpublished report no. CXR1085. CXR Biosciences, Dundee, United Kingdom. Submitted to WHO by Janssen Pharmaceutica NV.

Elcombe CR, Peffer RC, Wolf DC, Bailey J, Bars R, Bell D et al. (2014). Mode of action and human relevance analysis for nuclear receptor–mediated liver toxicity: a case study with phenobarbital as a model constitutive androstane receptor (CAR) activator. Crit Rev Toxicol. 44(1):64–82.

Elmore AR (2004a). Cell proliferation study in mice following dietary imazalil administration. Unpublished report no. C131-001. Integrated Laboratory Systems (ILS), Durham, NC, USA. Submitted to WHO by Janssen Pharmaceutica NV.

Elmore AR (2004b). BrdU assessment of rat livers following imazalil exposure. Unpublished report no. C131-002. Integrated Laboratory Systems (ILS), Durham, NC, USA. Submitted to WHO by Janssen Pharmaceutica NV.

Gur E, Nyska A, Crown S (1990). R 5767 imazalil: oral toxicity in dietary administration to rats; a 4-week preliminary range finding study. Unpublished preclinical research report no. LSRI MAK/208/IMA (MCW report no. R-5767). Life Science Research Israel Ltd, Ness Ziona, Israel. Submitted to WHO by ADAMA.

Gur E, Nyska A, Waner T (1991). R 5766 imazalil: toxicity in dietary administration to rats for 13 weeks. Unpublished preclinical research report no. LSRI MAK/209/IMA (MCW report no. R-5766). Life Science Research Israel Ltd, Ness Ziona, Israel. Submitted to WHO by ADAMA.

Hard GC (1998). Recent developments in the investigation of thyroid regulation and thyroid carcinogenesis. Environ Health Perspect. 106(8):1–21 [cited in IPCS, 2007].

Hill RN, Erdreich LS, Paynter OE, Roberts PA, Rosenthal SL, Wilkinson CF (1989). Thyroid follicular cell carcinogenesis. Fundam Appl Toxicol. 12(4):629–97 [cited in IPCS, 2007].

Hornung MW, Korte JJ, Olker JH, Denny JS, Knutsen C, Hartig PC et al. (2018). Screening the ToxCast Phase 1 Chemical Library for inhibition of deiodinase type 1 activity. Toxicol Sci. 162(2):570–81.

Hurley PM, Hill RN, Whiting RJ (1998). Mode of carcinogenic action of pesticides inducing thyroid follicular-cell tumours in rodents. Environ Health Perspect. 106(8):437–45 [cited in IPCS, 2007].

IARC (2001). Some thyrotropic agents. Lyon: International Agency for Research on Cancer (IARC Monographs on the Evaluation of Carcinogenic Risk to Humans, Vol. 79) [cited in IPCS, 2007].

IPCS (2007). Part 1: IPCS framework for analysing the relevance of a cancer mode of action for humans and case-studies. Part 2: IPCS framework for analysing the relevance of a non-cancer mode of action for humans. Geneva: World Health Organization, International Programme on Chemical Safety (Harmonization Project Document No. 4; http://www.inchem.org/documents/harmproj/harmproj/harmproj4.pdf, accessed September 2016).

Japundžić MM, Bastomsky CH, Japundžić IP (1976). Enhanced biliary excretion in rats treated with pregnenolone-16α-carbonitrile. Acta Endocrinol. 81:110–9.

LeBaron MJ, Geter DR, Rasoulpour RJ, Gollapudi BB, Thomas J, Murray J et al. (2013). An integrated approach for prospectively investigating a mode-of-action for rodent liver effects. Toxicol Appl Pharmacol. 270(2):164–73.

Lecureux L, Dieter MZ, Nelson DM, Watson L, Wong H, Gemzik B et al. (2009). Hepatobiliary disposition of thyroid hormone in Mrp2-deficient TR^- rats: reduced biliary excretion of thyroxine glucuronide does not prevent xenobiotic-induced hypothyroidism. Toxicol Sci. 108(2):482–91.

Masubuchi N, Hakusui H, Okazaki O (1997). Effects of proton pump inhibitors on thyroid hormone metabolism in rats: a comparison of UDP-glucuronyltransferase induction. Biochem Pharmacol. 54(11):1225–31 [cited in IPCS, 2007].

McClain RM (1989). The significance of hepatic microsomal enzyme induction and altered thyroid function in rats: implications for thyroid gland neoplasia. Toxicol Pathol. 17(2):294–306.

McClain RM (1995). Mechanistic considerations for the relevance of animal data on thyroid neoplasia to human risk assessment. Mutat Res. 333(1–2):131–42 [cited in IPCS, 2007].

Meek ME, Bucher JR, Cohen SM, Dellarco V, Hill RN, Lehman-McKeeman LD et al. (2003). A framework for human relevance analysis of information on carcinogenic modes of action. Crit Rev Toxicol. 33(6):591–654 [cited in IPCS, 2007].

Mertens JJWM (2011). Cell proliferation study in male Wistar rats after administration of imazalil in the diet for 1, 2, 7, 14, or 28 days. Unpublished report no. WIL-436011. WIL Research Laboratories, Inc., Ashland, OH, USA. Submitted to WHO by Janssen Pharmaceutica NV.

Munro A (1993). The paradoxical lack of interspecies correlation between plasma concentrations and chemical carcinogenicity. Regul Toxicol Pharmacol. 18:115–35.

NIEHS (2010). Dose–response approaches for nuclear receptor–mediated modes of action. CAR/PXR case study. Workshop, September 27–29, 2010. Research Triangle Park (NC): National Institute for Environmental Health Sciences (https://www.tera.org/Peer/NuclearReceptor/Nuclear%20Receptor%20Workshop%20Summary.pdf, accessed 3 January 2019).

O'Neill TP (2002). Cell proliferation study in male CD-1 mice after administered imazalil in the diet for 2 or 13 weeks. Unpublished report no. WIL-436001. WIL Research Laboratories, Inc., Ashland, OH, USA. Submitted to WHO by Janssen Pharmaceutica NV.

Oropeza-Hernandez LF, Lopez-Romero R, Albores A (2003). Hepatic CYP1A, 2B, 2C, 2E and 3A regulation by methoxychlor in male and female rats. Toxicol Lett. 144(1):93–103 [cited in IPCS, 2007].

Peffer RC, LeBaron MJ, Battalora M, Bomann WH, Werner C, Aggarwal M et al. (2018). Minimum datasets to establish a CAR-mediated mode of action for rodent liver tumours. Regul Toxicol Pharmacol. 96:106–20.

Piccirillo VJ (2000). Imazalil: one-month repeated dose oral toxicity study with 1 and 2 week interim sacrifices and 4 and 9 week recovery periods to evaluate thyroid effects. Unpublished report no. VJP 5452-00-1. VJP Consulting Inc., Sterling, VA, USA. Submitted to WHO by Janssen Pharmaceutica NV.

Sparrow S (1993). Peer review contract histopathology report. Unpublished report no. 93/JED003/0793. Pharmaco-LSR Ltd, Suffolk, United Kingdom. Submitted to WHO by Janssen Pharmaceutica NV.

Sundseth SS, Waxman DJ (1992). Sex-dependent expression and clofibrate inducibility of cytochrome P450 4A fatty acid omega-hydroxylases. Male specificity of liver and kidney CYP4A2 mRNA and tissue-specific regulation by growth hormone and testosterone. J Biol Chem. 267(6):3915–21 [cited in IPCS, 2007].

USEPA (1998). Assessment of thyroid follicular cell tumours. Washington (DC): United States Environmental Protection Agency, Risk Assessment Forum (EPA/630/R-97/002; https://www.epa.gov/osa/assessment-thyroid-follicular-cell-tumors, accessed 3 November 2018).

Vandenberghe J (2014). Statement on the interpretation of hepatocellular tumours in Swiss albino mice treated with imazalil for 23 months (Janssen study Tox2194) for purposes of cancer classification and cancer risk assessment. Unpublished report. Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Van Deun K (1999). Combined oral chronic toxicity/carcinogenicity study in the SPF Wistar rat. Unpublished report no. 3817 (study no. R023979). Department of Toxicology, Janssen Pharmaceutica NV, Turnhoutseweg, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Van Deun K, Vandenbergh J, Lammens L, Lampo A, Coussement W, Van Cauteren H (1994). Three-month oral mechanistic toxicity study with one month interim sacrifice in SPF albino Swiss mice. Unpublished report no. 3140. Department of Toxicology, Janssen Research Foundation, Turnhoutseweg, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Van Deun K, Lammens L, Benze J, Coussement W, Vandenberghe J, Lampo A et al. (1996a). 3-month dose range finding and mechanistic toxicity study in SPF Wistar rats. Unpublished report no. 3672. Department of Pharmacokinetics, Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Van Deun K, Lammens L, Benze J, Coussement W, Vandenberghe J, Lampo A et al. (1996b). 3-month dose range finding and mechanistic toxicity study with one month interim sacrifice in SPF Wistar rats. Unpublished study no. 3514. Department of Pharmacokinetics, Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Verbeek J, Verstyenen B, Vandenberghe J, Vinckier A, de Coster R, Lampo A et al. (2000). Imazalil: one-month repeated dose oral toxicity study in the Wistar rat with 1 and 2 week interim sacrifice and 4 and 9 week recovery periods to evaluate thyroid effects. Unpublished report no. R023979/5009. Department of Toxicology, Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Vermeir M, Lavrijsen K (1996). Study on the possible induction and/or inhibition of hepatic drug metabolizing enzymes by imazalil in male and female SPF Wistar rats, after oral administration through the diet for three months at levels of 800, 1,600 and 2,400 and 3,200 ppm. Unpublished study no. FK 2060. Department of Pharmacokinetics, Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Vermeir M, Lavrijsen K, Meuldermans W (1995). Study on the possible induction and/or inhibition of effects of hepatic drug metabolizing enzymes by imazalil in male and female SPF Wistar rats, after oral administration through the diet for one or three consecutive months at levels of 200, 400, 800 ppm. Unpublished report no. FK 1960. Department of Pharmacokinetics, Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Vermeir M, Lavrijsen K, Meuldermans W (2001). Study on the possible induction and/or inhibition of effects of hepatic drug metabolizing enzymes and of the hepatic 5′-monodeiodinase and thyroid peroxidase activities by imazalil in male SPF Wistar rats, after oral administration through the diet for one, two and four weeks at levels of 400, 1200 and 3200 ppm. Unpublished study no. FK 3378. Department of Pharmacokinetics, Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Vermeir M, Lavrijsen K, Van Leemput L (1994). Study on the possible induction and/or inhibition of hepatic drug metabolizing enzymes by imazalil in male and female SPF albino Swiss mice, after oral administration through the diet for one and three consecutive months. Unpublished study no. FK 1600. Department of Pharmacokinetics, Janssen Pharmaceutica NV, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Verstraeten A, Vandenberghe J, Lampo A, Van Deun K, Coussement W, Van Cauteren H (1993a). Imazalil base: carcinogenicity study in Swiss mice – administration through the diet for 2 years. Unpublished report no. 2194. Department of Toxicology, Janssen Research Foundation, Turnhoutsweg, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

Verstraeten A, Vandenberghe J, Lampo A, Van Deun K, Coussement W, Van Cauteren H (1993b). Imazalil: R 23979 – three-month oral toxicity study in albino Swiss mice. Unpublished report no. 2020. Department of Toxicology, Janssen Research Foundation, Turnhoutsweg, Beerse, Belgium. Submitted to WHO by Janssen Pharmaceutica NV.

KRESOXIM-METHYL

First draft prepared by
D. Kanungo[1] and Ian Dewhurst[2]

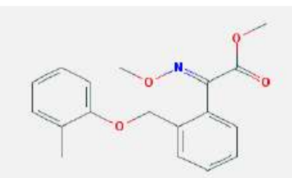
[1] Food Safety and Standards Authority of India, New Delhi, India
[2] Sunnycrest, Leavening, North Yorkshire, United Kingdom

Explanation 225
Evaluation for acceptable intake..226
1. Biochemical aspects 226
1.1 Absorption, distribution and excretion 226
1.2 Biotransformation 230
2. Toxicological studies 233
2.1 Acute toxicity 233
2.2 Short-term studies of toxicity 234
(a) Oral administration 234
(b) Dermal application 238
2.3 Long-term studies of toxicity and carcinogenicity 239
2.4 Genotoxicity 250
2.5 Reproductive and developmental toxicity 252
(a) Multigeneration studies 252
(b) Developmental toxicity 254
2.6 Special studies 255
(a) Neurotoxicity 255
(b) Mechanistic studies on the carcinogenic mode of action 256
(c) Other special studies 261
(d) Studies on metabolites 263
3. Observations in humans 264
3.1 Medical surveillance on manufacturing plant personnel 264
3.2 Direct observation 264
Comments 265
Toxicological evaluation 269
References 271

Explanation

Kresoxim-methyl (Fig. 1) is the International Organization for Standardization–approved common name for methyl-(*E*)-2-methoxyimino-2-[2-(2-methylphenoxymethyl)phenyl] acetate (International Union of Pure and Applied Chemistry), with the Chemical Abstracts Service number 143390-89-0.

Fig. 1. Chemical structure of kresoxim-methyl



Kresoxim-methyl is a broad-spectrum fungicide and a member of the strobilurin family, a class of biologically active compounds structurally related to strobilurin A, a natural product of the wood-decaying fungus *Strobilurus tenacellus.* It is intended for use as an agricultural spray in the control and treatment of fungal infections on crops and fruits. Strobilurins are known to bind bcl complex (complex III), one of the oxidoreductase enzymes in the electron transport chain in mitochondria.

Kresoxim-methyl was previously evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 1998, when an acceptable daily intake (ADI) of 0–0.4 mg/kg body weight (bw) was established on the basis of a no-observed-adverse-effect level (NOAEL) of 36 mg/kg bw per day in a 24-month study of toxicity and carcinogenicity in rats, with application of a 100-fold safety factor. It was considered to be unnecessary to establish an acute reference dose (ARfD).

Kresoxim-methyl was reviewed by the present Meeting as part of the periodic review programme of the Codex Committee on Pesticide Residues.

Newly submitted studies on acute toxicity, carcinogenicity in a different strain of rat, genotoxicity and neurotoxicity that were not made available to the 1998 Meeting were evaluated by the present Meeting.

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

Evaluation for acceptable intake

1. Biochemical aspects

1.1 Absorption, distribution and excretion

In a study of biokinetics, kresoxim-methyl labelled with ^{14}C on the phenyl A ring (phenoxy; radiochemical purity >98%) or B ring (phenyl; radiochemical purity >98%) or with ^{13}C on the carbon side-chain was administered to rats (aged about 7–13 weeks at the start of the study) by gavage as a suspension in 0.5% carboxymethylcellulose or intravenously as a 0.9% saline solution.

When groups of five male and five female rats were given [^{14}C-B ring]kresoxim-methyl by gavage at 50 or 500 mg/kg bw, with or without pretreatment with unlabelled kresoxim-methyl, or [^{14}C-A ring]kresoxim-methyl at a dose of 500 mg/kg bw, the compound was excreted predominantly in faeces. At the low dose of [^{14}C-B ring]kresoxim-methyl, faecal excretion represented 65–67% of the administered dose and urinary excretion represented 20–28% of the dose within 48 hours; less than 1% of the radiolabel was recovered in urine and faeces after this time. Pretreatment with unlabelled kresoxim-methyl at the low dose for 14 days did not change the excretion pattern. At the high dose, faecal excretion represented 80–81% of the administered dose and urinary excretion represented 8–13% of the dose within 48 hours. The total radiolabel recovered within 120 hours was 97% of the dose with [^{14}C-A ring]kresoxim-methyl and 90–96% of the dose with [^{14}C-B ring]kresoxim-methyl, with 62–78% of the dose with [^{14}C-A ring]kresoxim-methyl and 81% of the dose with [^{14}C-B ring]kresoxim-methyl excreted in faeces and 17–33% of the dose with [^{14}C-A ring]kresoxim-methyl and 9–13% of the dose with [^{14}C-B ring]kresoxim-methyl excreted in urine. No radiolabel was detected in exhaled air.

In the groups given [^{14}C-B ring]kresoxim-methyl, peak concentrations of radiolabel in plasma were reached 0.5–1 hour after dosing at the low dose and 8 hours after dosing at the high dose. The plasma level then declined, with a terminal half-life of 17–19 hours at the low dose and 22–31 hours at the high dose. The ratios of the area under the plasma concentration–time curve (AUC) for the high:low dose (10:1) were 2.3 for males and 2.1 for females (Table 1).

Radiolabel concentrations were determined in tissues 0.5, 8, 24, 96 and 120 hours after dosing. Except for the gastrointestinal tract, the highest residual concentration was found in the liver (0.1 µg equivalents [Eq]/g at 120 hours and 0.3–1.4 µg Eq/g at 24 hours after dosing at 50 mg/kg bw). The residual concentrations in other tissues were less than 0.1 µg Eq/g tissue at 120 hours after dosing at 50 mg/kg bw. The concentrations of radiolabel in the tissues were comparable in males and females, indicating a similar pattern of wide distribution and subsequent elimination.

Table 1. Pharmacokinetic parameters of radioactivity in plasma after single oral administration of ^{14}C-labelled kresoxim-methyl to male and female rats at 50 or 500 mg/kg bw

Sex	Dose (mg/kg bw)	C_{max} (µg/g)	T_{max} (h)	Terminal half-life (h)	AUC (µg·g/h)	Total clearance (µg/min)
Male	500	3.36	8.0	30.5	85.94	97.0
	50	1.59	1.0	19.1	36.88	22.6
Female	500	3.92	8.0	22.1	76.49	109.0
	50	2.58	0.5	16.9	36.22	23.0

AUC: area under the plasma concentration–time curve; bw: body weight; C_{max}: maximum concentration; T_{max}: time to reach maximum concentration
Source: Gans & Hildebrand (1994)

Groups of five male and five female rats given [^{14}C-B ring]kresoxim-methyl intravenously as a single dose of 5 mg/kg bw excreted 49–66% of the radiolabel in urine and 23–48% in faeces within 120 hours. Faecal excretion was considerably lower after parenteral administration than after ingestion (23–48% versus 80–81%), and the fraction of the dose excreted via the urine was correspondingly increased, indicating incomplete absorption and some excretion into the gastrointestinal tract.

Groups of four male and four female rats with cannulated bile ducts were given the [^{14}C-B ring]-labelled material as a single oral dose of 50 or 500 mg/kg bw. Biliary excretion accounted for 35–43% of the radiolabel at the low dose and 14–15% at the high dose within 48 hours. Excretion of the [^{14}C-A ring]-labelled material in bile was not examined.

If it is assumed that the amount of radioactivity excreted via bile and urine represents the bioavailable amount of ^{14}C-labelled kresoxim-methyl, then absorption rates of 22–28% for the high dose and 55–71% for the low dose can be calculated. Increasing the dose by a factor of 10 resulted in a decrease of absorption rate by a factor of about 2.5 in both sexes, indicating that absorption is saturated at high doses. This result is in agreement with the C_{max} results obtained from the plasma and blood level experiment, where C_{max} values increased with dose, but not dose proportionally (Gans & Hildebrand, 1994; Gans, 1995a).

To determine the tissue distribution of radioactivity after a single oral dose at different time points and doses, ^{14}C-labelled kresoxim-methyl (B ring) (lot/batch no. 445-25; chemical purity 97.2%; radiochemical purity 99.2%) was administered to groups of Wistar (Chbb:THOM(SPF)) rats (7 weeks of age at the time of administration; weight at dosing 210–243 g for males and 170–216 g for females) by oral gavage at a single dose of 50 or 500 mg/kg bw. The study was conducted with four sacrifice intervals per dose group. The high-dose group comprised three males and three females per time point, whereas the low-dose group comprised three females per time point only. The sacrifice intervals were based on the plasma toxicokinetics study (Gans & Hildebrand, 1994). In the low-dose group, the time points were T_{max} = 0.5 hour, 1/2 T_{max} = 8 hours, 1/4 T_{max} = 24 hours and 1/8 T_{max} = 96 hours. In the high-dose group, animals were sacrificed at T_{max} = 8 hours, 1/2 T_{max} = 20 hours, 1/4 T_{max} = 24 hours and 1/8 T_{max} = 96 hours. After sacrifice, remaining radioactivity was measured in the following organs of the high-dose group after solubilization: heart, carcass, fat (adipose) tissue, liver, bone, plasma, muscle, spleen, kidney, thyroid glands, gonads, skin, adrenal glands, brain, blood cells, lung, pancreas, bone marrow, intestinal tract and contents, and stomach and stomach contents. In the low-dose group (all females), only ovaries/uterus and bone marrow were analysed.

After oral administration of ^{14}C-labelled kresoxim-methyl at a dose of 500 mg/kg bw to male and female rats, there was a rapid absorption of radioactive material from the gastrointestinal tract. Radioactive material was distributed in all tissues and organs throughout the body (Tables 2 and 3).

Eight hours after administration of radiolabelled kresoxim-methyl to male and female rats, the highest tissue concentrations were found in the contents of the gastrointestinal tract, liver, kidney, adrenal glands and carcass, ranging from 37.50 to 14 090 µg Eq/g. In females, the concentration also

exceeded 30 µg Eq/g (30 parts per million [ppm]) in ovary/uterus, adipose tissue and pancreas. The lowest concentrations were measured in bone marrow and brain (0.40–2.52 µg Eq/g). Ninety-six hours after dosing, concentrations of radioactivity were below 50 µg Eq/g (50 ppm) in all organs and tissues. There was no evidence of accumulation of radioactive material after dosing with ^{14}C-labelled kresoxim-methyl (Tables 2 and 3).

Thirty minutes after oral administration of ^{14}C-labelled kresoxim-methyl to female rats at a dose of 50 mg/kg bw, there was radioactive material detectable in bone marrow (0.09 µg Eq/g) and in the ovary/uterus (36.88 µg Eq/g). The concentration of radioactivity declined continuously. Ninety-six hours after the administration, the concentration in both tissues was below 0.5 µg Eq/g (0.5 ppm). The results are shown in Table 4.

Table 2. Mean tissue concentrations of radioactivity after single oral administration of ^{14}C-labelled kresoxim-methyl to rats at a dose of 500 mg/kg bw

	Mean tissue concentration of radioactivity (µg Eq/g tissue)					
	8 hours		24 hours		96 hours	
Organ/tissue	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
Blood cells	3.33	7.06	2.33	2.56	0.99	1.24
Plasma/serum	22.19	23.68	6.77	4.05	0.91	0.93
Lung	19.18	18.99	7.69	7.93	4.58	4.80
Heart	10.66	11.44	6.36	6.84	4.79	5.89
Spleen	11.92	11.90	10.10	8.66	5.43	7.33
Kidney	57.32	59.23	20.35	10.88	3.05	3.16
Adrenals	53.91	55.07	36.60	36.12	36.15	33.50
Testes	7.95	–	4.22	–	2.63	–
Ovary/uterus	–	55.73	–	62.92	–	37.89
Muscle	24.71	8.53	23.45	21.09	30.03	16.17
Brain	1.46	2.52	0.43	0.32	0.21	0.22
Adipose tissue	12.78	48.76	21.16	17.91	9.61	8.94
Bone	6.62	8.82	6.57	7.39	7.67	5.21
Thyroid	29.72	12.62	10.99	11.90	8.92	10.42
Pancreas	27.96	213.28	43.15	65.18	14.10	11.09
Stomach contents	14 089.94	10 072.14	545.97	203.17	6.43	17.34
Stomach	2 347.85	1 943.42	128.20	104.69	15.66	47.61
Gut contents	5 625.08	6 631.68	590.79	460.74	17.80	16.95
Gut	726.72	681.95	381.89	375.57	24.53	21.47
Liver	58.01	63.33	17.30	13.03	3.85	4.01
Skin	21.81	22.22	14.62	12.23	11.19	7.95
Carcass	37.50	44.91	4.03	3.73	3.68	2.89
Bone marrow	1.67	0.40	0.41	0.32	0.24	0.23

bw: body weight; Eq: equivalents
Source: Gans (1995b)

Table 3. Mean tissue concentrations of radioactivity after single oral administration of ^{14}C-labelled kresoxim-methyl to rats at a dose of 500 mg/kg bw

	Mean tissue concentration of radioactivity (% of administered dose)					
	8 hours		**24 hours**		**96 hours**	
Organ/tissue	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
Blood cells	0.01	0.03	0.01	0.01	0.00	0.00
Plasma/serum	0.09	0.08	0.02	0.01	0.00	0.00
Lung	0.02	0.02	0.01	0.01	0.01	0.01
Heart	0.01	0.01	0.00	0.01	0.00	0.00
Spleen	0.01	0.01	0.01	0.00	0.00	0.00
Kidney	0.09	0.08	0.03	0.02	0.00	0.01
Adrenals	0.00	0.00	0.00	0.00	0.00	0.00
Testes	0.02	–	0.01	–	0.01	–
Ovary/uterus	–	0.02	–	0.03	–	0.02
Muscle	0.01	0.01	0.01	0.03	0.02	0.01
Brain	0.00	0.00	0.00	0.00	0.00	0.00
Adipose tissue	0.01	0.03	0.01	0.01	0.01	0.01
Bone	0.00	0.00	0.00	0.00	0.00	0.00
Thyroid	0.01	0.00	0.00	0.00	0.00	0.00
Pancreas	0.01	0.15	0.02	0.05	0.01	0.01
Stomach contents	31.93	19.93	1.10	0.19	0.02	0.04
Stomach	3.17	2.22	0.28	0.13	0.02	0.05
Gut contents	56.76	64.43	7.11	4.89	0.23	0.17
Gut	4.25	3.41	1.71	1.77	0.14	0.11
Liver	0.58	0.52	0.21	0.15	0.04	0.04
Skin	0.83	0.70	0.50	0.41	0.43	0.25
Carcass	3.43	4.45	0.41	0.40	0.38	0.31
Bone marrow	0.00	0.00	0.00	0.00	0.00	0.00

bw: body weight
Source: Gans (1995b)

Table 4. Mean tissue concentrations of radioactivity after single oral administration of ^{14}C-labelled kresoxim-methyl to female rats at a dose of 50 mg/kg bw

	Mean tissue concentration of radioactivity							
	0.5 hour		**8 hours**		**24 hours**		**96 hours**	
Organ/tissue	**µg Eq/g**	**%**	**µg Eq/g**	**%**	**µg Eq/g**	**%**	**µg Eq/g**	**%**
Ovary/uterus	36.88	0.21	4.83	0.02	0.91	0.00	0.48	0.00
Bone marrow	0.09	0.00	0.06	0.00	0.03	0.00	0.03	0.00

bw: body weight; Eq: equivalents
Source: Gans (1995b)

In view of the above, it is concluded that after oral administration of ^{14}C-labelled kresoxim-methyl at a dose of 500 mg/kg bw to male and female rats, there was a rapid but incomplete absorption of radioactive material from the gastrointestinal tract. Radioactive material was distributed in all tissues and organs throughout the body, but concentrations of radioactivity were below 50 µg Eq/g (50 ppm) in all organs and tissues at 96 hours after dosing. There was no evidence of accumulation of radioactive material after dosing with kresoxim-methyl, but clearance from several tissues and organs was slow (Table 2).

Thirty minutes after oral administration of ^{14}C-labelled kresoxim-methyl to female rats at a dose of 50 mg/kg bw, there was radioactive material detectable in bone marrow (0.09 µg Eq/g) and in the ovary/uterus (36.88 µg Eq/g). The concentration of radioactivity declined continuously. At 96 hours after administration, the concentration in both tissues was below 0.5 µg Eq/g (0.5 ppm) (Gans et al., 1995b).

The tissue distribution of radioactivity was determined by quantitative whole body autoradiography following a single oral administration of ^{14}C-labelled kresoxim-methyl (radiochemical purity 96.6%) to male and female albino Crl:(WI) BR rats (approximately 9 weeks of age at the start of administration; weight 207–216 g for males and 161–182 g for females) at a nominal dose of 50 mg/kg bw.

The absorption of ^{14}C-labelled kresoxim-methyl-derived material was low, and uptake into the tissues was limited. Other than the gastrointestinal tract, the highest concentrations of radioactivity were associated with the organs of metabolism and elimination (i.e. liver and kidney). As most of the administered radioactivity was confined to the gastrointestinal tract, lower concentrations were found in liver (5.269 and 4.939 µg Eq/g at 0.5 and 2 hours, respectively) and kidney (6.483 and 6.514 µg Eq/g, respectively). Of the remaining tissues where radioactivity was present (i.e. blood, bone marrow, brain, eye [including uveal tract], Harderian gland, lung, muscle [including myocardium], salivary glands, gonads [male rats], thymus and thyroid), ^{14}C-labelled kresoxim-methyl concentrations were below 1.6 µg Eq/g tissue. At 24 hours post-dosing, radioactivity was detected only in the contents of the gastrointestinal tract (at moderate levels) and in the liver and skin (at low levels). After 96 hours, radioactivity persisted at low levels in the contents of the gastrointestinal tract in both sexes, as well as in or on the skin of the females (Whitby, 1993).

1.2 Biotransformation

The samples collected in the experiments described above (Gans & Hildebrand, 1994) were analysed for metabolites of kresoxim-methyl. After oral administration, high proportions of parent compound were found in the faeces, but none was detected in the bile or in tissues (plasma, liver and kidney) sampled about 4 hours after administration of the low or high dose (Tables 5 and 6).

In total, 34 metabolites, including conjugates, were identified by nuclear magnetic resonance spectroscopy and mass spectrometry in rat excreta, with 20 in urine, eight in faeces and 17 in bile. The major metabolites identified in urine and faeces were M1, a hydrolytic product of the acetyl ester; M2, an oxidative metabolite of the aryl-methyl moiety of M1; and M9, a hydroxylated metabolite of the phenoxy ring of M1. M1 and M9 were the major metabolites identified in tissues. Glucuronidated conjugates were detected in notable quantities in the bile. There was no evidence that the metabolic pathways were induced by pretreatment with kresoxim-methyl. A small difference in the metabolite pattern in urine and bile was observed between males and females, the percentages of M1 and M9 in urine from females being greater than in urine from males.

In summary, the metabolic pathways of kresoxim-methyl consisted of hydrolytic cleavages of the ester, the oxime ether and the benzyl ether bonds; hydroxylation at the *para* position of the phenoxy ring; oxidation of the aryl-methyl group to benzyl alcohol and its subsequent oxidation to the corresponding carboxylic acid; and conjugation of the resulting hydroxy groups with glucuronate and sulfate (Kohl, 1994, 1995, 1998; Annex 1, reference *85*).

Table 5. Presence of parent compound and metabolites in excreta following administration of a single oral dose of ^{14}C-labelled kresoxim-methyl to rats

	Concentration (% of administered dose)							
	Faeces				Urine			
	50 mg/kg bw		500 mg/kg bw		50 mg/kg bw		500 mg/kg bw	
Substance	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
Parent	49.5	47.1	74.9	39.5	–	–	–	–
M1	2.1	–	0.1	7.1	0.4	2.7	2.8	2.2
M2	2.7	0.5	0.5	5.8	2.0	3.4	1.5	2.0
M4	1.1	0.5	0.3	2.5	Mix1	Mix1	Mix1	Mix1
M6	–	–	–	–	2.8	1.1	1.9	0.5
M8	–	–	–	–	0.1	0.4	–	Mix3
M9	5.2	6.0	0.9	13.3	5.5	11.0	2.7	4.9
M11	–	–	–	–	Mix2	–	Mix2	Mix3
M12	–	–	–	–	Mix2	–	Mix2	Mix3
M14	–	–	–	–	Mix1	Mix1	Mix1	Mix1
M15	1.3	2.7	0.1	3.4	–	–	–	–
M16	–	–	–	–	–	–	0.3	Mix3
M20	–	–	–	–	Mix1	Mix1	Mix1	Mix1
M24	–	–	0.1	–	–	–	0.1	0.4
M26	–	–	–	–	Mix2	–	Mix2	Mix3
Mix1	–	–	–	–	1.4	1.6	0.9	1.1
Mix2	–	–	–	–	0.9	–	0.8	–
Mix3	–	–	–	–	–	–	–	1.4
UK1	1.3	0.6	0.4	0.1	–	0.1	–	0.2
UK2	–	0.2	0.1	1.4	–	–	–	–
UK3	–	–	0.1	1.8	–	–	–	–
UK4	–	–	0.3	–	–	–	–	–
UK5	–	–	0.2	–	–	–	–	–
UK6	–	–	–	0.1	–	–	–	–

bw: body weight; Mix1: mixture of M4 + M14 + M20; Mix2: mixture of M11 + M12 + M26; Mix3: mixture of M8 + M11 + M12 + M26; UK: unknown compound
Source: Annex 1, reference *85*

Table 6. Presence of parent compound and metabolites in bile and tissues following administration of a single oral dose of ^{14}C-labelled kresoxim-methyl to rats[a]

	Concentration (% of administered dose)[b]							
	Bile		Plasma		Liver			
	50 mg/kg bw		50 mg/kg bw		50 mg/kg bw		500 mg/kg bw	
Substance	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
Parent	0	0	0	0	0	0	0	0
M1	1.7	1.9	0.386	0.304	0.13	0.07	0.07	0.12
M2	Mix5	Mix5	0.095	Mix8	0.08	0.04	0.04	0.04
M4	–	–	0.041	Mix8	0.03	0.02	0.04	0.02
M6	–	–	0.027	0.006	–	–	–	–
M9	1.1	1.3	0.173	0.164	0.17	0.07	0.06	0.09
M11	–	–	0.002	–	Mix4	Mix4	Mix4	Mix4

	Concentration (% of administered dose)[b]							
	Bile		**Plasma**		**Liver**			
	50 mg/kg bw		**50 mg/kg bw**		**50 mg/kg bw**		**500 mg/kg bw**	
Substance	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
M28	0.7	2.9	–	–	–	–	–	–
M31	0.5	1.1	–	–	–	–	–	–
M35	1.7	0.7	–	–	–	–	–	–
M44	0.4	0.3	–	–	–	–	–	–
M45	Mix5	Mix5	–	–	–	–	–	–
Mix4	–	–	0.115	–	0.08	0.02	0.02	0.01
Mix5	1.1	1.2	–	–	–	–	–	–
Mix6	6.3	3.6	–	–	–	–	–	–
Mix7	0.4	0.2	–	–	–	–	–	–
Mix8	–	–	–	0.169	–	–	–	–
UK1	–	–	–		0.02	–	–	–
UK2	–	–	–	0.024	0.02	0.01	–	–

bw: body weight; Mix4: mixture of M11 + M12 + M16 + M34 + M36 + M37; Mix5: mixture of M2 + M45; Mix6: mixture of M25 + M26 + M29 + M33 + M39; Mix7: mixture of M34 + M36 + M37; Mix8: mixture of M2 + M4; UK: unknown compound

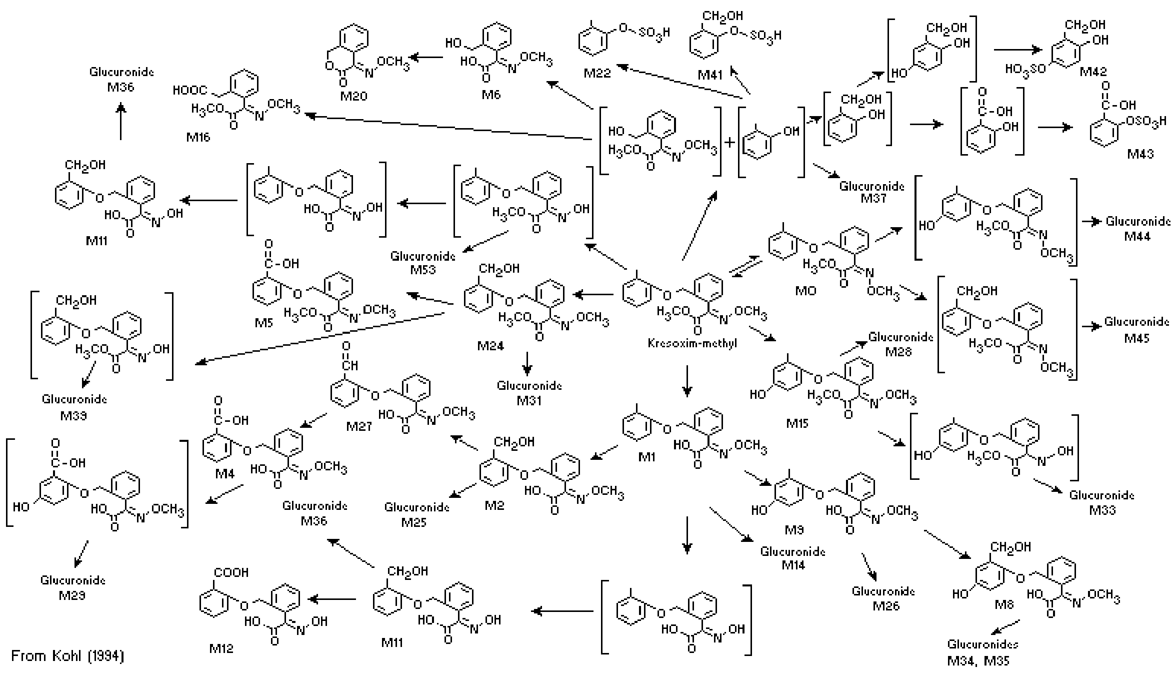
[a] The tissue samples were collected 3.5–4 hours after single-dose administration.

[b] The values in plasma are expressed as microgram equivalents per millilitre.

Source: Annex 1, reference *85*

The proposed metabolic pathway for kresoxim-methyl in rats is shown in Fig. 2.

Fig. 2. Metabolic pathway of kresoxim-methyl in rats



Source: Kohl (1994)

2. Toxicological studies

2.1 Acute toxicity

Several studies found kresoxim-methyl to have low acute toxicity when administered orally, dermally or through inhalation to rats (Table 7).

Table 7. Summary of acute toxicity studies with kresoxim-methyl

Species	Strain	Sex	Route	Purity (%)	LD_{50} (mg/kg bw) / LC_{50} (mg/L air)	Reference
Mouse	ICR	M + F	Oral	94.3	>5 000	Yamamoto (1994)
Rat	Chbb Wistar	M + F	Oral	93.7	>5 000	Kirsch & Hildebrand (1993a)
Rat	Wistar / CrI:WI (Han)	F	Oral	86.4	>2 000	Cords & Lammer (2011a)
Rat	Chbb Wistar	M + F	Dermal	93.7	>2 000	Kirsch & Hildebrand (1993b)
Rat	Wistar / CrI:WI (Han) SPF	M + F	Dermal	86.4	>2 000	Cords & Lammer (2011b)
Rat	Chbb Wistar	M + F	Inhalation	96.6	>5.6 (MMADs of 1.8–2.4 µm)	Gamer (1992)
Rat	Wistar / RccHan:WIST	M + F	Inhalation	86.4	>5.263 (MMADs of 4.1 and 4.0 µm)	Wittmer & Landsiedel (2011)

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

None of the studies included in Table 7 reported any death or abnormal clinical signs in mice or rats, and no abnormal changes in organs were seen at necropsy.

Kresoxim-methyl was not irritating to the skin or to the eyes of rabbits. It was also not sensitizing to the skin of guinea-pigs (Table 8).

Table 8. Summary of dermal and eye irritation and skin sensitization studies with kresoxim-methyl

Species	Strain	Sex	End-point	Purity (%)	Result	Reference
Rabbit	White Vienna	NS	Skin irritation	NS	Not irritating	Rossbacher (1992a)
Rabbit	New Zealand white CrI:KBL (NZW) SPF	F	Skin irritation	86.4	Not irritating	Wolf (2011a)
Rabbit	White Vienna	NS	Eye irritation	NS	Not irritating	Rossbacher (1992b)
Rabbit	New Zealand white	F	Eye irritation	86.4	Not irritating	Wolf (2011b)

Species	Strain	Sex	End-point	Purity (%)	Result	Reference
	CrI:KBL (NZW) SPF					
Guinea-pig	Dunkin Hartley HOE DHPK	NS	Skin sensitization	NS	Not sensitizing (Magnusson & Kligman test)	Rossbacher (1993)
Guinea-pig	Hartley CrI:HA	F	Skin sensitization	86.4	Not sensitizing (Buehler test)	Weiss-Fuchs (2011)

bw: body weight; F: female; NS: not specified

2.2 Short-term studies of toxicity

(a) Oral administration

Short-term studies of oral toxicity in mice, rats and dogs were conducted.

Mice

In a 28-day range-finding study, groups of five male and five female B6C3F1(Cr) mice (aged 49 days at the start of the study; mean body weight 24 g [23–25 g] for males and 19 g [18–20 g] for females) were given diets containing kresoxim-methyl (purity 96.6%) at a concentration of 0, 500, 2000 or 8000 parts per million (ppm) (equal to 0, 113, 485 and 2141 mg/kg bw per day for males and 0, 182, 798 and 3755 mg/kg bw per day for females, respectively). The animals were observed for clinical signs, death, feed consumption, body weight and clinical chemical, haematological and pathological end-points.

There were no deaths or signs of clinical toxicity. At the high dose, significantly reduced serum concentrations of triglycerides, cholesterol and lymphocytes were observed in males, and significantly increased relative liver weights (males: 13%; females: 12%) ($P < 0.01$) (Table 9) without any associated histopathological changes were observed in animals of both sexes. No compound-related lesions were observed on histopathological examination.

Table 9. Body weight and liver weights in mice administered kresoxim-methyl for 28 days

Parameter		Males				Females			
		0 ppm	500 ppm	2 000 ppm	8 000 ppm	0 ppm	500 ppm	2 000 ppm	8 000 ppm
Body weight	Mean (g)	24.48 –	24.50 (+0.08)[a]	24.84 (+1.47)	22.78 (−6.9)	18.54 –	18.44 (−0.54)	18.14 (−2.15)	18.78 (+1.29)
Absolute liver weight	Mean (g)	1.1 –	1.072 (−2.5)	1.141 (+3.72)	1.156 (+5.1)	0.902 –	0.854 (−5.3)	0.941 (+4.3)	1.021 (+13.2)
Relative liver weight	Mean (%)	4.489 –	4.378 (−2.5)	4.598 (+2.4)	5.071** (+13)	4.857 –	4.627 (−5)	5.186 (+7)	5.434** (+12)

ppm: parts per million; **: $P < 0.01$ (Kruskal-Wallis H test + Wilcoxon test)
[a] Per cent change relative to controls given in parentheses.
Source: Schilling & Hildebrand (1992a)

The NOAEL was 8000 ppm (equal to 2141 mg/kg bw per day), the highest dose tested (Schilling & Hildebrand, 1992a).

In a 3-month study, groups of 10 male and 10 female C57Bl/6N(Cr) mice (aged 55 days at the start of the study; mean body weight 22 g [20–24 g] for males and 18 g [17–19 g] for females) were given diets containing kresoxim-methyl (purity 98.7%) at a concentration of 0, 250, 1000, 4000 or 8000 ppm (equal to 0, 57, 230, 909 and 1937 mg/kg bw per day for males and 0, 80, 326, 1326 and 2583 mg/kg bw per day for females, respectively). The animals were observed for clinical signs, death, feed consumption, body weight, clinical chemistry parameters, including the activities of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT), and haematological and pathological end-points.

There were no deaths, signs of clinical toxicity or changes in haematological or clinical chemistry parameters. Dose-dependent reductions in terminal body weight, by 4.0% at 4000 ppm and 7.1% at 8000 ppm, and body weight gain, by 11% at 4000 ppm and 24% at 8000 ppm, were seen in males. Statistically significant increases in relative liver weight were observed in males at 4000 ppm (10.6%) and 8000 ppm (18.9%), without any associated compound-related histopathological changes (Table 10).

Table 10. Body weight and liver weights in mice administered kresoxim-methyl for 3 months

Parameter		Males					Females				
		0 ppm	**250 ppm**	**1 000 ppm**	**4 000 ppm**	**8 000 ppm**	**0 ppm**	**250 ppm**	**1 000 ppm**	**4 000 ppm**	**8 000 ppm**
Body weight	Mean (g)	30.9 –	30.4 (−1.6)	31.3 (+3.3)	29.7 (−4.0)	28.7 (−7.1)	23.6 –	23.5 (−0.4)	23.2 (−1.7)	23.7 (+0.4)	24.3 (+3.0)
Body weight gain	%	–	−3.2	+6.5	−11	−24	–	−5	−15	−10	+1.6
Absolute liver weight	Mean (g)	1.141 –	1.173 (+2.8)	1.168 (+2.4)	1.178 (+3.2)	1.219 (+6.8)	0.953 –	0.927 (−2.7)	0.938 (−1.6)	1.017 (+6.7)	1.018 (+6.8)
Relative liver weight	Mean (%)	4.396 –	4.645 (+5.6)	4.514 (+2.6)	4.866 (+10.6)**	5.227 (+18.9)**	5.026 –	4.886 (−2.8)	5.05 (+0.5)	5.308 (+5.6)	5.361 (+6.7)

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Dunnett test, two-sided)

[a] Per cent change relative to controls given in parentheses.

Source: Mellert & Hildebrand (1994a,b)

The NOAEL was 1000 ppm (equal to 230 mg/kg bw per day), based on decreases in body weight gain (>10%) in males at 4000 ppm (equal to 909 mg/kg bw per day) (Mellert & Hildebrand, 1994a,b).

Rats

In a 28-day range-finding study, groups of five male and five female Wistar (Cr) rats (Chbb:Thom(SPF); aged 42 days at the start of the study; mean body weight 178 g for males and 142 g for females) were given diets containing kresoxim-methyl (purity 96.55%) at a concentration of 0, 1000, 4000 or 16 000 ppm (equal to 0, 91, 365 and 1428 mg/kg bw per day for males and 0, 95, 375 and 1481 mg/kg bw per day for females, respectively). The rats were observed for clinical signs, death, feed consumption, body weight, clinical chemistry parameters, including the activities of serum ALT, AST, ALP and GGT, and haematological and pathological end-points.

There were no deaths, signs of clinical toxicity or changes in haematological parameters. The terminal body weights were slightly reduced in animals of both sexes at 4000 ppm (by 4% in males and

10% in females) and at 16 000 ppm (by 7% in males and 6% in females), and the absolute liver weights were slightly increased in males (by 8%) and females (9%) at 16 000 ppm; however, these changes were not statistically significant. Statistically significant increases in relative liver weights (18%) were observed in females at 16 000 ppm, and statistically significantly increased serum GGT activity and albumin concentration were observed in males at this dose. No compound-related lesions were observed on histopathological examination. There were no clear substance-related effects on thyroid stimulating hormone (TSH), triiodothyronine (T_3) or thyroxine (T_4).

The NOAEL was 4000 ppm (equal to 365 mg/kg bw per day), based on the increased serum GGT activity in males, increased albumin concentration in males and increased relative liver weight in females at 16 000 ppm (equal to 1428 mg/kg bw per day) (Schilling & Hildebrand, 1992b).

In a 90-day study, groups of 10 male and 10 female Wistar (Cr) rats (Chbb:Thom(SPF); aged 42 days at the start of the study; mean body weight 182 g for males and 141 g for females) were given diets containing kresoxim-methyl (purity 98.7%) at a concentration of 0, 500, 2000, 8000 or 16 000 ppm (equal to 0, 36, 146, 577 and 1170 mg/kg bw per day for males and 0, 43, 172, 672 and 1374 mg/kg bw per day for females, respectively). The rats were observed for clinical signs, death, feed consumption, body weight, clinical chemistry parameters, including the activities of serum ALT, AST, ALP and GGT, and haematological and pathological end-points. Feed consumption and body weight were determined once a week, and enzyme activities were determined after 6 weeks and at the end of the study.

There were no deaths, signs of clinical toxicity, changes in feed consumption or any compound-related changes in haematological parameters.

Slight but statistically significant decreases in terminal body weight (7–8% at 8000 ppm and 11–13% at 16 000 ppm) and body weight gain (7–10% at 8000 ppm and 13–15% at 16 000 ppm) were observed in males. Statistically significant increases in relative liver weight were observed in males at 16 000 ppm (10%) and in females at 2000 ppm and higher (10% at 2000 ppm, 7% at 8000 ppm and 12% at 16 000 ppm). Statistically significant increases in relative kidney weight were also observed in males, but the absolute weights were not increased. No compound-related histopathological lesions were observed in these or other organs in treated groups. Dose-dependent, statistically significantly increased activities of GGT were observed in males at 8000 ppm and higher, and statistically significantly decreased activities of ALP and ALT were observed in males at all doses and in females at 2000 ppm and higher. These reductions in enzyme activity were considered to be related to the slight decrease in feed consumption on the basis of a mechanistic study (Moss, 1994) on per cent reductions in intestinal and hepatic isozymes per total serum ALP activity (see section 2.6(c) below).

The NOAEL was 2000 ppm (equal to 146 mg/kg bw per day), based on decreased body weight and body weight gain and increased GGT activity in males at 8000 ppm (equal to 577 mg/kg bw per day) (Mellert & Hildebrand, 1994c).

Dogs

In a 3-month study, groups of six male and six female beagles (6–9 months old; mean body weights 11.3 kg [8.2–13.7 kg] for males and 9.8 kg [7.9–12.6 kg] for females) were given diets containing kresoxim-methyl (purity 94–95.9%) at a concentration of 0, 1000, 5000 or 25 000 ppm (equal to 0, 30, 150 and 776 mg/kg bw per day for males and 0, 34, 168 and 846 mg/kg bw per day for females, respectively). The animals were observed for clinical signs, death, feed consumption, body weight, clinical chemistry parameters, including the activities of serum ALT, AST, ALP and GGT, and haematological and pathological end-points. Blood samples for haematological and clinical chemistry analysis were collected prior to treatment (day −3) and during weeks 4 and 13 of treatment.

No deaths or ophthalmological abnormalities were observed. During the first 3 weeks, diarrhoea and vomiting were observed frequently in most animals at 25 000 ppm, and a slight but statistically significant reduction in body weight gain was observed in females at this dose throughout

the study. There were no treatment-related changes in haematological or urinary parameters; slight but statistically significant decreases in the concentration of total protein were observed in males at 25 000 ppm, and statistically significant decreases in the concentration of albumin were observed in females at 5000 ppm and in both sexes at 25 000 ppm. These changes were observed during week 4 of treatment but had disappeared by week 13. The changes in albumin and total protein concentrations might not be related to treatment, because they were slight, were transient and may have been a result of the vomiting and diarrhoea that occurred during the first weeks of the study. Dose-dependent increases in the absolute and relative weights of the liver were observed, but were not statistically significant. Histopathological examination revealed no compound-related lesions in tissues, including the liver.

The NOAEL was 5000 ppm (equal to 150 mg/kg bw per day), on the basis of vomiting and diarrhoea in animals of both sexes and reduced body weight gain in females at 25 000 ppm (equal to 776 mg/kg bw per day) (Mellert & Hildebrand, 1994d).

In a 12-month study, groups of six male and six female beagles (6–8 months old; mean body weight 11.0 kg [7.3–13.2 kg] for males and 9.9 kg [8.0–11.5 kg] for females) were given diets containing kresoxim-methyl (purity 93.7%) at a concentration of 0, 1000, 5000 or 25 000 ppm (equal to 0, 27, 140 and 710 mg/kg bw per day for males and 0, 30, 150 and 760 mg/kg bw per day for females, respectively). The animals were observed for clinical signs, death, feed consumption, body weight, ophthalmological end-points, clinical chemistry parameters, including the activities of serum ALT, AST, ALP and GGT, haematological parameters, including clotting time, and pathological end-points. Blood samples were collected for haematological and clotting analysis and clinical chemistry after 3, 6 and 12 months of treatment.

No deaths or ophthalmological abnormalities were observed. Diarrhoea and vomiting occurred infrequently in animals of both sexes at 25 000 ppm, and the body weights of males at this dose were statistically significantly reduced at study termination. The mean body weight of the test group males at 25 000 ppm was statistically significantly reduced from day 189, resulting in about a 12% reduction in body weight at the end of the study in comparison with the concurrent controls. The finding was predominantly caused by one male dog (No. 19.2), which had a 10% reduction in body weight, leading to an overall mean reduction of body weight gain. The details are shown in Tables 11, 12 and 13.

Table 11. Body weight and body weight changes of beagles during the study administration period

Dietary concentration (ppm)	Sex	Body weight (kg)				Body weight change during administration (day 0 vs day 364)	
		Day 0	Day 91	Day 182	Day 364	Mean (kg)	Increase (%)[a]
0	M	10.8	12.5	13.5	14.1	3.3	31
	F	10.0	10.9	11.5	12.2	2.2	22
1 000	M	11.2	12.3	13.1	13.7	2.5	22
	F	10.1	11.0	11.8	12.3	2.2	22
5 000	M	10.9	12.1	12.9	13.4	2.5	23
	F	10.0	11.2	12.0	12.4	2.4	24
25 000	M	11.2	11.8	12.5	12.5	1.3	12
	F	9.7	10.7	11.7	12.4	2.7	28

F: female; M: male; ppm: parts per million

[a] Percentage of body weight change relative to body weight at day 0.

Source: Hellwig & Hildebrand (1994)

Table 12. Body weight and body weight changes of individual male dogs at 25 000 ppm

Animal no.	Body weight (kg) on day 0	Body weight (kg) on day 364	% change
19.2	12.7	11.4	−10
23.2	10.6	13.0	22
37.2	12.2	12.7	4
53.2	10.8	12.6	17
59.2	9.5	12.9	36
Mean	11.2	12.5	12

Source: Hellwig & Hildebrand (1994)

Table 13. Body weight and body weight changes of individual male dogs in the control group

Animal no.	Body weight (kg) on day 0	Body weight (kg) on day 364	% change
27:2	13.2	13.8	4.5
39:2	11.7	14.7	26
43:2	11.1	14.8	33
47:2	10.7	13.2	23
65:2	7.3	14.1	93
Mean	10.8	14.1	35.9

Source: Hellwig & Hildebrand (1994)

There was no statistically significant reduction in body weight gain or feed consumption at any dose. Significant increases in the number of platelets were observed in males at all doses; the values for males at 25 000 ppm were within the range of historical control data, except for the mean value at the third month. There were no compound-related changes in clotting time. There were no compound-related changes in urinary or clinical chemistry parameters or in the activities of serum enzymes. Significant increases in relative liver weights were observed in males at 5000 ppm, but the absolute liver weights were not significantly increased. Histopathological examination revealed no treatment-related alterations in the liver or in any other tissue or organ examined.

In view of the above, the NOAEL was 5000 ppm (equal to 140 mg/kg bw per day), based on infrequent diarrhoea and vomiting occurring in animals of both sexes and the significantly reduced body weights of males at study termination at 25 000 ppm (equal to 710 mg/kg bw per day) (Hellwig & Hildebrand, 1994).

(b) Dermal application

Rats

Groups of five male and five female Wistar (Chbb) rats (aged about 8–10 weeks; body weight 200–300 g) received dermal applications of kresoxim-methyl (purity 94.3%) suspended in 0.5% carboxymethylcellulose at a dose of 0 or 1000 mg/kg bw per day under a semi-occlusive dressing (four layers of absorbent gauze and an elastic dressing) for 6 hours per day for 21 days. The rats were observed for clinical signs, death, feed consumption, body weight, clinical chemistry parameters, including the activities of serum ALT, AST, ALP and GGT, haematological parameters, including clotting times, and pathological end-points. Blood samples for haematological and clotting analysis and for clinical chemistry were collected at termination.

There were no compound-related effects on mortality rates, clinical signs, haematological parameters, clotting times or clinical chemistry parameters, including serum enzyme activities. There were no statistically significant changes in body weight gain or feed consumption in the treated group, and no signs of irritation were observed on treated skin of test or control animals. No effect on organ weights was observed, and histopathological examination revealed no treatment-related alterations in the liver or in any other tissue or organ examined.

The NOAEL was 1000 mg/kg bw per day, the highest dose tested (Kirsch & Hildebrand, 1994a).

2.3 Long-term studies of toxicity and carcinogenicity

Mice

In an 18-month study, groups of 50 male and 50 female C57Bl/6N (CrlBR) mice were given diets containing kresoxim-methyl (mean purity 96.3% during the first 12 months and 93.2% during the following 6 months) at a concentration of 0, 400, 2000 or 8000 ppm (equal to 0, 60, 304 and 1305 mg/kg bw per day for males and 0, 81, 400 and 1662 mg/kg bw per day for females, respectively). Satellite groups of 10 mice of each sex were treated concurrently for 12 months. The age of mice at the initiation of administration was 40–50 days, and the groups had a mean weight of 23.3 g (21.1–26.8 g) for males of the main group; 22.9 g (21.0–25.0 g) for males of the satellite group; 18.9 g (17.2–21.2 g) for females of the main group; and 18.8 g (16.9–20.7 g) for females of the satellite group. The animals were observed for clinical signs, death, feed consumption, body weight, and haematological and pathological end-points. Blood samples for haematology were collected during months 12 and 18 of treatment.

No compound-related effects were observed with respect to mortality rates, clinical signs, feed consumption or haematological parameters throughout the study. Survival rates at termination were 86%, 88%, 84% and 82% for males and 80%, 88%, 82% and 84% for females at 0, 400, 2000 and 8000 ppm, respectively. Statistically significant decreases in terminal body weights and body weight gains were observed in the main groups of males at 8000 ppm and females at 2000 and 8000 ppm during the final 6 months (Table 14).

Table 14. Body weight changes of mice receiving kresoxim-methyl in the diet for 18 months

Dietary concentration (ppm)	Body weight (g)[a]							
	Main group				Satellite group			
	Males		Females		Males		Females	
	Day 0	Day 546	Day 0	Day 546	Day 0	Day 371	Day 0	Day 371
0	23.4	39.3	19.1	36.9	23	41.5	19.1	37.7
	–	(68%)	–	(93%)	–	(80%)	–	(97%)
400	23.3	38.6	19.0	36.0	23	40.8	18.9	37.6
	–	(66%)	–	(89%)	–	(77%)	–	(99%)
2 000	23.3	39.0	18.7*	32.7**	22.6	40.9	18.7	36.5
	–	(67%)	–	(75%)	–	(81%)	–	(95%)
8 000	23.2	35.7*	18.8	29.7**	22.8	37.1	18.6	33.6
	–	(54%)	–	(58%)	–	(63%)	–	(81%)

ppm: parts per million; *: $P \leq 0.05$; **: $P \leq 0.01$ (analysis of variance + Dunnett's test, two-sided)
[a] Per cent change relative to day 0 given in parentheses.
Source: Mellert & Hildebrand (1994e)

Increased relative liver weights were observed in females in the satellite group examined at 12 months and in the main group examined at 18 months at 8000 ppm (Tables 15 and 16). Increased relative adrenal weights were observed in males at 12 and 18 months and in females at 18 months at 8000 ppm.

Table 15. Mean body weight and liver weights after 52 weeks (day 364)

		Males				Females			
Parameter		**0 ppm**	**400 ppm**	**2 000 ppm**	**8 000 ppm**	**0 ppm**	**400 ppm**	**2 000 ppm**	**8 000 ppm**
Body weight	Mean (g)	39.4	38.3	38.3	35.29	33.5	33.27	32.1	29.7
Absolute liver weight	Mean (g)	1.7	1.6	1.7	1.8	1.4	1.4	1.4	1.5
Relative liver weight	Mean (%)	4.4	4.1	4.5	5.0	4.1	4.3	4.6	5.1**

ppm: parts per million; **: $P < 0.01$ (Dunnett's test, two-sided)
Source: Mellert & Hildebrand (1994e)

Table 16. Mean body weight and liver weights after 78 weeks (day 546)

		Males				Females			
Parameter		**0 ppm**	**400 ppm**	**2 000 ppm**	**8 000 ppm**	**0 ppm**	**400 ppm**	**2 000 ppm**	**8 000 ppm**
Body weight	Mean (g)	35.0	34.6	34.8	31.9*	33.6	33.6	31.1	27.9**
Absolute liver weight	Mean (g)	1.61	1.66	1.55	1.6	1.6	1.5	1.5	1.6
Relative liver weight	Mean (%)	4.6	4.8	4.5	5.1	4.8	4.6	5.1	5.8**

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Dunnett's test, two-sided)
Source: Mellert & Hildebrand (1994e)

Histopathological examination at 12 months revealed no compound-related lesions in any group, but examination at 18 months revealed an increased incidence of centrilobular fatty infiltration (1/50 at 0 ppm and 8/50 at 8000 ppm) in the liver in females, an increased incidence and a greater degree of severity of hepatic amyloidosis (6/50 at 0 ppm and 16/50 at 8000 ppm) in females (Table 17) and an increased incidence of papillary necrosis of the kidney (2/50 at 0 ppm and 13/50 at 8000 ppm) in females. There was no treatment-related increase in the incidence of neoplastic lesions.

Table 17. Incidence of non-neoplastic findings in the liver of rats after 78 weeks

	Incidence of finding							
	Males				Females			
Finding	**0 ppm**	**400 ppm**	**2 000 ppm**	**8 000 ppm**	**0 ppm**	**400 ppm**	**2 000 ppm**	**8 000 ppm**
No. of livers examined	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*
Lymphoid infiltration	34	33	36	33	37	40	35	38
Haematopoiesis	4	0	1	1	4	1	0	2

Finding	Incidence of finding							
	Males				Females			
	0 ppm	400 ppm	2 000 ppm	8 000 ppm	0 ppm	400 ppm	2 000 ppm	8 000 ppm
Pigment storage	0	0	0	0	0	1	1	2
Focal fatty infiltration	4	1	2	7	4	2	1	2
Fatty infiltration centrilobular	6	5	2	3	1	2	4	8
Fatty infiltration diffuse	31	33	40	39	40	43	35	33
Amyloidosis	13	11	7	20	6	1	4	16
Storage, Kupffer's cells	0	1	1	0	0	0	0	0
Single-cell necrosis	0	0	0	1	0	0	0	0
Necrosis focal	3	4	3	6	5	0	3	2
Bile duct proliferation	0	1	0	0	0	1	1	0

ppm: parts per million
Source: Mellert & Hildebrand (1994e)

The NOAEL for toxicity was 2000 ppm (equal to 304 mg/kg bw per day), on the basis of reductions in body weight and body weight gain in both sexes and histopathological changes in the liver and increased liver weights in females at 8000 ppm (equal to 1305 mg/kg bw per day). The compound was not carcinogenic up to the highest dose tested (8000 ppm, equal to 1305 mg/kg bw per day) (Mellert & Hildebrand, 1994e).

Rats

In a 24-month toxicity study, groups of 20 male and 20 female Wistar (Chbb:THOM(SPF)) rats (aged 42 days; mean body weights 196 g for males and 147 g for females) were given diets containing kresoxim-methyl (purity 92.7–96.6%) at a concentration of 0, 200, 800, 8000 or 16 000 ppm (equal to 0, 9, 36, 370 and 746 mg/kg bw per day for males and 0, 12, 48, 503 and 985 mg/kg bw per day for females, respectively). The animals were observed for clinical signs, death, feed consumption, body weight, ophthalmological end-points, clinical chemistry parameters, including the activities of serum ALT, AST, ALP and GGT, and haematological, urinary and histopathological end-points. Blood samples for haematology and clinical chemistry were collected at 3, 6, 12, 18 and 24 months of treatment.

There were no treatment-related effects on mortality rates, clinical signs or ophthalmoscopic parameters. The survival rates of males until day 735 were 85%, 40%, 75%, 80% and 80% at 0, 200, 800, 8000 and 16 000 ppm, respectively. In females, the survival rates until day 375 were 75%, 75%, 60%, 80% and 85% at 0, 200, 800, 8000 and 16 000 ppm, respectively. The terminal body weights were slightly reduced in males at 16 000 ppm (by 4%) and statistically significantly reduced in females at 8000 ppm (by 13%) and 16 000 ppm (by 6%). Body weight gains were reduced in males (10% below controls) and females (15% below controls) at both 8000 and 16 000 ppm. No statistically significant change in feed consumption was observed. Slight but statistically significant reductions in mean corpuscular volume and mean corpuscular haemoglobin were observed in males at 16 000 ppm and in females at and above 200 ppm; however, these changes were within the background range and were not clearly dose dependent. The activity of serum ALT was statistically significantly decreased in animals

of both sexes at 8000 and 16 000 ppm, and that of serum ALP was statistically significantly decreased in animals of both sexes at and above 200 ppm. These reductions in enzyme activities are not toxicologically relevant (Moss, 1994; Mellert, Decardt & Hildebrand, 1997). The relative liver weights were statistically significantly increased in males at 8000 and 16 000 ppm, and the absolute liver weights were statistically significantly increased in males at the highest dose. Statistically significant, dose-related increases in GGT activity were also observed in males at and above 8000 ppm.

Microscopic examination revealed evidence of neoplasia in the liver. Increased incidences of hepatocellular carcinoma, but not hepatocellular adenoma, were observed in animals. The incidences are given in Table 18.

Table 18. Incidence of neoplastic and non-neoplastic findings in a 24-month toxicity study in rats (original report)

	Incidence of finding									
	Males					Females				
Finding	**0 ppm**	**200 ppm**	**800 ppm**	**8 000 ppm**	**16 000 ppm**	**0 ppm**	**200 ppm**	**800 ppm**	**8 000 ppm**	**16 000 ppm**
No. of animals examined	*20*	*20*	*20*	*20*	*20*	*20*	*20*	*20*	*20*	*20*
Liver										
Adenoma, hepatocellular	0	0	0	0	0	0	0	0	0	0
Carcinoma, hepatocellular *(one-sided P-value)*	0	1 *(NS)*	1 *(NS)*	3 *(NS)*	8 *(**)*	1	0 *(NS)*	2 *(NS)*	6* *(NS)*	6* *(NS)*
Chi-squared analysis		*0.00*	*0.00*	*1.41*	*7.46*		*0.00*	*0.00*	*2.70*	*2.70*
Hepatocellular hypertrophy	0	0	3	4	7*	1	1	0	1	8*
Eosinophilic foci	0	1	0	6	8*	1	0	0	–0	1
Bile duct proliferation	6	4	5	3	7	5	4	4	8	11*
Mixed-cell foci	0	0	2	4*	5*	0	0	0	0	2
Biliary cyst	0	1	2	1	4*	3	8	5	7	7
Pericholangitis	1	1	2	1	4	0	1	0	4	0
Kidney										
Tubular cast	0	0	0	1	0	2	1	2	6	10*
Tubular atrophy	2	1	2	1	0	4	1	5	8	12*

NS: not statistically significant; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$
Source: Mellert & Hildebrand (1994f)

The incidence and severity of hepatocellular hypertrophy were dose dependent and increased in animals of both sexes (males: 0/20 at 0 ppm, 0/20 at 200 ppm, 3/20 at 800 ppm, 4/20 at 8000 ppm and 7/20 at 16 000 ppm; females: 1/20 at 0 ppm,1/20 at 200 ppm, 0/20 at 800 ppm, 1/20 at 8000 ppm and 8/20 at 16 000 ppm); however, statistical significance was achieved only at 16 000 ppm in animals of both sexes. Statistically significant increases in the incidence and severity of eosinophilic foci (0/20 at 0 ppm, 6/20 at 8000 ppm and 8/20 at 16 000 ppm) and mixed-cell foci (0/20 at 0 ppm, 4/20 at 8000 ppm and 5/20 at 16 000 ppm) were observed in males at 16 000 ppm. Evidence of a proliferative response in bile duct cells was associated with increased incidences of biliary cysts in males at 16 000

ppm (0/20 in controls versus 4/20 at 16 000 ppm) and in females at 8000 and 16 000 ppm (3/20 in controls versus 7/20 at both 8000 and 16 000 ppm). Bile duct proliferation in females (5/20 in controls versus 8/20 and 11/20 at 8000 and 16 000 ppm, respectively) was observed with statistical significance at 16 000 ppm. Statistically significantly increased incidences of tubular casts of the kidneys (2/20 in controls versus 10/20 at 16 000 ppm) and tubular atrophy of the kidney (4/20 in controls versus 12/20 at 16 000 ppm) were seen in females at 16 000 ppm. Increased incidences of lesions in other tissues were age related or independent of dose and were not considered to be toxicologically relevant (Table 18).

Histopathological re-evaluation of liver tissue slides from this study was conducted by a reviewing pathologist (Harada, 1995). The results are shown in Table 19.

Table 19. Incidence of hepatocellular tumours and number of rats with hepatocellular tumours in a 24-month toxicity study: re-evaluation of liver tissues

	Incidence of liver tumours									
	Males					**Females**				
Tumour	**0 ppm**	**200 ppm**	**800 ppm**	**8 000 ppm**	**16 000 ppm**	**0 ppm**	**200 ppm**	**800 ppm**	**8 000 ppm**	**16 000 ppm**
No. of livers examined	*20*	*20*	*20*	*20*	*20*	*20*	*20*	*20*	*20*	*20*
Hepatocellular adenoma	0	0	0	2	2	0	0	1	4	4
Hepatocellular carcinoma	0	1	1	2	2	0	0	1	1	2
Total no. of animals with hepatocellular tumours	0	1	1	4	4	0	0	2	5*	6*#

ppm: parts per million; *: $P < 0.05$ (chi-squared test); #: $P < 0.05$ (Fisher exact probability test)
Source: Harada (1995)

A pathology working group (PWG) (Hildebrandt et al., 1995a) reviewed the original report (Mellert & Hildebrand, 1994f) as well as the reviewer's report (Harada, 1995). The objective of the PWG was to provide a consensus diagnosis for hepatocellular neoplasms. The PWG diagnosed the presence of both hepatocellular adenoma and hepatocellular carcinoma in the same animal. Multiple tumours of the same type were not recorded. The incidence of hepatocellular neoplasms is given in Table 20.

Other lesions, such as foci of cellular alteration of various types, hepatocellular hypertrophy (primarily periportal), cystic degeneration, angiectasis and/or biliary proliferative lesions, were commented on, particularly when such lesions had an impact on the interpretation of neoplasms. The PWG did not review all the liver slides or all examples of non-neoplastic liver lesions.

The results of the PWG review of proliferative hepatocellular lesions confirmed the increased incidence of hepatocellular neoplasms in male and female rats at 8000 and 16 000 ppm, as reported by the study and by the reviewing pathologist. The differences between the reviews lay primarily in the diagnosis of hepatocellular adenomas and carcinomas.

The NOAEL for toxicity was 800 ppm (equal to 36 mg/kg bw per day), on the basis of increased activity of serum GGT, increased relative liver weight, and increased incidence and degree of severity of eosinophilic and mixed-cell foci in males at 8000 ppm (equal to 370 mg/kg bw per day). The NOAEL for carcinogenicity was also 800 ppm (equal to 36 mg/kg bw per day), on the basis of an increased

incidence of hepatocellular tumours in animals of both sexes at 8000 ppm (equal to 370 mg/kg bw per day) (Mellert & Hildebrand, 1994f).

Table 20. Incidence of liver tumours in the 24-month toxicity study in rats

	Incidence of liver tumours									
	Males					Females				
	0 ppm	200 ppm	800 ppm	8 000 ppm	16 000 ppm	0 ppm	200 ppm	800 ppm	8 000 ppm	16 000 ppm
No. of animals	*20*	*20*	*20*	*20*	*20*	*20*	*20*	*20*	*20*	*20*
Hepatocellular adenoma	0	0	0	1	4	0	0	1	4	4
(one-sided P-value)	–	*(1)*	*(1)*	*(0.5)*	*(0.053)*	–	*(1)*	*(0.5)*	*(0.053)*	*(0.053)*
Hepatocellular carcinoma	0	1	1	3	3	0	0	1	2	3
(one-sided P-value)	–	*(0.5)*	*(0.5)*	*(0.115)*	*(0.115)*	–	*(1)*	*(0.5)*	*(0.244)*	*(0.115)*
Hepatocellular tumours	0	1	1	4	7**	0	0	2	5*	6**
(one-sided P-value)	–	*(0.5)*	*(0.5)*	*(0.053)*	*(0.004)*	–	*(1)*	*(0.244)*	*(0.024)*	*(0.01)*

ppm: parts per million; *: $P \leq 0.5$; **: $P \leq 0.01$ (Fisher's exact test, one-sided)
Source: Hildebrandt et al. (1995a)

In a 24-month study of carcinogenicity, groups of 50 male and 50 female Wistar (Chbb:THOM(SPF)) rats (aged 42 days; mean body weights 192 g for males and 149 g for females) were fed diets containing kresoxim-methyl (purity 92.7–96.6%) at a concentration of 0, 200, 800, 8000 or 16 000 ppm (equal to 0, 9, 36, 375 and 770 mg/kg bw per day for males and 0, 12, 47, 497 and 1046 mg/kg bw per day for females, respectively). The animals were observed for clinical signs, death, feed consumption, body weight, and haematological and histopathological end-points. Blood samples for haematology were collected at the end of the study.

There were no treatment-related effects on mortality rates or clinical signs. The survival rates of males until day 735 were 70%, 72%, 74%, 74% and 70% at 0, 200, 800, 8000 and 16 000 ppm, respectively. In females, the survival rates until day 375 were 66%, 70%, 78%, 74% and 78% at 0, 200, 800, 8000 and 16 000 ppm, respectively. The terminal body weights and body weight gains were statistically significantly reduced in animals of both sexes at 8000 ppm (9% and 13% in males and 13% and 20% in females, respectively) and 16 000 ppm (9% and 12% in males and 14% and 21% in females, respectively). No statistically significant change in feed consumption was observed. Statistically significantly increased relative liver weights were observed in males at 16 000 ppm. Microscopic examination revealed hepatic neoplasia; increased incidences of hepatocellular carcinoma were observed in animals of both sexes at 8000 and 16 000 ppm (males: 7/50 at 0 ppm, 5/50 at 200 ppm, 2/50 at 800 ppm, 18/50 at 8000 ppm and 11/50 at 16 000 ppm; females: 1/50 at 0 ppm, 1/50 at 200 ppm, 2/50 at 800 ppm, 13/50 at 8000 ppm and 16/50 at 16 000 ppm). The number of animals with adenoma plus carcinoma in the liver was statistically significantly increased among males at 8000 ppm (8/50 at 0 ppm, 19/50 at 8000 ppm and 13/50 at 16 000 ppm) and among females at 8000 and 16 000 ppm (1/50 in controls versus 15/50 and 17/50 at 8000 and 16 000 ppm, respectively). The incidence of hepatocellular hypertrophy was increased in males at 8000 and 16 000 ppm and in females at 16 000 ppm, but reached statistical significance only in males at 16 000 ppm (males: 3/50 at 0 ppm, 5/50 at 8000 ppm and 10/50 at 16 000 ppm; females: 5/50 at 0 ppm and 7/50 at 16 000 ppm). There were dose-

dependent increases in the incidences of eosinophilic foci (males: 1/50 at 0 ppm, 5/50 at 8000 ppm and 11/50 at 16 000 ppm; females: 3/50 at 0 ppm, 8/50 at 8000 ppm and 5/50 at 16 000 ppm) and mixed-cell foci in animals of both sexes (males: 4/50 at 0 ppm, 9/50 at 8000 ppm and 12/50 at 16 000 ppm; females: 0/50 at 0 ppm and 5/50 at 16 000 ppm); however, statistically significant results were observed only at 16 000 ppm. There was evidence of alterations in bile duct cells, including an increased incidence of bile duct proliferation in females at 16 000 ppm (10/50 in controls versus 28/50 at 16 000 ppm), cholangiofibrosis in females at 16 000 ppm (1/50 in controls versus 7/50 at 16 000 ppm) and biliary cysts in males at 8000 ppm (males: 1/50 at 0 ppm, 7/50 at 8000 ppm and 6/50 at 16 000 ppm; females: 8/50 at 0 ppm, 12/50 at 8000 ppm and 15/50 at 16 000 ppm). Other non-neoplastic lesions included tubular mineralization of the kidneys in males at 16 000 ppm (6/50 in controls versus 18/50 at 16 000 ppm). The tubular mineralization was dose related and considered to be related to treatment. The lesions observed in other tissues were considered to be independent of dose and age related. The details are given in Table 21.

Table 21. Incidence of neoplastic and non-neoplastic findings in the liver and kidney in rats in a 24-month carcinogenicity study (original report)

	Incidence of finding									
	Males					**Females**				
Finding	**0 ppm**	**200 ppm**	**800 ppm**	**8 000 ppm**	**16 000 ppm**	**0 ppm**	**200 ppm**	**800 ppm**	**8 000 ppm**	**16 000 ppm**
No. of animals examined	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*
Liver										
Adenoma	1	0	0	1	0	0	1	2	2	1
Carcinoma	7	5	2	18*	13	1	1	2	13**	16**
Adenoma + carcinoma	8	5	2	19*	13	1	2	4	15**	17**
Cellular hypertrophy	3	2	2	5	10**	5	3	4	2	7
Eosinophilic foci	1	0	3	5	11**	3	0	3	8	5*
Mixed-cell foci	4	1	2	9	12**	0	0	0	3	5
Bile duct proliferation	14	21	17	12	17	10	13	12	13	28**
Cholangiofibrosis	13	12	16	8	17	1	4	1	5	7
Biliary cyst	1	4	5	7	6	8	10	10	12	15
Kidney										
Tubular mineralization	6	3	6	10	18	48	50	50	50	50

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$
Source: Mellert & Hildebrand (1994g)

Histopathological re-evaluation of liver tissue slides from this study was conducted by a reviewing pathologist (Harada, 1995). The results are shown in Table 22.

Table 22. Incidence of hepatocellular tumours in rats in a 24-month carcinogenicity study

Tumour type	Incidence of liver tumours									
	Males					Females				
	0 ppm	200 ppm	800 ppm	8 000 ppm	16 000 ppm	0 ppm	200 ppm	800 ppm	8 000 ppm	16 000 ppm
No. of livers examined	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*
Hepatocellular adenoma	2	3	1	6	7	0	1	4	11**(**)	9**(**)
Hepatocellular carcinoma	4	3	1	9	7	1	0	1	6	7(*)
Total no. of animals with hepatocellular tumours	6	6	2	15*(**)	12	1	1	5	16**(**)	16(**)

*: $P < 0.05$; **: $P < 0.01$ (chi-squared test); (*): $P < 0.05$; (**): $P < 0.01$ (Fisher exact probability test)
Source: Harada (1995)

A PWG (Hildebrandt et al., 1995b) reviewed the original report (Mellert & Hildebrand, 1994g) as well as the reviewer's report (Harada, 1995). The objective of the PWG was to provide a consensus diagnosis for hepatocellular neoplasms. The PWG diagnosed the presence of both hepatocellular adenoma and hepatocellular carcinoma in the same animal. Multiple tumours of the same type were not recorded. The incidence of hepatocellular neoplasms is given in Table 23.

Table 23. Incidence of liver tumours in the 24-month carcinogenicity study in rats (re-evaluation)

Tumour type	Incidence of liver tumours									
	Males					Females				
	0 ppm	200 ppm	800 ppm	8 000 ppm	16 000 ppm	0 ppm	200 ppm	800 ppm	8 000 ppm	16 000 ppm
No. of animals	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*
Adenoma, hepatocellular	2	2	0	5	4	0	2	4	11**	11**
(one-sided P-value)	–	*(0.691)*	*(1)*	*(0.218)*	*(0.339)*	–	*(0.248)*	*(0.059)*	*(0.000)*	*(0.000)*
Carcinoma, hepatocholangiocellular	0	0	0	0	1	–	–	–	–	–
(one-sided P-value)	–	*(1)*	*(1)*	*(1)*	*(0.5)*	–	–	–	–	–
Carcinoma, hepatocellular	4	4	2	12*	8	1	0	1	7	7
(one-sided P-value)	–	*(0.643)*	*(0.898)*	*(0.027)*	*(0.178)*		*(1)*	*(0.753)*	*(0.030)*	*(0.030)*
Carcinoma, hepatocellular and/or cholangiocellular	4	4	2	12*	9	–	–	–	–	–
(one-sided P-value)	–	*(0.643)*	*(0.898)*	*(0.027)*	*(0.117)*	–	–	–	–	–
Hepatocellular tumours	6	6	2	16*	11	1	2	5	16**	17**
(one-sided P-value)	–	*(0.62)*	*(0.97)*	*(0.014)*	*(0.143)*	–	*(0.5)*	*(0.102)*	*(0.000)*	*(0.000)*

*: $P < 0.05$; **: $P < 0.01$ (Fisher's exact test, one-sided)
Source: Hildebrandt et al. (1995b)

The NOAEL for toxicity was 800 ppm (equal to 36 mg/kg bw per day), on the basis of reduced body weight and body weight gain and hepatic alterations at 8000 ppm (equal to 375 mg/kg bw per day). The NOAEL for carcinogenicity was also 800 ppm (equal to 36 mg/kg bw per day), on the basis of increased incidences of hepatocellular tumours at 8000 ppm (Mellert & Hildebrand, 1994g, 1995b,c).

A histopathological re-evaluation of the hepatocellular tumour incidence in the two studies (Mellert & Hildebrand, 1994f,g) in rats was conducted by a PWG (Hildebrandt et al., 1995c). The combined incidence of liver tumours is shown in Table 24. Concurrent reassessment revealed similar dose–response relationships in the occurrence of hepatocellular carcinoma, and the statistically significant results with the combined data clearly indicate the hepatic carcinogenic potential of kresoxim-methyl in rats (Van Ravenzwaay, 1996).

Table 24. Combined incidence of liver tumours in the 24-month carcinogenicity and chronic toxicity studies in rats

	Incidence of liver tumours									
	Males					**Females**				
Tumour type	**0 ppm**	**200 ppm**	**800 ppm**	**8 000 ppm**	**16 000 ppm**	**0 ppm**	**200 ppm**	**800 ppm**	**8 000 ppm**	**16 000 ppm**
Total no. of animals	*70*	*70*	*70*	*70*	*70*	*70*	*70*	*70*	*70*	*70*
Hepatocellular adenoma	2 (2.9%)	2 (2.9%)	0 (0%)	6 (8.6%)	8 (11.4%)	0 (0%)	2 (2.9%)	5* (7.1%)	15** (21%)	15** (21%)
(one-sided P-value)	*(PWG conclusion)*	*(0.690)*	*(1)*	*(0.137)*	*(0.048)*		*(0.248)*	*(0.029)*	*(0.000)*	*(0.000)*
Hepatocellular carcinoma	4 (5.7%)	5 (7.1%)	3 (4.3%)	15** (21.4%)	12 (17.1%)	1 (1.4%)	0 (0%)	2 (2.9%)	9** (12.8%)	10** (14.2%)
(one-sided P-value)		*(0.5)*	*(0.78)*	*(0.006)*	*(0.03)*		*(1)*	*(0.5)*	*(0.009)*	*(0.004)*
Hepatocellular tumours	6	7	3	20**	18**	1	2	7*	21**	23**
(one-sided P-value)		*(0.5)*	*(0.917)*	*(0.002)*	*(0.006)*		*(0.5)*	*(0.031)*	*(0.000)*	*(0.000)*

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Fisher's exact test, one-sided)
Source: Hildebrandt et al. (1995c)

The conclusion drawn in the Hildebrandt et al. (1995c) report is essentially the same as that drawn in the original reports – that is, kresoxim-methyl has a carcinogenic effect on the liver of male and female rats at doses of 8000 and 16 000 ppm. There was a slight numeric, but not statistically significant (level of significance did not reach $P < 0.01$), increase in the incidence of hepatocellular adenomas alone or in combination with hepatocellular carcinomas in females treated with 800 ppm, which was seen only when the incidences of the chronic toxicity and carcinogenicity studies were combined. There was no increase in the incidence of hepatocellular carcinomas alone. The PWG considered this to be an equivocal response.

The actual incidence at 800 ppm in the combined evaluation of the long-term rat studies was 5/70 females (for adenomas), or approximately 7% (Table 24). Weak statistical significance ($P < 0.05$, but not reaching the $P < 0.01$ level, which was considered by the PWG to be required for this common neoplasia) was achieved only because the control incidence was unusually low (0%). Contemporary

historical control data for liver adenomas in female Wistar rats in BASF's laboratories (Table 25) include a total of 38 long-term toxicity studies. In these studies, the number of liver adenomas in female control animals was 43/1620 animals, which is equivalent to 2.7%. This indicates that normally in a group of 70 female control animals, approximately two would have been found to have a liver adenoma. Clearly, there is no statistically, or biologically, significant difference between 2/70 (the historical mean) and 5/70 animals (the actual number at 800 ppm). Moreover, the historical data of BASF's laboratories also demonstrate that the maximum incidence of liver adenoma in control female Wistar rats can be as high as 20%, indicating that the 7% noted in the kresoxim-methyl studies at 800 ppm is well within the historical range. Similarly, the incidence of combined liver adenomas and carcinomas in the controls (1.4%) was also lower than the historical mean of 3.4%. Again, the actual study incidence at 800 ppm (10%) is well within the historical range (up to 22%). Therefore, it is concluded that a carcinogenic potential in the liver of Wistar rats exists only at doses of 8000 ppm and higher.

Table 25. Historical control data from the BASF test facility (1997)[a]

	Incidence of liver tumours					
	Males			Females		
Tumour type	**Mean**	**Minimum**	**Maximum**	**Mean**	**Minimum**	**Maximum**
Hepatocellular adenoma	120/1 620 (7.4%)	0/100 (0%)	15/50 (30%)	43/1 620 (2.7%)	0/100 (0%)	10/50 (20%)
Hepatocellular carcinoma	62/1 620 (3.8%)	0/50 (0%)	6/50 (12%)	12/1 620 (0.7%)	0/100 (0%)	3/60 (5%)
Hepatocellular tumours	182/1 620 (11.2%)	0/50 (0%)	16/50 (32%)	55/1 620 (3.4%)	0/100 (0%)	11/50 (22%)

[a] n = 38 studies; 1620 rats of each sex evaluated.
Source: Hildebrandt et al. (1995c)

To investigate the tumours seen in earlier carcinogenicity (Mellert & Hildebrand, 1994g) and chronic toxicity studies (Mellert & Hildebrand, 1994f), which were performed in Wistar rat strain Chbb:THOM (Dr. Karl Thomae GmbH, Biberach/Riss), another study was conducted using Wistar rat strain CrlGlxBrlHan:WI (Charles River Germany). The objective of this additional study was to determine whether the test substance exhibits a carcinogenic potential when administered in the diet to Wistar rats of the strain CrlGlxBrlHan:WI for the major part of their lifespan.

In this additional study, kresoxim-methyl (lot/batch no. COD-000225; purity 97.8%) was administered to Wistar rats (CrlGlxBrlHan:WI; aged 42 ± 1 days; body weight 154.6 ± 7.2 g and 156.0 ± 7.9 g for males at 0 and 16 000 ppm, respectively, and 127.6 ± 6.5 g and 126.3 ± 6.1 g for females at 0 and 16 000 ppm, respectively) in the feed at a concentration of 0 or 16 000 ppm (equal to 752.1 mg/kg bw per day for males and 1021.6 mg/kg bw per day for females) over a period of 24 months. Each group consisted of 50 animals of each sex. Feed consumption and body weight were determined once a week during the first 13 weeks and at 4-week intervals thereafter. The animals were examined for signs of toxicity or mortality at least once a day; moreover, detailed clinical examinations in an open field were conducted prior to the start of the administration period and weekly thereafter. Differential blood counts were determined for all surviving animals at the end of the study and also from all animals killed in extremis during the study. After about 24 months, the animals were subjected to gross pathological assessment, followed by histopathological examination.

Treatment did not affect clinical signs of toxicity, except for statistically significantly decreased feed consumption, body weight and body weight change. The survival of the animals was not adversely affected by the test substance administration. Clinical pathology showed no treatment-related effects regarding haematology. At the only dose tested, an increase in liver tumours (hepatocellular adenoma and carcinoma) was observed. This increase was statistically significant for hepatocellular carcinomas

in male animals and clearly above historical control values. For female animals, the tumour incidences were not statistically significantly increased and only slightly outside the historical control range in the case of hepatocellular adenoma (Tables 26 and 27).

Table 26. Neoplastic and non-neoplastic changes in the liver of rats administered kresoxim-methyl in the diet for 24 months

	Incidence of finding			
	Males		**Females**	
Finding	**0 ppm**	**16 000 ppm**	**0 ppm**	**16 000 ppm**
Number of animals	*50*	*50*	*50*	*50*
Non-neoplastic findings				
Hypertrophy peripheral	–	6*	–	12**
Eosinophilic foci	26	41**	7	30**
Basophilic foci	2	6	–	14**
Neoplastic findings				
Hepatocellular adenoma	0	3	0	4
Hepatocellular carcinoma	3	13**	1	3
Total tumours (adenoma + carcinoma)	3	16**	1	7*

ppm: parts per million; *: $P \leq 0.05$; **: $P \leq 0.01$ (Fisher's exact test, one-sided)
Source: Kamp et al. (2008)

Table 27. Historical control data: hepatocellular tumours[a]

	Incidence of liver tumours					
	Males			**Females**		
Tumour type	**Mean**	**Minimum**	**Maximum**	**Mean**	**Minimum**	**Maximum**
Hepatocellular adenoma	7/350 (2.0%)	0/50 (0.0%)	2/50 (4.0%)	3/350 (0.9%)	0/50 (0.0%)	3/50 (6.0%)
Hepatocellular carcinoma	3/350 (0.9%)	0/50 (0.0%)	2/50 (4.0%)	5/350 (1.4%)	0/50 (0.0%)	3/50 (6.0%)
Combined tumours	10/350 (2.9%)	0/50 (0.0%)	3/50 (6.0%)	8/350 (2.3%)	0/50 (0.0%)	3/50 (6.0%)

[a] Seven 24-month studies (oral diet) started at the test facility between 1999 and 2005; 50 Wistar rats (Charles River Germany) of each sex per study.
Source: BASF (2018b)

Additionally, non-neoplastic findings in the liver (eosinophilic foci and basophilic foci) were increased in both sexes compared with the control group (Table 26).

Overall, the liver carcinogenicity of kresoxim-methyl was confirmed for male rats; the slight increase in female hepatocellular tumours was not statistically significant. The strain CrlGlxBrlHan:WI was not less sensitive than the Wistar rat strain Chbb:THOM with respect to the carcinogenic potential of kresoxim-methyl in the liver of both male and female rats (Kamp et al., 2008).

2.4 Genotoxicity

Kresoxim-methyl was tested for genotoxicity in an adequate range of in vitro and in vivo assays. It gave a positive response only in one in vitro assay (the Syrian hamster embryo cell transformation assay) and was negative in all other in vitro assays and all in vivo assays. The details are provided in Table 28.

Table 28. Overview of genotoxicity tests with kresoxim-methyl

End-point	Test object	Concentration	Purity (%)	Results	Reference
In vitro					
Bacterial reverse mutation assay (Ames test)	*Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537; *Escherichia coli* strain WP2*uvr*A; plate incorporation and preincubation assay; with/without S9 mix	20, 100, 500, 2 500, 5 000 µg/plate	93.7	Negative	Engelhardt & Hoffmann (1993a)
Bacterial reverse mutation assay (Ames test)	*S. typhimurium* strains TA98, TA100, TA1535, TA1537; *E. coli* strain WP2*uvr*A; plate incorporation and preincubation assay; with/without S9 mix	0, 20, 100, 500, 2 500, 5 000 µg/plate	94.3	Negative	Engelhardt & Hildebrand (1994)
Bacterial reverse mutation assay (Ames test)	*S. typhimurium* TA98, TA100, TA1535, TA1537, standard plate test; *E. coli* WP2*uvr*A, preincubation test	33–6 000 µg/plate	86.4	Negative	Woitkowiak (2011)
Chromosomal aberration assay in mammalian cells	Human lymphocytes; with/without S9 mix	0, 2.5, 5, 10, 20, 40 µg/mL	98.7	Negative	Hoffmann & Engelhardt (1993)
Morphological transformation assay in mammalian cells	Syrian hamster embryo cells	0, 0.01, 0.025, 0.05, 0.1, 0.2, 0.3, 0.4 µg/mL (7-day experiment) 0, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 5.0 µg/mL (24-hour experiment)	97.8	Positive[a]	Schwindt & Landsiedel (2008)
Forward mutation assay in mammalian cells (HPRT test)	CHO-K1 cells; with/without S9 mix	0, 0.1, 0.5, 1, 2.15, 4.64, 5, 10, 21.5, 46.4, 50, 100 µg/mL	94.3	Negative	Polloth & Hoffmann (1994a)
Unscheduled DNA synthesis	Rat hepatocytes; with/without S9 mix	0, 0.33, 1, 3.3, 10, 33, 100 µg/mL	94.3	Negative	Polloth & Hoffmann (1994b)

End-point	Test object	Concentration	Purity (%)	Results	Reference
in mammalian cells					
In vivo					
Micronucleus test	NMRI mouse, male and female (single intraperitoneal administration; vehicle 0.5% aqueous carboxymethylcellulose)	0, 500, 1 000, 2 000 mg/kg bw	94.3	Negative	Engelhardt & Hoffmann (1993b)
Micronucleus test	CrI:NMRI male mice (single oral administration; vehicle corn oil)	0, 500, 1 000, 2 000 mg/kg bw	86.4	Negative	Schulz & Landsiedel (2011)
Micronucleus test	Wistar rat, male and female (single intraperitoneal administration; vehicle 0.5% aqueous carboxymethylcellulose)	0, 500, 1 000, 2 000 mg/kg bw	94.9	Negative	Hoffmann & Engelhardt (1997)
Ex vivo unscheduled DNA synthesis (and S-phase response)	Wistar rat, male (single oral gavage); analysis of rat hepatocytes prepared 18 hours after exposure	0, 20, 200, 1 000 mg/kg bw	94.3	Negative	Polloth & Hoffmann (1994c)
Ex vivo unscheduled DNA synthesis	Wistar rat, male (3-week dietary administration); analysis of rat hepatocytes prepared 16 hours after last exposure	0, 200, 16 000 ppm (0, 10, 800 mg/kg bw per day)	94.3	Negative	Polloth & Hoffmann (1994d)
Chromosomal aberration assay in germ cells	NMRI mouse, male (single oral gavage); analysis of spermatogonial cells	0, 500, 1 000, 2 000 mg/kg bw	95.0	Negative	Honarvar (2002)

bw: body weight; CHO: Chinese hamster ovary; DNA: deoxyribonucleic acid; HPRT: hypoxanthine–guanine phosphoribosyltransferase; MTF: morphological transformation frequency; RPE: relative plating efficacy; S9: 9000 × *g* supernatant fraction from rat liver homogenate

[a] Induces morphological transformations. No relevant increase in the number of transformed colonies was observed after an exposure period of 7 days. After an exposure time of 24 hours, a clear increase in the number of transformed colonies was observed beginning at the lowest tested concentration of 0.25 µg/mL, with an MTF of 1.12%, up to the highest response of 2.14% MTF at a concentration of 1.5 µg/mL. The RPE was reduced from about 0.2 µg/mL onward (RPE 79.8%) after the 7-day exposure and from about 2.0 µg/mL onward (RPE 66.9%) after the 24-hour exposure. Colony size and density were reduced from about 0.2 µg/mL onward after the 7-day exposure and from about 2.0 µg/mL onward after the 24-hour exposure. According to the results of the present in vitro study, the test substance kresoxim-methyl led to a clear, statistically significant increase in the number of morphologically transformed colonies, but in the 24-hour exposure only. All tested concentration groups (except 5.0 µg/mL, where the cytotoxicity was too strong to allow colony formation) showed a transformation frequency above 0.6%, which is the criterion for a positive response and is above the historical negative control range as well. A desired negative response in at least one of the lower concentrations could not be achieved, even though a wide concentration range was applied. In the experiment with the 7-day exposure, no relevant increase was observed. The increase in the transformation frequency induced by the positive control agent benzo(*a*)pyrene clearly demonstrated the sensitivity of the test method and of the metabolic activity of the Syrian hamster embryo cells. Thus, under the experimental conditions chosen here, kresoxim-methyl induces morphological transformation in Syrian hamster embryo cells in vitro.

When kresoxim-methyl was assessed for its potential to induce morphological transformation in Syrian hamster embryo cells, it was considered to cause a significant increase in transformed colonies. The Syrian hamster embryo transformation assay investigates morphological transformation,

not DNA or chromosomal damage, it is an old study design and there is currently no Organisation for Economic Co-operation and Development (OECD) test guideline for it.

2.5 *Reproductive and developmental toxicity*

(a) Multigeneration studies

Rats

In a two-generation study of reproductive toxicity, groups of 25 male and 25 female Wistar (Chbb:THOM(SPF)) rats (35–36 days old; mean body weights 142.9 g for males and 125.4 g for females) were fed diets containing kresoxim-methyl (purity >93.7%) at a concentration of 0, 50, 1000, 4000 or 16 000 ppm. The F_0 generation was exposed directly, the F_{1a} and F_{1b} generations directly and indirectly (through lactation) and the F_2 generation indirectly. The mean daily intakes of kresoxim-methyl are shown in Table 29.

Table 29. Mean daily intakes of kresoxim-methyl in a reproductive toxicity study in rats

Generation/sex/period		Mean intake (mg/kg bw per day)			
		50 ppm	1 000 ppm	4 000 ppm	16 000 ppm
F_0 males		5.1	102.6	411.0	1 623.1
F_0 female (premating)		5.6	106.7	437.2	1 741.1
F_0 female F_{1a} litter	Gestation period	4.6	91.7	483.8	1 482.5
	Lactation period	8.3	162.0	661.8	2 610.5
F_0 female F_{1b} litter	Gestation period	4.3	84.3	348.9	1 389.3
	Lactation period	7.1	143.2	587.2	2 409.0

bw: body weight; F_0: parental generation; F_1: first filial generation; ppm: parts per million
Source: Hellwig & Gelbke (1994a)

The parental rats were observed for clinical signs, death, feed consumption, body weight, and clinical chemistry, histopathological and reproductive parameters, including mating, fertility, gestation and live birth indices. The litters and pups were observed for viability, lactation, behaviour and developmental indices, including pinna unfolding and opening of the auditory canal and eyes. The functional tests included grip strength, startle reflex and pupillary reflex. Reproductive organs and the pituitary, liver and kidney were examined histopathologically. The clinical chemistry end-points included assays for serum ALT, AST, ALP and GGT activities.

No compound-related clinical signs or deaths were observed in the F_0, F_1 or F_2 generation throughout the study. F_0 and F_1 parental animals showed no effects on mating, fertility, gestation or live birth indices, but statistically significant reductions in feed consumption were observed in F_0 and F_1 males during treatment and in F_0 and F_1 females during gestation and lactation at 16 000 ppm. Statistically significant reductions in body weight were seen at doses of 4000 ppm and higher in F_0 and F_1 males and in F_0 and F_1 females during gestation and lactation of the F_{1a} and F_{1b} generations. Statistically significant reductions in body weight gain were also observed in F_0 and F_1 males at these doses and in F_0 females at 16 000 ppm during the premating period before the first gestation. Statistically significant reductions in the activities of ALT and ALP were observed in F_0 and F_1 parents of both sexes, although these reductions may not be toxicologically relevant (Moss, 1994; Mellert & Hildebrand, 1995d). The activity of GGT was statistically significantly increased in F_0 males at 4000 ppm and higher and in F_1 animals of both sexes at 16 000 ppm. Statistically significantly decreased numbers of fat storage cells were observed in the livers of F_0 and F_1 males at 4000 ppm and higher; however, this change may have occurred as a result of the reduced feed consumption at higher doses.

Statistically significant increases in relative kidney weights were observed in F_0 males at 16 000 ppm and in F_0 females and F_1 males at 4000 ppm and higher. No treatment-related morphological lesions were observed in the liver or kidney.

No compound-related changes in clinical signs, sex ratio, viability index or lactation index were seen in pups of the F_{1a}, F_{1b} or F_{2a} generations. Body weights and body weight gains during lactation were statistically significantly decreased in F_{1a}, F_{1b} and F_{2a} pups at 4000 ppm and higher. A statistically significantly lower percentage of F_{1b} pups at these doses had pinna unfolding; statistically significant retardations in opening of the auditory canal and eyes were also observed in F_{1b} and F_{2a} pups at 4000 ppm, but these were not dose dependent (Table 30). There were no differences in the results of reflex tests between controls and treated animals in any generation. Necropsy of pups revealed no external abnormalities.

Table 30. Physical development and reflex indices in pups from the F_0 females for the F_{1b} generation

Parameter			0 ppm	50 ppm	1 000 ppm	4 000 ppm	16 000 ppm
Pinna unfolding	Litters tested	*N*	25	24	25	23	24
	Pups tested	*N*	362	349	380	333	364
	Pups meeting criteria	*N*	329	325	347	251	289
		%	91	93	91	75	79
	Pups meeting criteria/litter	Mean %	92.9	92.5	89.7	77.0**	79.5*
		SD	24.78	22.33	28.80	35.09	34.26
Auditory canal opening	Litters tested	*N*	25	24	25	23	24
	Pups tested	*N*	197	191	197	179	186
	Pups meeting criteria	*N*	195	189	186	175	175
		%	99	99	94	98	94
	Pups meeting criteria/litter	Mean %	99.0	99.0	94.5	97.8	93.8
		SD	3.46	5.10	20.44	4.84	18.85
Eye opening	Litters tested	*N*	25	24	25	23	24
	Pups tested	*N*	197	191	197	179	186
	Pups meeting criteria	*N*	193	174	172	154	170
		%	98	91	87	86	91
	Pups meeting criteria/litter	Mean %	98.0	91.1	87.5	86.4*	91.7
		SD	6.92	23.16	29.32	23.81	22.62

N: number; ppm: parts per million; SD: standard deviation; *: $P \leq 0.05$; **: $P \leq 0.01$ (Wilcoxon test, one-sided)
Source: Hellwig & Gelbke (1994a)

The NOAEL for parental toxicity was 1000 ppm (equal to 84.3 mg/kg bw per day), based on reduced body weight and body weight gain, increased serum GGT activity and increased relative kidney weights at 4000 ppm (equal to 348.9 mg/kg bw per day). The NOAEL for reproductive toxicity was 16 000 ppm (equal to 1389.3 mg/kg bw per day), the highest dose tested, as no reproductive effects were observed at any dose. The NOAEL for offspring toxicity was 1000 ppm (equal to 84.3 mg/kg bw

per day), based on retarded growth in pups leading to a lower rate of F_{1b} pups per litter with pinna unfolding at 4000 ppm (equal to 348.9 mg/kg bw per day) (Hellwig & Gelbke, 1994a).

(b) Developmental toxicity

Rats

Groups of 25 female Wistar (Chbb:THOM(SPF)) rats were given kresoxim-methyl (purity >93.7%) suspended in 0.5% carboxymethylcellulose by gavage at a dose of 0, 100, 400 or 1000 mg/kg bw per day on days 6–15 of gestation.

No treatment-related changes in clinical signs, mortality rates, body weight or feed consumption were observed in maternal animals. There were no differences in conception rate, mean numbers of corpora lutea, total implantations, resorptions, preimplantation or postimplantation losses, or live fetuses. No statistically significant differences in fetal sex ratio, placental weight or fetal weight were observed between control and treated groups.

External examination revealed three fetuses with external malformations: one fetus at 100 mg/kg bw per day had anasarca and a cleft palate, one fetus at 400 mg/kg bw per day was acaudate and one fetus at 1000 mg/kg bw per day had meningocele and unilateral microphthalmia; however, the incidence of these malformations was within the range for historical controls. One fetus at 1000 mg/kg bw per day had hydrocephalus, but this incidence was also within the historical control range. An increased incidence of incompletely ossified thoracic vertebral bodies was seen in 13% of all fetuses and 56% of litters (statistically significant) at 1000 mg/kg bw per day (Table 31); the mean historical control values were 8% (0–49%) of all fetuses and 23% (0–100%) of litters.

Table 31. Ossification status of thoracic vertebrae in a developmental toxicity study in rats

Parameter			**0 mg/kg bw per day**	**100 mg/kg bw per day**	**400 mg/kg bw per day**	**1 000 mg/kg bw per day**
Litters evaluated		*N*	24	23	22	25
Fetuses evaluated		*N*	168	167	156	172
Live fetuses		*N*	168	167	156	172
Thoracic vertebral body/bodies incompletely ossified	Fetal incidence	*N*	12	12	12	23
		%	7.1	7.2	7.7	13
	Litter incidence	*N*	5	9	7	14*
		%	21	39	32	56
	Affected fetuses/litter	Mean %	6.9	6.9	7.9	14.2*
		SD	17.55	10.19	13.34	16.08
Thoracic vertebral body/bodies only one ossification centre	Fetal incidence	*N*	0	0	1	0
		%	0	0	0.6	0
	Litter incidence	*N*	0	0	1	0
		%	0	0	4.5	0
	Affected fetuses/litter	Mean %	0	0	0.5	0
		SD	0	0	2.37	0
Thoracic vertebral	Fetal incidence	*N*	0	0	1	0
		%	0	0	0.6	0

Parameter			0 mg/kg bw per day	100 mg/kg bw per day	400 mg/kg bw per day	1 000 mg/kg bw per day
body/bodies not ossified	Litter incidence	*N*	0	0	1	0
		%	0	0	4.5	0
	Affected fetuses/litter	Mean %	0	0	0.6	0
		SD	0	0	3.05	0

bw: body weight; *N*: number; SD: standard deviation; *: $P \leq 0.05$
Source: Hellwig & Gelbke (1994b)

The NOAEL for maternal toxicity was 1000 mg/kg bw per day, the highest dose tested. The NOAEL for embryo and fetal toxicity was 400 mg/kg bw per day, on the basis of a slight increase in incidence of reduced ossification in fetuses above the mean historical control value at 1000 mg/kg bw per day (Hellwig & Gelbke, 1994b).

Rabbits

Groups of 15 female Himalayan rabbits were given kresoxim-methyl (purity 96.6%) suspended in 0.5% carboxymethylcellulose by gavage at a dose of 0, 100, 400 or 1000 mg/kg bw per day on days 7–19 of gestation.

No compound-related changes in clinical signs, death, body weight or feed consumption were observed in maternal animals, and there were no compound-related changes in conception rate, mean numbers of corpora lutea, total implantations, resorptions, preimplantation or postimplantation losses, or live fetuses. No significant differences in fetal sex ratio, placental weight or fetal weight were observed between control and treated groups.

External examination revealed one fetus with microcephaly and brachygnathia at 100 mg/kg bw per day, but the incidence was within the range of historical control data. Eight fetuses (0/15, 2/15, 2/15 and 3/15 at 0, 100, 400 and 1000 mg/kg bw per day, respectively) had soft tissue malformations: at 100 mg/kg bw per day, one had a septal defect, and one had agnesis of the gall bladder (2.5% incidence); at 400 mg/kg bw per day, two had a septal defect (1.9%); and at 1000 mg/kg bw per day, one had a septal defect, dilatation of the aortic arch and an aorta descendens, one had hydrocephaly and one had agnesis of the gall bladder (4.1%). The percentage of soft tissue malformations in historical controls was 2.2–3.1%. The incidences of ventricular septal defects in the treated groups were comparable to historical control values. Increased incidences of fused sternebrae were observed in 3/15 controls, 11/15 at 100 mg/kg bw per day, 7/15 at 400 mg/kg bw per day and 9/15 at 1000 mg/kg bw per day; the increase at 100 mg/kg bw per day was statistically significant, but was within the historical control range. Increased total numbers of fetal malformations were also observed in treated groups, but again at incidence rates comparable with those of historical controls (0%, 4.9%, 3.8% and 4.1% at 0, 100, 400 and 1000 mg/kg bw per day, respectively, versus 2.9–3.5% for historical controls).

The NOAEL for both maternal and embryo/fetal toxicity was 1000 mg/kg bw per day, the highest dose tested (Hellwig & Hildebrand, 1993).

2.6 Special studies

(a) Neurotoxicity

Acute neurotoxicity

Kresoxim-methyl (purity 93.7%) was administered to groups of 10 male and 10 female Wistar (Chbb:THOM(SPF)) rats (42 days old; mean body weight 190 g for males and 147 g for females) as a

single oral administration by gavage at a dose of 0, 500, 1000 or 2000 mg/kg bw. The vehicle was 0.5% carboxymethylcellulose solution, and the dosing volume was 10 mL/kg bw.

The animals were observed for up to 2 weeks after dosing. Body weight was determined weekly. The general state of health was checked at least daily, and the animals were additionally examined and palpated once a week. Functional observational batteries and motor activity measurements were carried out on all animals 6 days prior to dosing, at day 0 (within a few hours of dosing) and 7 and 14 days after dosing. Five animals of each sex per dose were fixed by in situ perfusion and subjected to neuropathological examination.

No substance-related signs of neurotoxicity or any other signs of general toxicity were observed.

The NOAEL for both systemic toxicity and neurotoxicity under the conditions of this study was 2000 mg/kg bw, the highest dose tested (Mellert, Kaufmann & Hildebrand, 1996a).

Subchronic neurotoxicity

Kresoxim-methyl (purity 94.3%) was administered to groups of 10 male and 10 female Wistar (Chbb:THOM(SPF)) rats (49 days old; mean body weight 227 g for males and 167 g for females) for 3 months at a dietary concentration of 0, 1000, 4000 or 16 000 ppm (equal to 0, 72, 292 and 1180 mg/kg bw per day for males and 0, 84, 341 and 1354 mg/kg bw per day for females, respectively). Feed consumption and body weight were determined at least once a week. A check of the general state of health of the animals was made at least daily. Furthermore, the animals were examined and palpated once a week. In addition to this, general clinical examination and functional observational batteries and motor activity measurements were carried out on all animals before the start of the administration period and during weeks 4, 8 and 13 of the administration period. Five animals of each sex per dose were fixed by in situ perfusion and subjected to neuropathological examination.

In the 16 000 ppm group, there was a reduction in feed consumption in both sexes, which was statistically significant on some days only. The values were 8% (males) and 13% (females) below the control values. There was also a reduction in body weight in both sexes, which was statistically significant on some days only. The values were up to 9% below the control values. There was a decrease in body weight gain in both sexes, which was statistically significant on some days only. The values were 17% (males) and 32% (females) below the control values.

There were no substance-related effects in other groups. No signs of neurotoxicity were detected at any of the doses.

The NOAEL for neurotoxicity was 16 000 ppm (equal to 1180 mg/kg bw per day), the highest dose tested. The NOAEL for systemic toxicity was 4000 ppm (equal to 292 mg/kg bw per day), based on a reduction of body weight gain at 16 000 ppm (equal to 1180 mg/kg bw per day) (Mellert, Kaufmann & Hildebrand, 1996b).

(b) Mechanistic studies on the carcinogenic mode of action

Tumour initiating potential

Groups of 10 Wistar (Chbb:THOM) rats of each sex were subjected to a partial hepatectomy and 14 hours later received kresoxim-methyl (purity 94.3% and 99.4%) in a single gavage dose of 2388 mg/kg bw suspended in 0.5% carboxymethylcellulose. For promotion, phenobarbital was incorporated in the diet at a concentration of 500 ppm (equivalent to 25 mg/kg bw) for 8 weeks. Liver slices were examined histologically on slides stained with haematoxylin and eosin (H&E) or stained immunochemically for the placental form of glutathione *S*-transferase (GST-P). The incidences of hepatocellular alteration (foci) and of GST-P-positive foci were used to estimate initiating potential. *N*-Nitrosomorpholine was used as the positive control.

Hepatocellular hypertrophy was found in almost all of the phenobarbital-treated animals, and GST-P-positive foci and foci of hepatocellular alteration were found in nearly all animals treated with the positive control. The number of animals with GST-P-positive foci in groups treated with kresoxim-methyl was comparable with that of vehicle controls. The numbers of foci per liver in promoted animals were 0–3 in those given kresoxim-methyl, 0–10 in vehicle controls and 3–100 in positive controls.

The results suggest that kresoxim-methyl does not have tumour initiating potential in rats in this test (Gamer & Hildebrand, 1995a,b).

Tumour promoting potential

In a medium-term study of promotion, male Fischer (F344/DuCrj) rats (aged 4 weeks; body weight 55–65 g) were initiated with a single intraperitoneal injection of *N*-nitrosodiethylamine at a dose of 200 mg/kg bw. The animals were then maintained on basal diet ad libitum for 14 days. Five groups of 16 male rats were fed diets containing 0, 200, 800, 8000 or 16 000 ppm kresoxim-methyl (purity 95.4%) for 6 weeks, with average intakes of 0, 10.78, 42.47, 430.6 and 886 mg/kg bw per day, respectively (not adjusted for purity). The remaining 16 male rats were fed a diet containing 500 ppm phenobarbital (28 mg/kg bw per day) as a positive control for 6 weeks. The animals were subjected to a two-thirds partial hepatectomy after the first week of feeding with kresoxim-methyl or phenobarbital and were observed for clinical signs, death, feed consumption and body weight. The liver was examined grossly and histopathologically.

There were no compound-related deaths or clinical signs of toxicity. Body weight and feed consumption in groups given kresoxim-methyl were comparable with those of controls. Significant increases in the absolute and relative weights of the liver were observed in groups given kresoxim-methyl at 800 ppm and higher. Treatment with phenobarbital caused significant increases in body weight, feed consumption and relative liver weight. Quantification of hepatic foci with a computer-assisted image analyser revealed significant, dose-related increases in the number and area of GST-P-positive hepatocellular foci in groups given kresoxim-methyl at and above 8000 ppm, as well as in the phenobarbital-treated positive controls.

The NOAEL for foci promotion in F344 rats was 800 ppm (equal to 42.47 mg/kg bw per day) (Harada, Kawakatsu & Nagayoshi, 1997).

Hepatic cell proliferation

A series of studies was conducted to investigate the effect of kresoxim-methyl on hepatic cell proliferation in rats, by measuring S-phase DNA synthesis, an indicator of cell proliferation. Incorporation of 5-bromo-2′-deoxyuridine (BrdU) into DNA was measured by immunohistochemical staining.

In the first study, groups of five young (64 days old) male Wistar (Chbb:Thom(SPF)) rats were given diets containing kresoxim-methyl (purity 94.3%) at a concentration of 0, 200 or 16 000 ppm (equal to 0, 15 and 1100 mg/kg bw per day, respectively) for 3 weeks. Osmotic minipumps filled with BrdU were implanted subcutaneously 1 week before necropsy. The animals were observed for clinical signs, death, feed consumption and body weight. The livers were examined grossly and immunohistopathologically. Samples of the hepatic lobule and the jejunum were taken as positive tissues for proliferation and were stained with H&E and immunochemically with an antibody against BrdU. Immunopositive and H&E-counterstained hepatocyte nuclei from 11 fields for each of three lobes were counted.

No treatment-related changes in body weight, feed consumption or clinical signs were seen. A slight increase in liver weights was observed at 16 000 ppm, but no treatment-related gross lesions or histopathological changes were observed in the livers of treated rats. A statistically significant increase in the number of hepatocytes in which BrdU was incorporated into the DNA of S-phase cells was

observed in the periportal zone (zone 1) and the intermediate zone (zone 2) of the hepatic lobule in the group at 16 000 ppm. No significant increase in cell proliferation was observed in the group at 200 ppm (Polloth & Hildebrand, 1994a).

In a supplementary study with a similar design, groups of five young (56 days of age) male Wistar (Chbb:THOM) rats received kresoxim-methyl (purity 94.9%) in the diet at a concentration of 0, 800 or 8000 ppm (equal to 0, 61 and 603 mg/kg bw per day, respectively) for 3 weeks.

Results similar to those observed at 16 000 ppm in the first study were observed at 8000 ppm. Statistically significant increases in cell proliferation were observed in zones 1 and 2 of the hepatic lobule at 8000 ppm, but not at 800 ppm

Based on these findings, the NOAEL for hepatic cell proliferation was 800 ppm (equal to 61 mg/kg bw per day) (Mellert, Bahnemann & Hildebrand, 1997).

In a study of the hepatic proliferating activity of kresoxim-methyl (purity 94.3%) in the livers of older rats, groups of five male Wistar (Chbb) rats (aged 16 months) were given diets containing kresoxim-methyl (purity 94.3%) at a concentration of 0, 200 or 16 000 ppm (equal to 0, 15 and 1140 mg/kg bw per day, respectively) for 3 weeks. The design of the study was similar to those described above.

No compound-related changes in clinical signs or body weight were seen, and no compound-related lesions in the liver were observed by microscopic examination with H&E staining. A statistically significant increase in cell proliferation was observed in zone 1 of the hepatic lobule at 16 000 ppm, which was comparable with that observed in the young rats (Polloth & Hildebrand, 1994b).

In a study of the hepatic proliferating activity of kresoxim-methyl in the livers of rats treated for various periods, groups of five male Wistar (Chbb) rats (42 days old) were given diets containing kresoxim-methyl (purity 92.7%) at a concentration of 0 or 16 000 ppm (equal to 1660 mg/kg bw per day for 1 week; 1362 mg/kg bw per day for 6 weeks; and 1150 mg/kg bw per day for 13 weeks) for 1, 6 or 13 weeks. Groups were allowed to recover for 2 or 3 weeks.

Significant increases in cell proliferation were observed in the treated groups after 1 week (zones 1, 2 and 3) and after 6 weeks (zone 1). The increase in zone 1 in the group treated for 1 week was greater than that in the group treated for 6 weeks. This compound-related enhancement of cell proliferation was significantly reversed in the groups allowed to recover. The zonal distribution of increased cell proliferation revealed a selective effect of kresoxim-methyl on hepatocytes in zone 1 (Mellert, Bahnemann & Hildebrand, 1996).

Based on the findings of these studies, it was concluded that the cell proliferating activity of kresoxim-methyl is not influenced by the age of the test animals.

In a study of unscheduled DNA synthesis and S-phase response in rat hepatocytes, groups of three male Wistar (Chbb) rats received kresoxim-methyl (purity 94.3%) in a single oral gavage dose of 0, 20, 200 or 1000 mg/kg bw. 2-Acetylaminofluorene was used as a positive control at a dose of 50 mg/kg bw in the assay of unscheduled DNA synthesis and at 1000 mg/kg bw in the assay of S-phase response. Hepatocytes were prepared by in situ hepatic perfusion 18 hours after treatment. The isolated hepatocytes were cultured with [^{3}H]thymidine for 18 hours, and S-phase response and unscheduled DNA synthesis were evaluated autoradiographically in the labelled cells.

Exposure of rats to kresoxim-methyl in vivo was not cytotoxic to liver cells. Slight but dose-dependent increases in the number of cells in S-phase were observed in all treated groups, with 1% at

0 mg/kg bw, 1.37% at 20 mg/kg bw, 2.78% at 200 mg/kg bw and 2.58% at 1000 mg/kg bw, as well as in the positive control group (5.87%).

The results suggest that kresoxim-methyl induced a moderate increase in S-phase DNA synthesis at 200 mg/kg bw and has a weak potential for enhancing hepatic cell proliferation (Polloth & Hildebrand, 1994c).

Morphology of hepatic proliferation

Groups of three female Wistar (Chbb) rats (12 weeks old) received diets containing kresoxim-methyl (purity 94.3%) at a concentration of 0, 200 or 16 000 ppm (equal to 0, 15 and 1200 mg/kg bw per day, respectively) for 3 weeks. At termination, the livers were fixed in situ by perfusion, and the peroxisomes in the liver were examined by light and electron microscopy after staining with diaminobenzidine to detect catalase activity.

There were no compound-related changes in clinical signs, body weight or feed consumption; reduced body weight gain was observed at 16 000 ppm. No compound-related lesions were observed in the liver, and no difference was seen between treated and control animals in the numbers of peroxisomes.

In view of the above, kresoxim-methyl did not induce peroxisome proliferation under the conditions of this study (Mellert, Kaufmann & Hildebrand, 1995a).

Groups of three female Wistar (Chbb) rats (15 months old) received diets containing kresoxim-methyl (purity 94.3%) at a concentration of 0, 200 or 16 000 ppm (equivalent to 0, 20 and 1600 mg/kg bw per day, respectively) for 3 weeks and were then fixed in situ by perfusion. Liver samples were examined by light and electron microscopy.

There were no compound-related changes in clinical signs or body weight, and no compound-related lesions were observed in the liver on light microscopic examination. Electron microscopy showed that the amount, shape and size of hepatocyte mitochondria in the treated group were comparable with those of controls (Mellert, Kaufmann & Hildebrand, 1995b).

Induction of hepatic metabolic enzyme activities

Groups of 10 male and 10 female Wistar (Chbb:THOM) rats were fed diets containing kresoxim-methyl (purity 94.3%) at a concentration of 0, 200 or 16 000 ppm (equal to 0, 13 and 973 mg/kg bw per day for males and 0, 15 and 1186 mg/kg bw per day for females, respectively) for 3 weeks. The animals were observed for clinical signs, death, body weight and feed consumption. Indicators of hepatic enzymes were measured, including the activities of GGT and drug metabolizing enzymes, the concentration of glutathione in liver homogenates, and the content of cytochrome P450 in microsomes.

Significant increases in the activities of GGT and 7-pentoxyresorufin *O*-depentylase and in P450 content were observed in males at 16 000 ppm. The pattern of induction of drug metabolizing enzyme activities resembled that reported for phenobarbital in other studies. In females, only a tendency towards induction was observed (Mellert et al., 1996a).

The above studies are summarized in Table 32.

Table 32. Summary of mechanistic investigations on the carcinogenic mode of action of kresoxim-methyl

Study **Doses** **(Batch / purity)**	**Results**	**Reference**
Rat foci initiation hepatocarcinogenesis study, single oral gavage 0, 2 388 mg/kg bw (N36 / 94.3%; 26833/147 / 99.4%)	No tumour-initiating activity.	Gamer & Hildebrand (1995a,b)
Rat medium-term promotion hepatocarcinogenesis study, male Fischer rats 0, 200, 800, 8 000, 16 000 ppm (0, 10.78, 42.47, 430.6, 886 mg/kg bw per day) (N112 / 95.4%)	Treatment significantly increased the area (mm²) and number of DEN-initiated GST-P-positive liver foci per square centimetre at 8 000 and 16 000 ppm. NOAEL = 800 ppm (42.47 mg/kg bw per day)	Harada, Kawakatsu & Nagayoshi (1997)
Rat 3-week S-phase response study, diet, 64-day-old male rats 0, 200, 16 000 ppm (intake not determined; equivalent to 0, 20, 1 600 mg/kg bw per day) (N36 / 94.3%)	Increased S-phase response (BrdU incorporation in DNA) of rat hepatocytes at 16 000 ppm, especially of zone 1 (3-fold increase). No effect at 200 ppm. No effects on body weight, feed intake, clinical parameters or liver histology at 200 or 16 000 ppm, except for slight increase in liver weight at 16 000 ppm.	Polloth & Hildebrand (1994a)
Rat 3-week S-phase response study, diet, male Wistar rats 0, 800, 8 000 ppm (0, 61, 603 mg/kg bw per day) (N36 / 94.9%)	Increased S-phase response in zone 1 hepatocytes at 8 000 ppm, no increased S-phase response at 800 ppm.	Mellert, Bahnemann & Hildebrand (1997)
Rat 3-week S-phase response study, diet; male Wistar rats 0, 200, 16 000 ppm (0, 15, 1 140 mg/kg bw per day) (N36 / 94.3%)	Increased S-phase response (BrdU incorporation in DNA) of rat hepatocytes at 16 000 ppm, especially of zone 1 (3-fold increase). No effect at 200 ppm. No effects on body weight, feed intake, clinical parameters, liver weight or liver histology at 200 or 16 000 ppm.	Polloth & Hildebrand (1994b)
Rat 1-, 6-, 13-week S-phase response, diet, including reversibility, male Wistar rats 0, 16 000 ppm (week 1: 1 660 mg/kg bw per day; week 6: 1 362 mg/kg bw per day; week 13: 1 150 mg/kg bw per day) (N36 / 92.7%)	Enhanced S-phase response over whole 13-week period, peak after 1 week (all three zones affected, with increased liver weight), highest S-phase response in zone 1 after 6- and 13-week treatment; S-phase response was clearly below control values in rats subjected to 2-week or 5-week off-treatment period, after 1-week or 13-week treatment, respectively, indicating counter-regulation of cell proliferation and reversibility.	Mellert, Bahnemann & Hildebrand (1996)
Rat ex vivo UDS / S-phase response Single gavage administration 0, 20, 200, 1 000 mg/kg bw (N36 / 94.3%)	Increased S-phase response at 200 and 1 000 mg/kg bw (no evidence of UDS).	Polloth & Hildebrand (1994c)

Study Doses (Batch / purity)	Results	Reference
Rat 3-week oral diet; electron microscopic investigation of the liver, female Wistar rats 0, 200, 16 000 ppm (0, 15, 1 200 mg/kg bw per day) (N36 / 94.3%)	No induction of peroxisome proliferation.	Mellert, Kaufmann & Hildebrand (1995a)
Rat 3-week oral diet; electron microscopic investigation of liver, 15-month-old Wistar rats 0, 200, 16 000 ppm (intake not determined; equivalent to 0, 20, 1 600 mg/kg bw per day) (N36 / 94.3%)	No changes in mitochondria were noted.	Mellert, Kaufmann & Hildebrand (1995b)
Rat 3-week oral diet; enzyme induction investigation in liver microsomes, male and female Wistar rats 0, 200, 16 000 ppm (males: 0, 13, 973 mg/kg bw per day; females: 0, 15, 1 186 mg/kg bw per day) (N36 / 94.3%)	Significant increase at 16 000 ppm in males for GGT, weak phenobarbital-type enzyme inducer based on slightly increased CYP450 content and PROD activity in males (statistically significant) and females (non-significant). No changes in ω-oxidation of lauric acid, EROD, cyanide-insensitive palmitoyl-CoA oxidation or glutathione content. No substance-related effects at 200 ppm.	Mellert et al. (1996a)

BRdU: 5-bromo-2′-deoxyuridine; bw: body weight; CoA: coenzyme A; CYP450: cytochrome P450; DEN: *N*-nitrosodiethylamine; DNA: deoxyribonucleic acid; EROD: ethoxyresorufin *O*-deethylase; GGT: gamma-glutamyl transferase; GST-P: placental form of glutathione *S*-transferase; ppm: parts per million; PROD: 7-pentoxyresorufin *O*-depentylase; UDS: unscheduled DNA synthesis

(c) Other special studies

Mechanism of decreased serum enzyme activities

As marked reductions in the activities of serum ALP and ALT were reported in short- and long-term studies of toxicity in rats, a series of experiments was conducted in which groups of five male and five female rats were fed diets containing kresoxim-methyl at a concentration of 8000 ppm (equivalent to 800 mg/kg bw per day) for 2 weeks.

In the first experiment, ALP activity was determined in serum samples and extracts of liver and small intestine. There was a marked fall in intestinal ALP activity in serum, with no significant change in the contribution of the tissue-nonspecific (liver/bone) isoenzyme. The author indicated that the reduction in serum ALP activity observed in the kresoxim-methyl-treated groups was mostly due to a reduction in the contribution of the intestinal isoenzyme.

In the second experiment, serum ALP activity was markedly reduced after fasting and was increased by feeding a diet supplemented with olive oil. In the third experiment, addition of sera collected from treated animals to sera collected from untreated animals did not suppress ALP activity, indicating the absence of an inhibitor. The observed reduction in serum ALP activity was therefore probably due to a slight alteration in feed absorption in treated rats. These studies did not conform to GLP (Moss, 1994).

In a second study to investigate the reduced enzyme activities, groups of 10 male and 10 female Wistar rats were fed diets containing kresoxim-methyl (purity 94.9%) at a concentration of 0 or 16 000 ppm (equal to 910 mg/kg bw per day for males and 1100 mg/kg bw per day for females) for 2 weeks. The animals were observed for clinical signs, death, body weight and feed consumption. ALT and ALP activities in serum and urine were assayed at the end of the study.

There were no compound-related changes in clinical signs or mortality rates. Statistically significantly decreased feed consumption (about 10% below controls) was observed in treated animals of both sexes. A slight but statistically significant decrease in body weight (2.9% below control value) was observed in treated males. Statistically significantly reduced activities (about 25% compared with the corresponding control value) of ALT and ALP in serum were observed in animals of both sexes treated with 16 000 ppm of the test substance, but no change in the activities of either enzyme in urine was observed. No change in urinary creatinine or urinary volume was observed in treated animals, indicating no change in renal function. Thus, the reduced enzyme activity observed in sera of kresoxim-methyl-treated rats was not caused by a change in renal excretion of the enzymes (Mellert, Decardt & Hildebrand, 1997).

Dietary administration of kresoxim-methyl to rats after repeated pretreatment with dimethoate

In a study to determine whether repeated pretreatment of rats with an organophosphate substantially enhances the toxicity of kresoxim-methyl, a group of three male and three female Wistar rats (main group) and a group of four female rats (satellite group) were treated with dimethoate at a dose of 40 mg/kg bw per day by gavage for 3 days. In the satellite group, cholinesterase activities were monitored.

The administration of dimethoate (40 mg/kg bw per day) for 3 days led to clinical signs of toxicity, such as tremors, lateral position and reduced feed consumption and body weight. The clinical signs observed were expected and are typical for substances inhibiting cholinesterases. After cessation of treatment with dimethoate, all signs were reversible either with or without subsequent treatment with kresoxim-methyl at a dose of 800 or 8000 ppm (equal to 84 and 597 mg/kg bw per day for males and 78 and 605 mg/kg bw per day for females, respectively) for 6 days.

It was observed that repeated pretreatment of rats with dimethoate did not enhance the toxicity of kresoxim-methyl under the conditions of the study. Substantially lowered cholinesterase activities in serum or erythrocytes have therefore no influence on the toxicological profile of kresoxim-methyl (Mellert et al., 1996b).

Effects on the vital functions of animals: general pharmacology

To study the effects of kresoxim-methyl on the vital functions of animals and general pharmacology, the acute effects of kresoxim-methyl on the native behaviour of NMRI mice were investigated at 0.5, 1, 2, 4 and 6 hours after a single oral administration of 1000, 2000 or 5000 mg/kg bw. The behaviour of animals in their home cage as well as in a new environment and their responses to handling were assessed.

At 30 minutes after the administration of the highest dose of kresoxim-methyl, 2/3 mice showed a slight change in their body carriage (hunched posture). However, this finding was also seen in 1/3 mice at 1 hour after treatment with 2000 mg/kg bw and 1/3 mice treated with 1000 mg/kg bw at 2 hours after dosing. In the control group, 2/3 animals administered vehicle only were also recorded to have a hunched posture at 2 hours after treatment. Therefore, this observation is considered not to be related to the test article.

At 4 and 8 hours after treatment, no abnormal findings were recorded in any animals of any group (Dubach-Powell et al., 1994).

(d) Studies on metabolites

The data on the acute toxicity and genotoxicity of some metabolites of kresoxim-methyl (Table 33) are presented in Tables 34 and 35. None of the metabolites is more acutely toxic than the parent compound.

Table 33. Identification of metabolites of kresoxim-methyl

Metabolite	Reg. No.	Chemical name
M1	262451	(*E*)-2-Methoxyimino-2-[2-(*o*-tolyloxymethyl)phenyl]acetic acid
M2	291685	2-[2(2-Hydroxymethylphenoxymethyl)phenyl]-2-methoxyiminoacetic acid
M9	292932	2-[2-(4-Hydroxy-2-methylphenoxymethyl)phenyl]-2-methoxyiminoacetic acid
M15	339774	2-[2-(5-Hydroxy-2-methylphenoxymethyl)phenyl]-2-methoxyiminoacetic acid

Reg. No.: Registration Number

Table 34. Acute toxicity of some metabolites of kresoxim-methyl

Metabolite	Species	Strain	Sex	Route	LD_{50} (mg/kg bw)	Reference
M1	Rat	Chbb Wistar	M + F	Oral	~2 000	Kirsch & Hildebrand (1995)
M2	Rat	Chbb Wistar	M + F	Oral	>5 000	Kirsch & Hildebrand (1994a)
M9	Rat	Chbb Wistar	M + F	Oral	>5 000	Kirsch & Hildebrand (1994b)

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

Table 35. Genotoxicity of some metabolites of kresoxim-methyl

Metabolite	End-point	Test object	Concentration	Results	Reference
M1	Bacterial reverse mutation assay (Ames test)	*Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537; *Escherichia coli* strain WP2*uvr*A; with/without S9 mix	20–5 000 µg/plate (standard plate test) 4–2 500 µg/plate (preincubation test)	Negative	Engelhardt & Hoffmann (1995a)
M1	Syrian hamster embryo cell assay	Syrian golden hamster	7 days: 15–250 µg/mL 24 hours: 100–500 µg/mL	Negative	Engelhardt & Leibold (2005)
M2	Bacterial reverse mutation assay (Ames test)	*S. typhimurium* strains TA98, TA100, TA1535, TA1537; *E. coli* strain WP2*uvr*A; with/without S9 mix	4–5 000 µg/plate (*S. typhimurium*) 20–5 000 µg/plate (*E. coli*)	Negative	Engelhardt & Hoffmann (1995b,c)
M9	Bacterial reverse mutation assay (Ames test)	*S. typhimurium* TA98, TA100, TA1535, TA1537 (standard plate test); *E. coli* WP2*uvr*A	4–5 000 µg/plate (*S. typhimurium*) 20–5 000 µg/plate (*E. coli*)	Negative	Engelhardt (1995)

Metabolite	End-point	Test object	Concentration	Results	Reference
M15	Bacterial reverse mutation assay (Ames test)	*S. typhimurium* TA98, TA100, TA1535, TA1537 (standard plate test); *E. coli* WP2*uvr*A	20–5 000 µg/plate (standard plate test) 4–2 500 µg/plate (preincubation test)	Negative	Engelhardt & Hoffmann (1996)

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

3. Observations in humans

3.1 Medical surveillance on manufacturing plant personnel

All persons handling crop protection products are surveyed by regular medical examinations. There are no specific parameters available for monitoring the effects of kresoxim-methyl. Thus, the medical monitoring programme is designed as a general health check-up, with special interest in the primary target organs presumed to be relevant by analogy from animal experiments. The surveillance programme includes a general physical examination, including neurological status, red and white blood cell counts and liver enzymes. Adverse health effects suspected to be related to kresoxim-methyl exposure have not been observed (BASF, 2018a).

In 1996, a special cross-sectional health examination study of employed men assigned to kresoxim-methyl production was conducted. Personnel at the kresoxim-methyl manufacturing site in Guaratinguetá, Brazil, were examined twice at an interval of 6 months. Employees were subdivided into two main groups: those having no direct contact ($N = 47$) and those with sporadic or daily contact with the final product ($N = 28$). Health end-points of interest were selected based on the results of chronic toxicity studies with animals, which reported indications of liver injury in mice, rats and dogs at high kresoxim-methyl doses. Comparisons of laboratory data were based on analysis of variance and regression analyses incorporating a kresoxim-methyl dose score as the primary explanatory variable and age, smoking history, alcohol intake and body mass index as concomitant variables.

No findings indicative of kresoxim-methyl-induced changes in four liver function indicators (GGT, AST, ALT and ALP) were seen at about 6 months and 1 year after start-up of the manufacturing process. Individual review of health complaints and conditions also did not reveal a connection between any of these occurrences and assignment to the kresoxim-methyl operations. Under the conditions of exposure, which were not quantitatively characterized, there was no evidence of kresoxim-methyl-induced health effects.

The above medical data were compiled by the BASF medical department in April 2008 (BASF, 2018a). Moreover, the personnel who are working with kresoxim-methyl are examined regularly (according to national law). The detailed medical data of persons employed at BASF are covered by data privacy. Adverse health effects have not been observed (BASF, 2018b).

3.2 Direct observation

Some cases of slight irritation of the skin, eyes, mouth or respiratory tract (including rhinitis and cough) have been reported to BASF in persons exposed to kresoxim-methyl in combination with other products. These reports could not be verified, and it is not clear whether kresoxim-methyl was the cause of these irritations (BASF, 2018a).

Data on exposure of the general public to kresoxim-methyl are not available. BASF is not aware of any epidemiological studies performed by third parties (BASF, 2018a). No published literature is available on epidemiological studies or the effect of kresoxim-methyl on humans (BASF, 2018b).

Comments

Biochemical aspects

In rats, orally administered radiolabelled kresoxim-methyl was rapidly, but incompletely, absorbed from the gastrointestinal tract. The extent of oral absorption was about 55–71% at the low dose (50 mg/kg bw) in both sexes, based on recovery of the radiolabel in bile and urine, and 22–28% at the high dose (500 mg/kg bw). Kresoxim-methyl was excreted mainly in the faeces (70% of the low dose and 80% of the high dose), with about 40% excreted via the bile at the low dose and 15% at the high dose within 48 hours. Lesser amounts were excreted in urine (about 20% of the low dose and 10% of the high dose). Peak levels of the radiolabel in plasma were reached 0.5–1 hour after the low dose and 8 hours after the high dose. The plasma half-life of radiolabel was 17–19 hours at the low dose and 22–31 hours at the high dose (Gans & Hildebrand, 1994).

Radioactive material was distributed in all tissues and organs throughout the body, and the total radioactivity in the organs was less than 2% of the administered dose 96 hours after dosing. The highest radioactivity was associated with the gastrointestinal tract, liver and kidney. There was no evidence of accumulation of radioactive material after dosing with ^{14}C-labelled kresoxim-methyl (Whitby, 1993; Gans, 1995b).

After oral administration in the rat, absorbed kresoxim-methyl was rapidly and completely metabolized. The metabolic pathways of kresoxim-methyl consisted of hydrolytic cleavages of the ester, the oxime ether and the benzyl ether bonds; hydroxylation at the *para* position of the phenoxy ring; oxidation of the aryl-methyl group to benzyl alcohol and its subsequent oxidation to the corresponding carboxylic acid; and conjugation of the resulting hydroxy groups with glucuronate and sulfate. The major metabolites identified in urine and faeces were M1, a hydrolytic product of the acetyl ester; M2, an oxidative metabolite of the aryl-methyl moiety of M1; and M9, a hydroxylated metabolite of the phenoxy ring of M1. M1 and M9 were the major metabolites identified in tissues (Kohl, 1994, 1995, 1998).

Toxicological data

The acute toxicity of kresoxim-methyl in rats was studied by the oral route (LD_{50} > 2000 mg/kg bw) (Kirsch & Hildebrand, 1993a; Yamamoto, 1994; Cords & Lammer, 2011a), the dermal route (LD_{50} > 2000 mg/kg bw) (Kirsch & Hildebrand, 1993b; Cords & Lammer, 2011b) and inhalation (LC_{50} > 263 mg/L air) (Wittmer & Landsiedel, 2011). Kresoxim-methyl is not irritating to the skin of rabbits (Rossbacher, 1992a; Wolf, 2011a) or irritating to the eyes of rabbits (Rossbacher, 1992b; Wolf, 2011b). Kresoxim-methyl is not a skin sensitizer in guinea-pigs in the Magnusson and Kligman maximization test (Rossbacher, 1993) or in the Buehler test (Weiss-Fuchs, 2011).

In repeated-dose toxicity studies in mice, rats and dogs, the predominant target organ was the liver.

In a 28-day range-finding study in mice administered kresoxim-methyl in the diet at a concentration of 0, 500, 2000 or 8000 ppm (equal to 0, 113, 485 and 2141 mg/kg bw per day for males and 0, 182, 798 and 3755 mg/kg bw per day for females, respectively), the NOAEL was 8000 ppm (equal to 2141 mg/kg bw per day), the highest dose tested (Schilling & Hildebrand, 1992a).

In a 3-month study in mice administered kresoxim-methyl in the diet at a concentration of 0, 250, 1000, 4000 or 8000 ppm (equal to 0, 57, 230, 909 and 1937 mg/kg bw per day for males and 0, 80, 326, 1326 and 2583 mg/kg bw per day for females, respectively), the NOAEL was 1000 ppm (equal to 230 mg/kg bw per day), based on decreases in body weight gain (>10%) in males at 4000 ppm (equal to 909 mg/kg bw per day) (Mellert & Hildebrand, 1994a,b).

In a 28-day range-finding study in rats administered kresoxim-methyl in the diet at a concentration of 0, 1000, 4000 or 16 000 ppm (equal to 0, 91, 365 and 1428 mg/kg bw per day for males and 0, 95, 375 and 1481 mg/kg bw per day for females, respectively), the NOAEL was 4000 ppm (equal to 365 mg/kg bw per day), based on the increased serum GGT activity in males, increased albumin

concentration in males and increased relative liver weight in females at 16 000 ppm (equal to 1428 mg/kg bw per day) (Schilling & Hildebrand, 1992b).

In a 90-day study in rats administered kresoxim-methyl in the diet at a concentration of 0, 500, 2000, 8000 or 16 000 ppm (equal to 0, 36, 146, 577 and 1170 mg/kg bw per day for males and 0, 43, 172, 672 and 1374 mg/kg bw per day for females, respectively), the NOAEL was 2000 ppm (equal to 146 mg/kg bw per day), based on decreased body weight and body weight gain and increased GGT activity in males at 8000 ppm (equal to 577 mg/kg bw per day) (Mellert & Hildebrand, 1994c).

In a 90-day study in dogs administered kresoxim-methyl in the diet at a concentration of 0, 1000, 5000 or 25 000 ppm (equal to 0, 30, 150 and 776 mg/kg bw per day for males and 0, 34, 168 and 846 mg/kg bw per day for females, respectively), the NOAEL was 5000 ppm (equal to 150 mg/kg bw per day), on the basis of vomiting and diarrhoea in both sexes and reduced body weight gain in females at 25 000 ppm (equal to 776 mg/kg bw per day) (Mellert & Hildebrand, 1994d).

In a 12-month study in dogs administered kresoxim-methyl in the diet at a concentration of 0, 1000, 5000 or 25 000 ppm (equal to 0, 27, 140 and 710 mg/kg bw per day for males and 0, 30, 150 and 760 mg/kg bw per day for females, respectively), the NOAEL was 5000 ppm (equal to 140 mg/kg bw per day), based on infrequent diarrhoea and vomiting occurring in both sexes and significantly reduced body weights of males at study termination at 25 000 ppm (equal to 710 mg/kg bw per day) (Hellwig & Hildebrand, 1994).

The overall NOAEL for dogs was 5000 ppm (equal to 150 mg/kg bw per day), and the overall lowest-observed-adverse-effect level (LOAEL) was 25 000 ppm (equal to 710 mg/kg bw per day).

In an 18-month assay for carcinogenicity in mice, kresoxim-methyl was administered at a dietary concentration of 0, 400, 2000 or 8000 ppm (equal to 0, 60, 304 and 1305 mg/kg bw per day for males and 0, 81, 400 and 1662 mg/kg bw per day for females, respectively). The NOAEL for toxicity was 2000 ppm (equal to 304 mg/kg bw per day), on the basis of reductions in body weight and body weight gain in both sexes and histopathological changes in the liver and increased liver weights in females at 8000 ppm (equal to 1305 mg/kg bw per day). Kresoxim-methyl was not carcinogenic in mice up to 8000 ppm (equal to 1305 mg/kg bw per day), the highest dose tested (Mellert & Hildebrand, 1994e).

In a 24-month toxicity study in Wistar (Chbb:THOM(SPF)) rats administered kresoxim-methyl in the diet at a concentration of 0, 200, 800, 8000 or 16 000 ppm (equal to 0, 9, 36, 370 and 746 mg/kg bw per day for males and 0, 12, 48, 503 and 985 mg/kg bw per day for females, respectively), the NOAEL for toxicity was 800 ppm (equal to 36 mg/kg bw per day), on the basis of increased activity of serum GGT, increased relative liver weight, and increased incidence and degree of severity of eosinophilic and mixed-cell foci in males at 8000 ppm (equal to 370 mg/kg bw per day). An increased incidence of hepatocellular tumours was observed in both sexes at 8000 ppm (equal to 370 mg/kg bw per day) (Mellert & Hildebrand, 1994f). This study was not powered to characterize the carcinogenic potential of kresoxim-methyl.

In a 24-month study of carcinogenicity in Wistar (Chbb:THOM(SPF)) rats administered kresoxim-methyl at a dietary concentration of 0, 200, 800, 8000 or 16 000 ppm (equal to 0, 9, 36, 375 and 770 mg/kg bw per day for males and 0, 12, 47, 497 and 1046 mg/kg bw per day for females, respectively), the NOAEL for toxicity was 800 ppm (equal to 36 mg/kg bw per day), on the basis of reduced body weight and body weight gain and hepatic alterations at 8000 ppm (equal to 375 mg/kg bw per day). It should be noted that this study was not designed to assess chronic toxicity, and the evaluation of non-neoplastic effects was less thorough than would be required for this purpose. A robust NOAEL for carcinogenicity could not be clearly identified, as the increase in the incidence of hepatocellular tumours in female rats at 800 ppm (equal to 36 mg/kg bw per day) was equivocal, with the incidences varying in the different pathology evaluations performed; clear increases in liver tumours were evident in both sexes at 8000 and 16 000 ppm (Mellert & Hildebrand, 1994g, 1995b,c).

The Meeting concluded that the analysis of the liver tumours produced by kresoxim-methyl in rats was best performed by a benchmark dose (BMD) analysis using the combined tumour incidences in the chronic toxicity study with 20 rats per group (Mellert & Hildebrand, 1994f) and the

carcinogenicity study with 50 rats per group (Mellert & Hildebrand, 1994g). The two studies were performed concurrently in the same laboratory, with the same batch of animals, with no differences in study design that would likely have an impact on the evaluation of liver carcinogenicity. Of the three pathology evaluations performed on these liver tumours, the Meeting concluded that the analysis by the PWG was the most robust and should be utilized for the BMD analysis. The lowest lower limit on the benchmark dose for a 10% response ($BMDL_{10}$) for liver tumours in female rats identified using "PROASTweb" software was 29.1 mg/kg bw per day, and this was selected as the point of departure for consideration in the risk assessment.

In a limited 24-month carcinogenicity study, kresoxim-methyl was administered to the CrlGlxBrlHan:WI strain of Wistar rats by feed at a concentration of 0 or 16 000 ppm (equal to 0 and 752.1 mg/kg bw per day for males and 0 and 1021.6 mg/kg bw per day for females, respectively). At 16 000 ppm, an increase in liver tumours (hepatocellular adenoma and carcinoma) was observed in both sexes (Kamp et al., 2008).

The Meeting concluded that kresoxim-methyl is carcinogenic in rats, but not in mice.

Kresoxim-methyl was tested for genotoxicity in an adequate range of in vitro and in vivo assays (Engelhardt & Hoffmann, 1993a,b; Hoffmann & Engelhardt, 1993, 1997; Engelhardt & Hildebrand, 1994; Polloth & Hoffmann, 1994a,b,c,d; Honarvar, 2002; Schwindt & Landsiedel, 2008; Schulz & Landsiedel, 2011; Woitkowiak, 2011). No evidence of genotoxicity was found. In the Syrian hamster embryo assay (morphological transformation assay), exposure after 24 hours increased cell transformation only at cytotoxic concentrations (Schwindt & Landsiedel, 2008).

The Meeting concluded that kresoxim-methyl was unlikely to be genotoxic.

A series of mechanistic studies was conducted with kresoxim-methyl, including tests for tumour initiating and promoting potential. In a study on tumour initiating activity, kresoxim-methyl did not increase the number of GST-P-positive hepatocellular foci in rats at a single dose of 2388 mg/kg bw (Gamer & Hildebrand, 1995a,b). In a study on the promoting potential of kresoxim-methyl, rats received an initiating dose of *N*-nitrosodiethylamine and then a diet containing 0, 200, 800, 8000 or 16 000 ppm (equal to 0, 10.78, 42.47, 430.6 and 886 mg/kg bw per day, respectively) of kresoxim-methyl for 6 weeks. The NOAEL for the promoting effect was 800 ppm (equal to 42.47 mg/kg bw per day), based on dose-dependent increases in GST-P-positive hepatocellular foci, indicating a promoting effect of kresoxim-methyl on hepatocarcinogenesis at 8000 ppm (equal to 430.6 mg/kg bw per day) (Harada, Kawakatsu & Nagayoshi, 1997).

Four in vivo studies were conducted to investigate the effect of kresoxim-methyl on hepatic cell proliferation in rat liver by measuring BrdU incorporation into hepatocyte DNA during S-phase DNA synthesis. The overall NOAEL for hepatic cell proliferation was 800 ppm (equal to 61 mg/kg bw per day) (Polloth & Hildebrand, 1994a,b; Mellert, Bahnemann & Hildebrand, 1996, 1997).

Overall, the above mechanistic data support a threshold-based mode of action for carcinogenesis.

Several additional studies were conducted to investigate unscheduled DNA synthesis and S-phase response in rat hepatocytes (Polloth & Hildebrand, 1994c), the morphology of hepatic proliferation in rats (Mellert, Kaufmann & Hildebrand, 1995a), effects on hepatocyte mitochondria (Mellert, Kaufmann & Hildebrand, 1995b), the induction of hepatic metabolic enzyme activities (Mellert et al., 1996a) and the mechanism of decreased serum enzyme activities in rats (Moss, 1994; Mellert, Decardt & Hildebrand, 1997). The Meeting concluded that these studies did not contribute to the risk assessment of kresoxim-methyl.

In view of the lack of genotoxicity, the absence of carcinogenicity in mice and the fact that only hepatocellular tumours were observed and that these were increased in both sexes of rats by a threshold-dependent mode of action, the Meeting concluded that kresoxim-methyl is unlikely to pose a carcinogenic risk to humans from the diet.

In a two-generation study of reproductive toxicity in which rats were fed diets containing kresoxim-methyl at a concentration of 0, 50, 1000, 4000 or 16 000 ppm (equal to 0, 5.1, 102.6, 411.0

and 1623.1 mg/kg bw per day for males and 0, 4.3, 84.3, 348.9 and 1389.3 mg/kg bw per day for females, respectively), the NOAEL for parental toxicity was 1000 ppm (equal to 84.3 mg/kg bw per day), based on reduced body weight and body weight gain, increased serum GGT activity and increased relative kidney weights at 4000 ppm (equal to 348.9 mg/kg bw per day). The NOAEL for reproductive toxicity was 16 000 ppm (equal to 1389.3 mg/kg bw per day), the highest dose tested, as no reproductive effects were observed at any dose. The NOAEL for offspring toxicity was 1000 ppm (equal to 84.3 mg/kg bw per day), based on retarded growth in pups leading to a lower rate of F_{1b} pups per litter with pinna unfolding at 4000 ppm (equal to 348.9 mg/kg bw per day) (Hellwig & Gelbke, 1994a).

In a developmental toxicity study in rats using gavage dosing at 0, 100, 400 or 1000 mg/kg bw per day on days 6–15 of gestation, the NOAEL for maternal toxicity was 1000 mg/kg bw per day, the highest dose tested. The NOAEL for embryo and fetal toxicity was 400 mg/kg bw per day, on the basis of a slight increase in incidence of reduced ossification in fetuses at 1000 mg/kg bw per day (Hellwig & Gelbke, 1994b).

In a developmental toxicity study in rabbits using gavage dosing at 0, 100, 400 or 1000 mg/kg bw per day on days 7–19 of gestation, the NOAEL for maternal and embryo/fetal toxicity was 1000 mg/kg bw per day, the highest dose tested (Hellwig & Hildebrand, 1993).

The Meeting concluded that kresoxim-methyl is not teratogenic in rats or rabbits.

In an acute neurotoxicity study in rats administered a single oral kresoxim-methyl dose of 0, 500, 1000 or 2000 mg/kg bw by gavage, the NOAEL for systemic toxicity and neurotoxicity was 2000 mg/kg bw, the highest dose tested (Mellert, Kaufmann & Hildebrand, 1996a).

In a 90-day study of neurotoxicity in rats given diets containing kresoxim-methyl at a concentration of 0, 1000, 4000 or 16 000 ppm (equal to 0, 72, 292 and 1180 mg/kg bw per day for males and 0, 84, 341 and 1354 mg/kg bw per day for females, respectively), the NOAEL for neurotoxicity was 16 000 ppm (equal to 1180 mg/kg bw per day), the highest dose tested (Mellert, Kaufmann & Hildebrand, 1996b).

The Meeting concluded that kresoxim-methyl is not neurotoxic.

Toxicological data on metabolites and/or degradates

The acute oral toxicity of the rat and plant metabolites M1, M2 and M9 was investigated, and the LD_{50} was 2000 mg/kg bw or greater for all three metabolites (Kirsch & Hildebrand, 1994a,b, 1995). M1, M2, M9 and M15 are not genotoxic (Engelhardt, 1995; Engelhardt & Hoffmann, 1995a,b,c, 1996; Engelhardt & Leibold, 2005).

M9 is found in urine at over 10% of the dose and is derived from M1, and therefore their toxicity could be considered to be covered by the toxicity of the parent compound.

M2 is present at less than 6% in faeces and urine of rats. For chronic toxicity, the threshold of toxicological concern (TTC) approach (Cramer class III) could be applied for M2 and its conjugate, expressed as M2.

Human data

No adverse health effects suspected to be related to kresoxim-methyl exposure have been observed in persons handling crop protection products.

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

No information on accidental or intentional poisoning in humans was identified.

No epidemiological studies are available (BASF, 2018a,b).

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

Toxicological evaluation

The Meeting established an ADI of 0–0.3 mg/kg bw, derived from a $BMDL_{10}$ of 29.1 mg/kg bw per day from the 2-year chronic toxicity and carcinogenicity studies in rats, on the basis of liver tumours produced by a threshold-based mode of action. A safety factor of 100 was applied.

The Meeting concluded that the ADI could be applied to the metabolites M1 and M9 and their conjugates.

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

Levels relevant to risk assessment of kresoxim-methyl

Species	Study	Effect	NOAEL	LOAEL
Mouse	Eighteen-month study of toxicity and carcinogenicity[a]	Toxicity	2 000 ppm, equal to 304 mg/kg bw per day	8 000 ppm, equal to 1 305 mg/kg bw per day
		Carcinogenicity	8 000 ppm, equal to 1 305 mg/kg bw per day[b]	–
Rat	Two-year study of toxicity[a]	Toxicity	800 ppm, equal to 36 mg/kg bw per day	8 000 ppm, equal to 370 mg/kg bw per day
	Two-year study of carcinogenicity[a]	Toxicity	800 ppm, equal to 36 mg/kg bw per day	8 000 ppm, equal to 375 mg/kg bw per day
		Carcinogenicity	29.1 mg/kg bw per day ($BMDL_{10}$)	–
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	16 000 ppm, equal to 1389.3 mg/kg bw per day[b]	–
		Parental toxicity	1 000 ppm, equal to 84.3 mg/kg bw per day	4 000 ppm, equal to 348.9 mg/kg bw per day
		Offspring toxicity	1 000 ppm, equal to 84.3 mg/kg bw per day	4 000 ppm, equal to 348.9 mg/kg bw per day
	Developmental toxicity study[c]	Maternal toxicity	1 000 mg/kg bw per day[b]	–
		Embryo and fetal toxicity	400 mg/kg bw per day	1 000 mg/kg bw per day
	Acute neurotoxicity study[c]	Neurotoxicity	2 000 mg/kg bw[b]	–
	Ninety-day neurotoxicity study[a]	Neurotoxicity	1 180 mg/kg bw per day[b]	–

Species	Study	Effect	NOAEL	LOAEL
Rabbit	Developmental toxicity study[c]	Maternal toxicity	1 000 mg/kg bw per day[b]	–
		Embryo and fetal toxicity	1 000 mg/kg bw per day[b]	–
Dog	Ninety-day and 1-year studies of toxicity[a,d]	Toxicity	5 000 ppm, equal to 150 mg/kg bw per day	25 000 ppm, equal to 710 mg/kg bw per day

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

Acceptable daily intake (ADI) (applies to kresoxim-methyl, M1 and M9 and their conjugates, expressed as kresoxim-methyl)

0–0.3 mg/kg bw

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 kresoxim-methyl

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapidly, but incompletely, absorbed (~60%)
Dermal absorption	No data
Distribution	Widely distributed
Potential for accumulation	None
Rate and extent of excretion	In faeces (70% of the low dose and 80% of the high dose); in urine (about 20% of the low dose and 10% of the high dose)
Metabolism in animals	Extensive
Toxicologically significant compounds in animals and plants	Kresoxim-methyl
Acute toxicity	
Rat, LD_{50}, oral	>2 000 mg/kg bw
Rat, LD_{50}, dermal	>2 000 mg/kg bw
Rat, LC_{50}, inhalation	>5.263 mg/L
Rabbit, dermal irritation	Not irritating
Rabbit, ocular irritation	Not irritating
Guinea-pig, dermal sensitization	Not sensitizing (Buehler & maximization)

Short-term studies of toxicity	
Target/critical effect	Diarrhoea, vomiting, reduced body weight gain
Lowest relevant oral NOAEL	150 mg/kg bw per day (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	Liver; hepatotoxicity and tumours (rat)
Lowest relevant $BMDL_{10}$	29.1 mg/kg bw per day (rat)
Carcinogenicity	Not carcinogenic in mice, carcinogenic in rats[a]
Genotoxicity	
	No evidence of genotoxicity in vitro or in vivo[a]
Reproductive toxicity	
Target/critical effect	No reproductive effects
Lowest relevant parental NOAEL	84.3 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	84.3 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	1 389.3 mg/kg bw per day, highest dose tested (rat)
Developmental toxicity	
Target/critical effect	Reduced ossification (rat)
Lowest relevant maternal NOAEL	1 000 mg/kg bw per day, highest dose tested (rat and rabbit)
Lowest relevant embryo/fetal NOAEL	400 mg/kg bw per day (rat)
Neurotoxicity	
Acute neurotoxicity NOAEL	2 000 mg/kg bw, highest dose tested (rat)
Subchronic neurotoxicity NOAEL	1 180 mg/kg bw per day, highest dose tested (rat)
Developmental neurotoxicity NOAEL	No data

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

Summary

	Value	Study	Safety factor
ADI	0–0.3 mg/kg bw[a]	Two-year chronic toxicity and carcinogenicity studies in rats	100
ARfD	Unnecessary	–	–

[a] Applies to kresoxim-methyl, M1 and M9 and their conjugates, expressed as kresoxim-methyl.

References

BASF (2018a). Report on medical surveillance on manufacturing plant personnel. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

BASF (2018b). Kresoxim methyl – BASF replies to questions from WHO during the CXLs evaluation. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Cords SM, Lammer EM (2011a). BAS 490 F (kresoxim-methyl) – acute oral toxicity study in rats. Unpublished report no. 2010/1091718. Bioassay – Labor für biologische Analytik GmbH, Heidelberg, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Cords SM, Lammer EM (2011b). BAS 490 F (kresoxim-methyl) – acute dermal toxicity study in rats. Unpublished report no. 2010/1091719. Bioassay – Labor für biologische Analytik GmbH, Heidelberg, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Dubach-Powell JR, Kleinlogel H, Gisin M, Luetkemeier H, Weber K (1994). Effects on the vital functions of animals – general pharmacology of Reg. No. 242 009. Unpublished report no. 1994/11219. RCC – Research & Consulting Co. Ltd, Itingen, Switzerland. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Engelhardt G (1995). Study of Reg. No. 292 932 (ZHT test substance no.: 94/263) in the Ames *Salmonella*/mammalian-microsome mutagenicity test and *Escherichia coli*/mammalian-microsome reverse mutation assay (standard plate test and preincubation test). Unpublished report no. 1995/10026. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Engelhardt G, Hildebrand B (1994). Report on the study of Reg. No. 242009 (ZHT test substance no.: 91/180-2) in the Ames test (*Salmonella*/mammalian-microsome mutagenicity test – standard plate test and preincubation test). Unpublished report no. 1994/10753. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Engelhardt G, Hoffmann HD (1993a). Report on the study of Reg. No. 242 009 in the Ames *Salmonella*/mammalian-microsome mutagenicity test and *Escherichia coli*/mammalian-microsome reverse mutation assay (standard plate test and preincubation test). Unpublished report no. 1993/10249. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Engelhardt G, Hoffmann HD (1993b). Cytogenetic study in vivo of Reg. No. 242 009 in mice – micronucleus test – single intraperitoneal administration. Unpublished report no. 1993/11104. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Engelhardt G, Hoffmann HD (1995a). Report on the study of Reg. No. 262 451 (ZHT test substance no.: 94/520) in the Ames *Salmonella*/mammalian-microsome mutagenicity test and *Escherichia coli*/mammalian-microsome reverse mutation assay (standard plate test and preincubation test). Unpublished report no. 1995/10409. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Engelhardt G, Hoffmann HD (1995b). Study of Reg. No. 291685 (ZHT test substance no.: 94/262) in the Ames *Salmonella*/mammalian-microsome mutagenicity test and *Escherichia coli*/mammalian-microsome reverse mutation assay (standard plate test and preincubation test). Unpublished report no. 1995/10027. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Engelhardt G, Hoffmann HD (1995c). Amendment no. 1 to study of Reg. No. 291 685 (ZHT test substance no.: 94/262) in Ames *Salmonella*/mammalian-microsome mutagenicity test and *Escherichia coli*/mammalian-microsome reverse mutation assay (standard plate test and preincubation test). Unpublished report no. 1995/10482. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Engelhardt G, Hoffmann HD (1996). Report on the study of Reg. No. 339 774, BF 490-15 in the Ames *Salmonella*/mammalian-microsome mutagenicity test and *Escherichia coli*/mammalian-microsome reverse mutation assay (standard plate test and preincubation test). Unpublished report no. 1996/10353. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Engelhardt G, Leibold E (2005). The low pH 6.7 in vitro cell transformation assay with Reg. No. 262 451 (metabolite of BAS 490 F) in Syrian hamster embryo cells (SHE assay). Unpublished report no. 2005/1004865. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Gamer AO (1992). Study on the acute inhalation toxicity LC_{50} of Reg. No. 242 009 as a dust aerosol in rats – 4-hour exposure. Unpublished report no. 1992/10759. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Gamer AO, Hildebrand B (1995a). Study of foci initiating activity of Reg. No. 242 009 in Wistar rats. Unpublished report no. 1995/10244. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Gamer AO, Hildebrand B (1995b). Amendment no. 1 to the report: Study of foci initiating activity of Reg. No. 242 009 in Wistar rats. Unpublished report no. 1995/10567. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Gans G (1995a). Amendment no. 1 to the report: Study of the biokinetics of ^{14}C-Reg. No. 242 009 (BAS 490 .. F) in rats. Unpublished report no. 1995/10458. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Gans G (1995b). Study of the tissue distribution of ^{14}C-Reg. No. 242 009 (BAS 490 .. F) in rats. Unpublished report no. 1995/10543. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Gans G, Hildebrand B (1994). Study of the biokinetics of ^{14}C-Reg. No. 242 009 (BAS 490 F) in rats. Unpublished report no. 1994/10992. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Harada T (1995). Histopathological re-evaluation of liver tissue slides from a two year carcinogenicity study (project no. 70C0180/91006) and a two year chronic toxicity study (project no. 70C0180/91007) with Regd. No. 242 009 in Wistar rat. Unpublished report no. 94/10951. Institute of Environmental Toxicology, Kodaira, Tokyo, Japan. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Harada T, Kawakatsu H, Nagayoshi E (1997). BAS 490F (Reg. No. 242009): medium term promotion hepato-carcinogenesis study in rats. Unpublished report no. 97/10918. Institute of Environmental Toxicology, Kodaira, Tokyo, Japan. Submitted to WHO by BASF AG, Limbergerhof, Germany.

Hellwig J, Gelbke HP (1994a). Reproduction toxicity study with Reg. No. 242 009 in Wistar rats – continuous dietary administration over 2 generations (2 litters in the first and 1 litter in the second generation). Unpublished report no. 1994/10950. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Hellwig J, Gelbke HP (1994b). Study of the prenatal toxicity of Reg. No. 242 009 in Wistar rats after oral administration (gavage). Unpublished report no. 1994/10833. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Hellwig J, Hildebrand B (1993). Study of the prenatal toxicity of Reg. No. 242 009 in rabbits after oral administration (gavage). Unpublished report no. 1993/10980. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Hellwig J, Hildebrand B (1994). Report on the study of the toxicity of Reg. No. 242 009 in beagle dogs – administration via the diet over 12 months. Unpublished report no. 1994/10832. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Hildebrandt PK, Pappritz G, Harada T, Goodman DG, Hardisty JF, Kuttler K et al. (1995a). Pathology working group (PWG) report compound Reg. No. 242 009 – 24 month chronic toxicity study (project no. 70C0180/91007) in Wistar rats. Unpublished report no. 1995/10979. Pathco Inc., Ijamsville, MD, United States of America (USA). Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Hildebrandt PK, Pappritz G, Harada T, Goodman DG, Hardisty JF, Kuttler K et al. (1995b). Pathology working group (PWG) report compound Reg. No. 242 009 – 24-month carcinogenicity study (project no. 70C0180/91006) in Wistar rats. Unpublished report no. 1995/10977. Pathco Inc., Ijamsville, MD, USA. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Hildebrandt PK, Pappritz G, Harada T, Goodman DG, Hardisty JF, Kuttler K et al. (1995c). Pathology working group (PWG) report compound Reg. No. 242 009 – 24-month chronic toxicity (project no. 70C0180/91007) and carcinogenicity (project no. 70C0180/91006) studies in Wistar rats. Unpublished report no. 1995/10980. Pathco Inc., Ijamsville, MD, USA. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Hoffmann HD, Engelhardt G (1993). In vitro cytogenetic investigations of Reg. No.: 242 009 in human lymphocytes. Unpublished report no. 1993/10493. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Hoffmann HD, Engelhardt G (1997). Cytogenetic study in vivo with Reg. No. 242 009 in the rat micronucleus test – single intraperitoneal administration. Unpublished report no. 1997/10312. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Honarvar N (2002). Chromosome aberration assay in mouse spermatogonial cells with Reg. No. 242 009 – application: once, orally. Unpublished report no. 2002/1011621. RCC Cytotest Cell Research GmbH, Rossdorf, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Kamp H , Mellert W, Strauss V, Kuttler K, Fabian E, Van Ravenzwaay B (2008). BAS 490 F (kresoxim-methyl): carcinogenicity study in Wistar rats – CrlGlxBrlHan:Wl – administration via the diet over 24 months.

Unpublished report no. 2008/1028083. BASF SE, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Kirsch P, Hildebrand B (1993a). Study on the acute oral toxicity of Reg. No. 242 009 in rats. Unpublished report no. 1993/10730. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Kirsch P, Hildebrand B (1993b). Study on the acute dermal toxicity of Reg. No. 242 009 in rats. Unpublished report no. 1993/11108. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Kirsch P, Hildebrand B (1994a). Study of the dermal toxicity of Reg. No. 242 009 in Wistar rats – application to the intact skin over 3 weeks (21 applications). Unpublished report no. 1994/11070. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Kirsch P, Hildebrand B (1994a). Study on the acute oral toxicity of Reg. No. 291 685 in rats. Unpublished report no. 1994/11172. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Kirsch P, Hildebrand B (1994b). Study on the acute oral toxicity of Reg. No. 292 932 in rats. Unpublished report no. 1994/11171. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Kirsch P, Hildebrand B (1995). Study on the acute oral toxicity of Reg. No. 262 451 in rats. Unpublished report no. 1995/10213. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Kohl W (1994). The metabolism of ^{14}C-BAS 490 F (^{14}C-Reg. No. 242 009) in rats. Unpublished report no. 1994/10981. BASF AG Agrarzentrum Limburgerhof, Limburgerhof, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Kohl W (1995). Addendum no. 1 to report: The metabolism of ^{14}C-BAS 490 F (^{14}C-Reg. No. 242 009) in rats. Unpublished report no. 1995/10366. BASF AG Agrarzentrum Limburgerhof, Limburgerhof, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Kohl W (1998). Addendum no. 2 to study code P90-M009: The metabolism of ^{14}C-BAS 490 F (^{14}C-Reg. No. 242 009) in rats. Unpublished report no. 1998/11159. BASF AG Agrarzentrum Limburgerhof, Limburgerhof, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W, Hildebrand B (1994a). Subchronic oral toxicity study with Reg. No. 242 009 in C 57 BL mice – administration in the diet for 3 months. Unpublished report no. 1994/10495. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W, Hildebrand B (1994b). Amendment no. 1 to the report: Subchronic oral toxicity study with Reg. No. 242 009 in C57BL mice – administration in the diet for 3 months. Unpublished report no. 1994/10942. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W, Hildebrand B (1994c). Subchronic toxicity study with Reg. No. 242 009 in Wistar rats – administration in the diet over 3 months. Unpublished report no. 1994/10954. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W, Hildebrand B (1994d). Report on the study of the toxicity of Reg. No. 242 009 in beagle dogs – administration via the diet over 3 months. Unpublished report no. 1994/10834. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W, Hildebrand B (1994e). Carcinogenicity study with Reg. No. 242 009 in C57BL mice – administration in the diet for 18 months. Unpublished report no. 1994/10919. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W, Hildebrand B (1994f). Chronic toxicity study with Reg. No. 242 009 in Wistar rats – administration in the diet for 24 months. Unpublished report no. 1994/10951. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W, Hildebrand B (1994g). Carcinogenicity study with Reg. No. 242 009 in Wistar rats – administration in the diet for 24 months. Unpublished report no. 1994/10953. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W, Hildebrand B (1995a). Amendment no. 1 to the report: Chronic toxicity study with Reg. No. 242 009 in Wistar rats – administration in the diet for 24 months. Unpublished report no. 1995/10810. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W, Hildebrand B (1995b). Amendment no. 1 to the report: Carcinogenicity study with Reg. No. 242 009 in Wistar rats – administration in the diet for 24 months. Unpublished report no. 1995/11544. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W, Hildebrand B (1995c). Amendment no. 2 to the report: Carcinogenicity study with Reg. No. 242 009 in Wistar rats – administration in the diet for 24 months. Unpublished report no. 1995/10811. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W, Hildebrand B (1995d). Test study on enzyme activity after treatment with Reg. No. 242 009 in Wistar rats – dietary administration for 3 weeks and recovery of 2 weeks. Unpublished report no. 1995/10853. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W, Bahnemann R, Hildebrand B (1996). Reg. No. 242009 – S phase response study in male Wistar rats including reversibility – administration in the diet up to 13 weeks. Unpublished report no. 96/10053. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Limbergerhof, Germany.

Mellert W, Bahnemann R, Hildebrand B (1997). S-phase-response study with BAS 490 .. F (Reg. No. 242 009) in Wistar rats after administration in the diet for 3 weeks (supplementary study to project no. 83M0180/910149). Unpublished report no. 1997/10318. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W, Decardt K, Hildebrand B (1997). BAS 490..F (Reg. No. 242 009) – study of enzyme excretion in urine of Wistar rats after repeated administration in the diet. Unpublished report no. 1997/10317. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W, Kaufmann W, Hildebrand B (1995a). Reg. No. 242 009 – electron microscopic examinations of liver samples to assess mitochondria from old Wistar rats treated for 3 weeks in the diet. Unpublished report no. 1995/11105. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W, Kaufmann W, Hildebrand B (1995b). Reg. No. 242 009 – electron microscopic examinations of liver samples to assess peroxisomes from Wistar rats treated for 3 weeks in the diet. Unpublished report no. 1995/11106. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W, Kaufmann W, Hildebrand B (1996a). Reg. No. 242 009 – Acute oral neurotoxicity study in Wistar rats. Unpublished report no. 1996/10410. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W, Kaufmann W, Hildebrand B (1996b). Reg. No. 242 009 – Subchronic oral neurotoxicity study in Wistar rats – administration in the diet for 3 months. Unpublished report no. 1996/10411. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W, Decardt K, Leibold E, Hildebrand B (1996a). Reg. No. 242 009 – examination of enzyme activities in the liver of Wistar rats – administration in the diet for 3 weeks. Unpublished report no. 1996/10100. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W, Decardt K, Kittel B, Hildebrand B (1996b). Kresoxim-methyl (Reg. No. 242 009): pilot study – dietary administration to rats after repeated pretreatment with dimethoate. Unpublished report no. 1996/10246. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Moss DW (1994). Effects of Reg. No. 242 009 on enzyme levels in rat serum. Unpublished report no. 1994/10578. Royal Postgraduate Medical School, Hammersmith Hospital, London, United Kingdom. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Pappritz G (1995a). Amendment: BASF project no. 70C0180/91006, Reg. No. 242 009 – 24-month carcinogenicity study in rats. Unpublished report no. 1995/10686. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Polloth C, Hildebrand B (1994a). S-phase-response study with Reg. No. 242 009 in Wistar rats after administration in the diet for 3 weeks. Unpublished report no. 1994/10922. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Polloth C, Hildebrand B (1994b). S-phase response study with Reg. No. 242 009 in 16-month old Wistar rats after administration in the diet for 3 weeks. Unpublished report no. 1994/10984. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Polloth C, Hildebrand B (1994c). Report: ex vivo unscheduled DNA synthesis (UDS) assay and S phase response in rat hepatocytes with Reg. No. 242009. Unpublished report no. 94/10867. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Limbergerhof, Germany.

Polloth C, Hildebrand B (1994d). Amendment to the report: S-phase-response study with Reg. No. 242 009 in Wistar rats after administration in the diet for 3 weeks. Unpublished report no. 1994/11177. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Polloth C, Hoffmann HD (1994a). Gene mutation test in Chinese hamster ovary cells (HPRT locus assay) with Reg. No. 242 009. Unpublished report no. 1994/10350. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Polloth C, Hoffmann HD (1994b). In vitro – unscheduled DNA synthesis (UDS) assay in rat hepatocytes with Reg. No. 242 009. Unpublished report no. 1994/10351. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Polloth C, Hoffmann HD (1994c). Ex vivo – unscheduled DNA synthesis (UDS) assay and S-phase-response in rat hepatocytes with Reg. No. 242 009. Unpublished report no. 1994/10867. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Polloth C, Hoffmann HD (1994d). Ex vivo – unscheduled DNA synthesis (UDS) assay in rat hepatocytes with Reg. No. 242 009 after administration in the diet for 3 weeks. Unpublished report no. 1994/10894. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Rossbacher R (1992a). Study on the acute dermal irritation/corrosion of Reg. No. 242 009 in the rabbit. Unpublished report no. 1992/11663. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Rossbacher R (1992b). Study on the acute eye irritation of Reg. No. 242 009 in the rabbit. Unpublished report no. 1992/11664. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Rossbacher R (1993). Report on the maximization test for the sensitizing potential of Reg. No. 242 009 in guinea pigs. Unpublished report no. 1993/10014. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Schilling K, Hildebrand B (1992a). Study on the oral toxicity of Reg. No. 242 009 in B6C3F1 mice – administration in the diet over 4 weeks (range-finding study). Unpublished report no. 1992/10539. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Schilling K, Hildebrand B (1992b). Study on the oral toxicity of Reg. No. 242 009 in Wistar rats – administration in the diet over 4 weeks (range-finding). Unpublished report no. 1992/10551. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Schulz M, Landsiedel R (2011). BAS 490 F (kresoxim-methyl) – micronucleus test in bone marrow cells of the mouse. Unpublished report no. 2011/1174047. BASF SE, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Schwindt KR, Landsiedel R (2008). The low pH 6.7 cell transformation assay with BAS 490 F (kresoxim-methyl) in Syrian hamster embryo cells (SHE assay). Unpublished report no. 2007/1044609. BASF SE, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Van Ravenzwaay B (1996). Kresoxim-methyl: mechanism and assessment of liver tumor induction. Unpublished report no. 96/10078. BASF AG, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Limbergerhof, Germany.

Weiss-Fuchs U (2011). BAS 490 F (kresoxim-methyl) – skin sensitisation study (Buehler test). Unpublished report no. 2010/1091722. Seibersdorf Labor GmbH, Seibersdorf, Austria. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Whitby BR (1993). (^{14}C)-Reg. No. 242 009: quantitative whole-body autoradiography following oral administration to the rat. Unpublished report no. 1993/11013. Hazleton UK, Harrogate, North Yorkshire United Kingdom. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Wittmer E, Landsiedel R (2011). BAS 490 F (kresoxim-methyl) – acute inhalation toxicity study in Wistar rats – 4-hour dust aerosol exposure (head-nose only). Unpublished report no. 2011/1150078. BASF SE, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Woitkowiak C (2011). BAS 490 F (kresoxim-methyl) – *Salmonella typhimurium* / *Escherichia coli* – reverse mutation assay. Unpublished report no. 2011/1135894. BASF SE, Ludwigshafen/Rhein, Germany. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Wolf T (2011a). BAS 490 F (kresoxim-methyl) – acute dermal irritation/corrosion in rabbits. Unpublished report no. 2010/1091721. Seibersdorf Labor GmbH, Seibersdorf, Austria. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Wolf T (2011b). BAS 490 F (kresoxim-methyl) – acute eye irritation/corrosion in rabbits. Unpublished report no. 2010/1091720. Seibersdorf Labor GmbH, Seibersdorf, Austria. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Yamamoto T (1994). Report: Study on the acute oral toxicity of Reg. No. 242009 in mice. Unpublished report no. 94. Biosafety Research Center, Foods, Drugs and Pesticides, Shizuoka, Japan. Submitted to WHO by BASF AG, Limbergerhof, Germany.

LAMBDA-CYHALOTHRIN (addendum)

First draft prepared by
Esther de Jong[1] and Angelo Moretto[2]

[1] Dutch Board for the Authorisation of Plant Protection Products and Biocides, Ede, the Netherlands
[2] Department of Biomedical and Clinical Sciences, University of Milan, and International Centre for Pesticides and Health Risk Prevention, ASST Fatebenefratelli Sacco, Milan, Italy

Explanation....279
Evaluation for acceptable intake....280
1. Biochemical aspects....280
1.1 Absorption and excretion....280
1.2 Biotransformation....281
2. Toxicological studies....285
2.1 Short-term studies of toxicity....285
(a) Dermal application....285
(b) Exposure by inhalation....286
2.2 Genotoxicity....287
2.3 Special studies....287
(a) Developmental neurotoxicity....287
(b) Studies on metabolites and impurities....288
3. Observations in humans....290
Comments....291
Toxicological evaluation....292
References....292

Explanation

Lambda-cyhalothrin was previously evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 2000, 2004 and 2007. In the 2007 evaluation, an acceptable daily intake (ADI) of 0–0.02 mg/kg body weight (bw) and an acute reference dose (ARfD) of 0.02 mg/kg bw were established.

Following a request for additional maximum residue levels by the Codex Committee on Pesticide Residues, lambda-cyhalothrin was placed on the agenda of the present Meeting, which assessed additional toxicological information available since the last review.

The new studies with lambda-cyhalothrin consisted of a biliary elimination and biotransformation study, a 21-day dermal toxicity study, a 21-day inhalation toxicity study, two bacterial gene mutation studies and a preliminary developmental neurotoxicity study.

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

Evaluation for acceptable intake

1. Biochemical aspects

1.1 Absorption and excretion

A new biliary elimination and biotransformation (see section 1.2) study (Tomlinson, 2011) was carried out with lambda-cyhalothrin (batch no. 200911001; purity 99.5%). Groups of 4–5 male and 3–4 female bile duct–cannulated Han Wistar rats were administered cyclopropyl- or phenoxy-labelled [^{14}C]lambda-cyhalothrin at a single oral dose of 1 or 12.5 mg/kg bw. To characterize the absorption and excretion of total radioactivity, urine, bile and faeces were collected at intervals for up to 4 days. Urine was collected before dosing, at 0–8 and 8–12 hours and then at daily intervals until termination. Faeces and cage wash were collected before dosing and then at daily intervals. Bile was collected before dosing and at 0–0.5, 0.5–1, 1–2, 2–4, 4–8, 8–12, 12–24, 24–30, 30–48, 48–72 and 72–96 hours after dosing.

The per cent absorption of radioactivity over 48 hours after dosing was estimated from the radioactivity recovered from bile duct–cannulated rats in urine, bile, cage wash and carcass (Tables 1 and 2). Absorption of radioactivity following administration of [phenoxy-^{14}C]lambda-cyhalothrin was approximately 2- to 3-fold higher than absorption following administration of [cyclopropyl-^{14}C]lambda-cyhalothrin. In addition, with [phenoxy-^{14}C]lambda-cyhalothrin, mean absorption at the high dose of 12.5 mg/kg bw (11–12%) was around half that at the low dose of 1 mg/kg bw (19–24%), indicating that saturation occurred with increasing doses.

Table 1. Absorption of radioactivity following administration of [cyclopropyl-^{14}C]lambda-cyhalothrin to rats

	Absorption (% of radioactive dose)			
	1 mg/kg bw		**12.5 mg/kg bw**	
Matrix	**Males (*n* = 4)**	**Females (*n* = 3)**	**Males (*n* = 4)**	**Females (*n* = 3)**
Faeces (0–96 h)	93	93	94	95
Urine (0–96 h)	0.8	1.5	1.2	1.0
Bile (0–96 h)	9.6	6.3	6.3	5.5
Cage wash	0.3	0.1	0.6	0.2
Carcass	0.6	0.3	0.5	<0.1
Gastrointestinal tract	0.1	<0.1	<0.1	<0.1
Total recovery	105	101	103	102
% absorbed	11	8.2	8.3	6.9

bw: body weight; *n*: number
Source: Tomlinson (2011)

The majority of the administered dose was found in the faeces of both male and female rats, with 93–95% for the low and high doses of [cyclopropyl-^{14}C]lambda-cyhalothrin and 83–90% for the low and high doses of [phenoxy-^{14}C]lambda-cyhalothrin. The majority of the administered radioactivity (>90%) was excreted by 48 hours post-dosing. There was 1.2% or less radioactivity remaining in the carcass or gastrointestinal tract in all treatment groups, indicating that excretion was essentially complete by 96 hours post-dosing (Tomlinson, 2011).

Table 2. Absorption of radioactivity following administration of [phenoxy-^{14}C]lambda-cyhalothrin to rats

	Absorption (% of radioactive dose)			
	1 mg/kg bw		12.5 mg/kg bw	
Matrix	**Males (*n* = 5)**	**Females (*n* = 3)**	**Males (*n* = 4)**	**Females (*n* = 4)**
Faeces (0–96 h)	84	83	88	90
Urine (0–96 h)	9.7	8.1	5.8	6.7
Bile (0–96 h)	6.3	12	3.3	3.3
Cage wash	1.7	2.5	1.4	1.1
Carcass	1.0	1.2	0.4	0.5
Gastrointestinal tract	0.1	0.1	<0.1	0.1
Total recovery	103	107	99	101
% absorbed	19	24	11	12

bw: body weight; *n*: number
Source: Tomlinson (2011)

1.2 Biotransformation

The nature and identity of metabolites present in samples of urine, bile and faeces of rats collected in the above study (Tomlinson, 2011) were determined by thin-layer chromatography. The structures of the metabolites found are shown in Table 3.

Table 3. Molecular structure of metabolites found in rat

Component name	**Structure**	**Matrices**
R119321 Lambda-cyhalothrin		Faeces
R211133 Compound XV		Bile
R119890 Compound 1a		Bile

Component name	Structure	Matrices
R173948 Compound XI		Bile Urine
R110649 Compound IV		Bile
R41207 Compound V		Urine Bile
R175447 Compound XXIII		Urine Bile
R633325 Compound XIII		Urine
R231173 Compound XIX		Bile

Source: Tomlinson (2011)

The majority of the administered dose was excreted via faeces as lambda-cyhalothrin (R119321), reflecting the unabsorbed parent, which represented 77–91% of the dose. No individual unconjugated metabolite accounted for more than 1% of the administered dose in urine or bile samples prior to hydrolysis. In urine and bile samples from rats administered [cyclopropyl-^{14}C]lambda-cyhalothrin, the components detected were identified as R119890 (0.3–0.5% of the dose), R173948 (up to 0.1%) and R211133 (<0.1%). Several unidentified components were detected, none of which exceeded 0.1% of the dose individually. In urine and bile samples from rats administered [phenoxy-^{14}C]lambda-cyhalothrin, the most abundant metabolites were identified as R41207 (0.4–1.0% of the dose) and R175447 (0.1–0.4%). R110649 and R211133 (0.1–0.3% of the dose) were quantified as a single component, as they could not be separated in solvent system 1. Metabolite R633325 accounted for up to 0.2% of the dose. No significant differences were observed between male and female rats. For each radiolabel, metabolites detected in urine, bile and faeces were qualitatively and quantitatively similar. No significant differences were observed following administration of a single dose of 1 or 12.5 mg/kg bw to male or female rats.

A summary of the metabolites detected in excreta subjected to enzyme hydrolysis is presented in Tables 4 and 5. Metabolites present in samples hydrolysed with glucuronidase and sulfatase were consistent with free metabolites detected in unhydrolysed samples. The quantitative differences between samples before and after hydrolysis indicated that metabolites were excreted as conjugates. Increased amounts of R173948 (0.2–0.3% of the dose; likely a glucuronide conjugate), R119890 (1.4–2.1% of the dose; likely a glucuronide conjugate) and R211133 (0.8–1.2% of the dose; likely a sulfate conjugate) were observed following hydrolysis of bile samples obtained after administration of [cyclopropyl-^{14}C]lambda-cyhalothrin to bile duct–cannulated rats at 1 mg/kg bw with glucuronidase and sulfatase. On hydrolysis of urine and bile samples obtained from rats administered [phenoxy-^{14}C]lambda-cyhalothrin at a dose of 1 mg/kg bw, substantial increases in the amounts of R175447 (7.5–9.3% of the dose; likely a sulfate conjugate) and R211133 (1.8–3.2% of the dose; likely a sulfate conjugate) were observed. Minor increases in the amounts of free R633325 and R41207 were also seen. Hydrolysis also released minor amounts of R231173, which was not seen in unhydrolysed samples.

Table 4. Summary of metabolites in bile (0–24 hours) subjected to enzyme hydrolysis following administration of [cyclopropyl-^{14}C]lambda-cyhalothrin to bile duct–cannulated rats in a single oral dose of 1 mg/kg bw

	% of administered dose			
	Males		Females	
Compound	Glucuronidase	Glucuronidase and sulfatase	Glucuronidase	Glucuronidase and sulfatase
R173948	0.20	0.31	0.17	0.15
R119890	2.13	1.99	1.49	1.38
R211133	1.22	0.97	1.09	0.77
Remainder[a]	0.88	1.03	0.45	0.55
Origin[b]	3.32	3.44	2.64	2.98
Post-extraction solids	0.17	0.17	0.10	0.10
Total identified	3.55	3.27	2.75	2.30
Total unidentified	4.20	4.47	3.09	3.53
Total accounted for	7.92	7.91	5.94	5.93
Losses/gains	0.00	0.01	0.00	0.01
Total	7.92	7.92	5.94	5.94

[a] Remainder comprises diffuse areas of radioactivity within the chromatogram that cannot be assigned to discrete radioactive components.
[b] Origin refers to unidentified polar material not eluted from the baseline of the thin-layer chromatogram.
Source: Tomlinson (2011)

The biotransformation of lambda-cyhalothrin in rats proceeds via ester cleavage of the parent molecule to yield R119890 and R116795 (a postulated intermediate that was not observed in the study; Fig. 1). R119890 is subsequently hydroxylated to R173948, both of which are excreted as free metabolites and conjugates that are likely to be glucuronides. R116795 can be oxidized to give R110649, R41207 and R175447. Hydrolysis of the cyano group may also occur, giving R633325. All metabolites arising from the biotransformation of the phenoxy half of the molecule are excreted as both free and conjugated metabolites. Hydroxylation of the intact molecule to yield R211133 is also observed. Ester cleavage of R211133 gives R119890 and intermediates leading to R231173 and R175447. These metabolites were all excreted as conjugates, which are likely to be sulfates. The most abundant metabolites of lambda-cyhalothrin were R119890 (up to 2.1% of the dose in bile), R175447

(up to 9.3% of the dose in urine and bile) and R211133 (up to 3.2% of the dose in bile), which are mainly excreted as conjugates (Tomlinson, 2011).

Table 5. Summary of metabolites in urine (0–48 hours) and bile (0–24 hours) subject to enzyme hydrolysis following administration of [phenoxy-^{14}C]lambda-cyhalothrin to bile duct–cannulated rats in a single oral dose of 1 mg/kg bw

	% of administered dose					
	Males			Females		
	Urine	Bile		Urine	Bile	
Compound	**Glucuronidase**	**Glucuronidase**	**Glucuronidase and sulfatase**	**Glucuronidase**	**Glucuronidase**	**Glucuronidase and sulfatase**
R633325[a]	0.26	ND	ND	0.18	ND	ND
R175447	6.84	0.58	0.70	5.89	3.16	3.36
R231173	ND	ND	0.32	ND	ND	0.31
R41207	0.81	0.40	0.55	0.65	1.25	1.65
R211133	ND	1.83	1.37	ND	3.16	2.48
Unidentified (Rf 0.09)	ND	ND	ND	ND	ND	0.17
Unidentified (Rf 0.11)	0.30	ND	ND	0.22	ND	ND
Remainder[b]	0.00	1.41	1.24	0.00	1.39	0.61
Origin[c]	0.93	0.99	1.03	0.88	2.35	2.75
Post-extraction solids	0.22	0.10	0.10	0.10	0.23	0.23
Total identified	7.91	2.81	2.94	6.72	7.57	7.80
Total unidentified	1.23	2.40	2.27	1.10	3.71	3.53
Total accounted for	9.36	5.31	5.31	7.92	11.54	11.56
Losses/gains	0.01	0.01	0.01	0.01	0.00	−0.02
Total	9.37	5.32	5.32	7.93	11.54	11.54

ND: not detected; Rf: retention factor

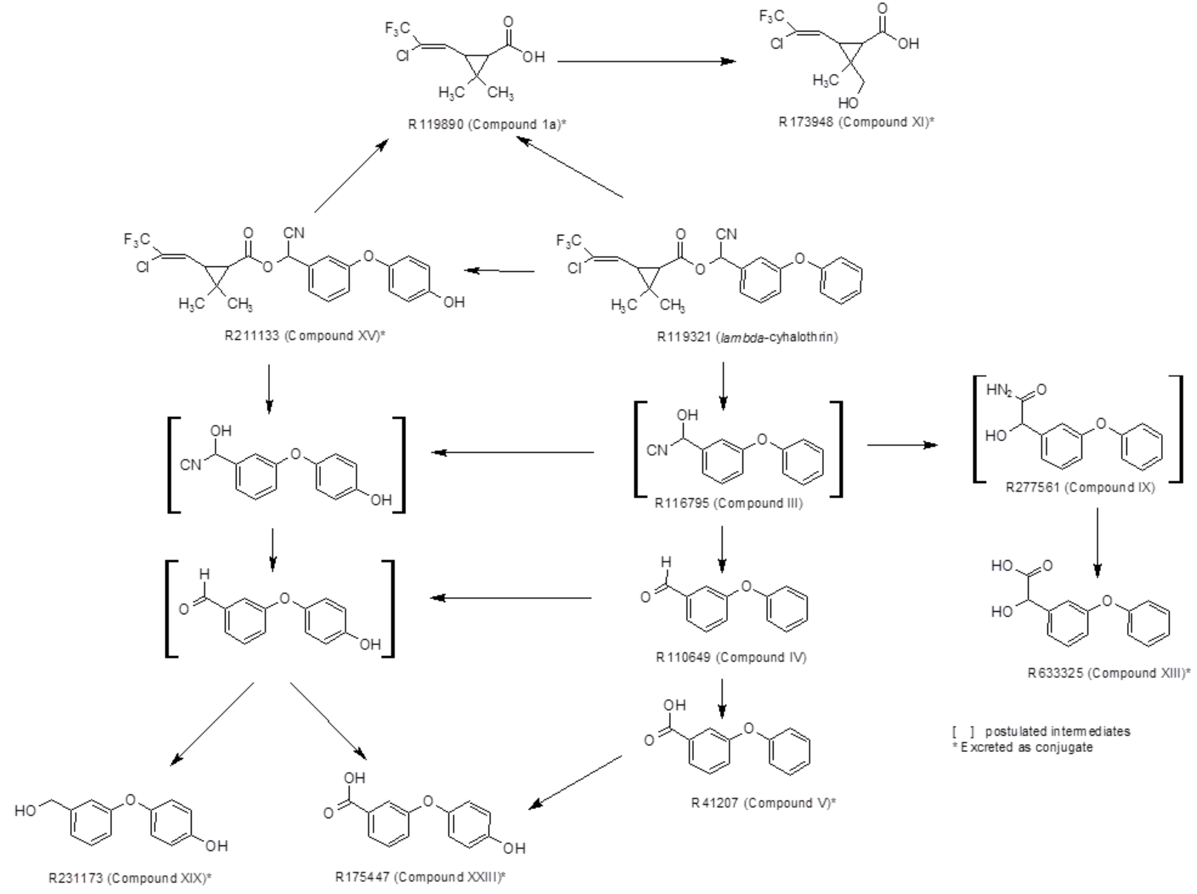
[a] Component may also contain R277561, but its presence could not be confirmed in any solvent system used; therefore, it is quantified as R633325 only.

[b] Remainder comprises diffuse areas of radioactivity within the chromatogram that cannot be assigned to discrete radioactive components.

[c] Origin refers to unidentified polar material not eluted from the baseline of the thin-layer chromatogram.

Source: Tomlinson (2011)

Fig. 1. Biotransformation pathways based on identified metabolites of lambda-cyhalothrin



Source: Tomlinson (2011)

2. Toxicological studies

2.1 Short-term studies of toxicity

(a) Dermal application

Rats

In a dermal toxicity study, groups of five male and five female Alpk:APfSD rats were given 6-hour dermal applications of lambda-cyhalothrin (batch no. P13; purity 96.6%) at a dose of 0, 1, 10 or 100 mg/kg bw per day (reduced to 50 mg/kg bw per day after two or three applications) for 21 consecutive days. Animals were observed twice daily, prior to dosing and at decontamination, for signs of mortality, toxicity and the presence of dermal irritation. Feed consumption per rat was estimated for a 24-hour period between days −1 and 1, days 6 and 7, days 13 and 14 and days 20 and 21. After termination, blood samples were taken for haematological and clinical chemistry evaluation. The weights of adrenal glands, brain, kidneys, ovaries, testes and liver were measured. Histopathological evaluation was carried out on the adrenal glands, brain, kidneys, liver, sciatic nerve, spinal cord, skin and spleen.

Two males receiving 100 mg/kg bw per day were found dead on day 4 of the study. The remaining animals were subsequently dosed with 50 mg/kg bw per day. There were no mortalities in the other groups. Clinical signs, including bizarre behaviour, reduced stability, dehydration and reduced splay reflex, were observed in the high-dose group. There were no significant signs of skin irritation at any dose. There was no significant difference in body weight gain between control animals and those dosed with 1 mg/kg bw per day. Males dosed with 10 mg/kg bw per day showed a significantly lower body weight gain compared with controls (−23%) until day 13. This recovered

despite continued treatment, and final body weight was within 4% of control values. Surviving males dosed at 50 mg/kg bw per day showed a significantly lower body weight gain compared with controls throughout the study, which resulted in a 19% reduction in final body weight at day 21 compared with controls. Females in this group were less severely affected, but did show a lower body weight gain for the first half of the study compared with controls. Feed consumption was slightly reduced in high-dose males (−14%). There were no treatment-related differences in haematological parameters, clinical chemistry, organ weights or histopathological findings.

The no-observed-adverse-effect level (NOAEL) was 10 mg/kg bw per day, based on the clinical signs and body weight effects at 50 mg/kg bw per day. No indication of skin irritation was observed in the study (Leah, 1989).

(b) Exposure by inhalation

In an inhalation toxicity study, groups of 10 male and 10 female Alpk:APfSD rats were exposed nose-only to lambda-cyhalothrin (batch no. ADH 553 225 367; purity 81.5%) for 6 hours/day, 5 days/week, for 21 days. The mean particulate concentrations measured gravimetrically throughout the study were 0, 0.3, 3.3 and 16.7 µg/L lambda-cyhalothrin. Clinical signs were observed during and following exposure and also daily on non-exposure days. Individual body weights were recorded prior to exposure on days 1, 2 and 3 (males), days 1, 2 and 5 (females) and days 7 and 15 (both sexes). Feed consumption was measured at the same time as body weights. Ophthalmoscopic examination was carried out prior to exposure and on day 21. Urine analysis was carried out on samples collected from five males and five females at day 20. After termination, blood samples were taken for haematological and clinical chemistry evaluation. Organ weights were measured, and histopathological evaluation was performed.

The test atmospheres had study mean mass median aerodynamic diameters of 1.91, 1.48 and 1.47 µm, respectively, for the low, intermediate and high doses, and respective geometric standard deviations of 2.24, 1.82 and 1.68.

No mortality was observed. Clinical signs, including salivation and lacrimation, were observed in animals exposed to 3.3 and 16.7 µg/L lambda-cyhalothrin. In addition, paw flicking and tail erections were observed at these doses. Body weight gain was reduced throughout the study in both sexes at 16.7 µg/L lambda-cyhalothrin compared with controls, final body weights being 15% and 12% below control values in males and females, respectively. Effects of a similar nature, although smaller in magnitude, were seen throughout the study at 3.3 µg/L lambda-cyhalothrin; final weights were 5% and 6% below control values in males and females, respectively. Males and females exposed to 16.7 µg/L lambda-cyhalothrin had statistically significantly reduced feed consumption up to day 18 and a slight reduction in feed consumption between days 18 and 22. Reduced feed consumption was also seen in males exposed to 3.3 µg/L lambda-cyhalothrin during the first week of the study. Ophthalmoscopic examination revealed a dose-related increase in the incidence of punctate foci on the cornea in males and females exposed to 3.3 and 16.7 µg/L lambda-cyhalothrin.

There were slight increases in the plasma aspartate transaminase (+19%) and alkaline phosphatase activities (+22%) of females exposed to 16.7 µg/L lambda-cyhalothrin. Small reductions were seen in the plasma cholesterol levels of females at 3.3 and 16.7 µg/L lambda-cyhalothrin (−16% and −20%, respectively) and in the plasma triglyceride levels of males exposed to 16.7 µg/L lambda-cyhalothrin (−19%). Urine volume was statistically significantly reduced and specific gravity slightly raised in both sexes exposed to 3.3 and 16.7 µg/L lambda-cyhalothrin. There were reductions in protein levels of males exposed to 3.3 and 16.7 µg/L lambda-cyhalothrin and of females exposed to 16.7 µg/L lambda-cyhalothrin. A statistically significant increase in relative liver weight was observed in mid- and high-dose males; however, the effect remained slight, with a 7% increase in mid-dose males and a 12% increase in high-dose males, and no histopathological findings were observed in the liver.

The NOAEL was 0.3 µg/L lambda-cyhalothrin, based on the observed clinical signs and ophthalmoscopic findings at 3.3 µg/L lambda-cyhalothrin (Hext, 1990).

2.2 Genotoxicity

The genotoxicity of lambda-cyhalothrin was previously evaluated in 2007. It was tested in a range of guideline-compliant assays, including an Ames test, an in vitro mammalian gene mutation test, an in vitro chromosomal aberration study, an in vitro unscheduled DNA synthesis test and an in vivo micronucleus assay. The results of all studies were concluded to be negative. Two additional Ames tests were provided to the current Meeting (Callander & Priestley, 1989; Sokolowski, 2012). The results of these two new studies are in line with the previous evaluation. The results of the genotoxicity tests, including those previously evaluated in 2007, are summarized in Table 6.

Table 6. Overview of genotoxicity with lambda-cyhalothrin[a,b]

End-point	Test object	Concentration	Purity (%)	Results	Reference[c]
In vitro					
Reverse mutation	*Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538	1.6–5 000 µg/plate (±S9)	96.5	Negative	Callander (1984)
Reverse mutation	*S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538	1.6–5 000 µg/plate (±S9)	81.5	Negative	Callander & Priestley (1989)*
Reverse mutation	*S. typhimurium* strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* WP2 *uvr*A and WP2 *uvr*A (pKM101)	3–5 000 µg/plate (±S9)	90.5	Negative	Sokolowski (2012)*
Gene mutation	Mouse lymphoma L5178Y$Tk^{+/-}$	125–4 000 µg/mL (±S9)	96.6	Negative	Cross (1985)
Chromosomal aberration	Human lymphocytes	10^{-9}–10^{-2} mol/L (±S9)	96.5	Negative	Sheldon, Howard & Richardson (1985)
Unscheduled DNA synthesis	Rat primary hepatocytes	17–5 000 µg/mL	96.6	Negative	Trueman (1989)
In vivo					
Micronucleus formation	Mouse bone marrow	22 and 35 mg/kg bw (intraperitoneal)	96.5	Negative	Sheldon et al. (1984)[d]

bw: body weight; DNA: deoxyribonucleic acid; GLP: good laboratory practice; NCE: normochromatic erythrocytes; PCE: polychromatic erythrocytes; S9: 9000 × *g* supernatant fraction from rat liver homogenate

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

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

[c] Newly submitted studies are marked with an asterisk (*). All other studies were evaluated at the 2007 JMPR.

[d] Doses were 44% and 70% of the median lethal dose at 7 days (based on mortality observed within 7 days after a single intraperitoneal injection of lambda-cyhalothrin). A reduction in the PCE/NCE ratio indicated that lambda-cyhalothrin had reached the bone marrow.

2.3 Special studies

(a) Developmental neurotoxicity

In the JMPR evaluation from 2007, a developmental neurotoxicity study (Milburn, 2004) was reported. The NOAEL for maternal toxicity was 60 parts per million (ppm) (equal to 4.9 mg/kg bw per day), on the basis of reduced body weight gain during gestation. The NOAEL for offspring

toxicity was 60 ppm (equal to 10.7 mg/kg bw per day), on the basis of reduced body weight gain during lactation. No evidence for developmental neurotoxicity was observed.

A preliminary dose range–finding developmental neurotoxicity study is also available that has not been previously reported and is therefore included below.

In the preliminary dose range–finding developmental neurotoxicity study, groups of 10 time-mated female Alpk:APfSD (Wistar-derived) rats received lambda-cyhalothrin (batch no. P31 [BX E624] R119321; purity 87.7%) in the diet at a concentration of 0, 25, 60 or 150 ppm (equal to 0, 2.0, 4.7 and 10.7 mg/kg bw per day during gestation and 0, 4.0, 9.4 and 22.7 mg/kg bw per day during lactation, respectively). The dosing period commenced on gestation day 7 and finished on lactation day 22. The dams were allowed to rear the ensuing litters to lactation day 22. Satellite groups of six time-mated female rats were also fed diets containing 25, 60 or 150 ppm lambda-cyhalothrin. The following end-points were evaluated for maternal animals: clinical condition, body weight and feed consumption daily throughout gestation and lactation. The following end-points were measured for the offspring: clinical condition, body weight (days 1, 5, 8, 11, 15 and 22 postpartum), number and sex. Blood samples were collected from the maternal animals and pups in the satellite groups on gestation days 8, 15 and 22 and on lactation days 1, 5 and 11, and plasma levels of lambda-cyhalothrin were determined.

Maternal animals in the 150 ppm group had statistically significantly lower (−6%) body weights during gestation, and feed consumption was also lower during this phase. On postpartum day 1, body weights of male and female pups were statistically significantly lower in the 150 ppm group, but the growth of these pups thereafter was not different from that of control animals. All other end-points evaluated during the in-life phase were unaffected by treatment (e.g. clinical condition, body weights and feed consumption during lactation, pup survival and clinical condition, litter size and sex distribution).

Lambda-cyhalothrin was detected in the plasma of the maternal animals and pups at all time points evaluated. In general, plasma levels increased in both pups and dams with increasing dietary concentration of lambda-cyhalothrin.

Based on the results of the preliminary study, doses of 0, 2, 60 and 150 ppm were considered to be acceptable for the main study (Williams, 2001).

(b) Studies on metabolites and impurities

Metabolite R119890/PP890

Studies of the acute toxicity, acute irritation and sensitization potential, and genotoxicity of metabolite R119890/PP890 are summarized in Tables 7, 8 and 9, respectively.

Table 7. Studies of acute toxicity of R119890/PP890

Species	Strain	Sex	Route	Purity (%)	LD_{50}/LC_{50}	Reference
Rat	Alderley Park SPF albino	M + F	Oral	nr	>4 990 mg/kg bw[a]	Oliver (1984)[b]
Rat	Alderley Park SPF albino	M + F	Dermal	nr	>2 000 mg/kg bw[c]	Oliver (1984)[b]
Rat	Alpk:AP	M + F	Inhalation	>99	>1.1 mg/L[d]	Hext (1987)[b]

bw: body weight; F: females; GLP: good laboratory practice; LC_{50}: median lethal concentration; LD_{50}: median lethal dose; M: males; nr: not reported

[a] Clinical signs consisted of decreased activity, piloerection, stains around the nose, signs of urinary incontinence and upward curvature of the spine.

[b] Studies not carried out under GLP.

[c] Clinical signs consisted of diarrhoea, stains around the nose, signs of urinary incontinence and signs of skin irritation, including slight desquamation, scabs at the edge of the application area and small scattered scabs.

[d] Nose-only exposure; clinical signs consisted of hunched posture, piloerection and stains around the snout.

Table 8. Studies of acute dermal and eye irritation and skin sensitization potential of R119890/PP890

Species	Strain	End-point	Purity (%)	Result	Reference
Rabbit	New Zealand white	Skin irritation	nr	Slightly irritating[a]	Chester (1984)
Rabbit	New Zealand white	Eye irritation	nr	Moderately irritating	Chester (1984)
Guinea-pig	Dunkin-Hartley	Skin sensitization (maximization test)	nr	Non-sensitizing	Chester (1984)

nr: not reported

[a] The application sites were assessed for erythema and oedema at 1, 25, 45 and 65 hours after the end of the application period instead of at 24, 48 and 72 hours.

Table 9. Studies of genotoxicity of R119890/PP890

End-point	Test object	Concentration[a]	Purity (%)	Results	Reference
In vitro					
Reverse mutation	*S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538	1.6–5 000 µg/plate (±S9)	99.5	Negative	Callander (1984)

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

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

Metabolite R41207

A study of the acute toxicity of metabolite R41207 is summarized in Table 10.

Table 10. Study of acute toxicity of R41207

Species	Strain	Sex	Route	Purity (%)	LD_{50}	Reference
Rat	Alderley Park SPF albino	M + F	Oral	nr	>3 000 mg/kg bw[a]	Smith (1981)

bw: body weight; F: females; LD_{50}: median lethal dose; M: males; nr: not reported

[a] Clinical signs consisted of subdued behaviour, piloerection, urinary incontinence, signs of dehydration and areas of fur stained.

Metabolite R79406

Studies of the acute toxicity and dermal and eye irritation potential of metabolite R79406 are summarized in Tables 11 and 12, respectively.

Table 11. Studies of acute toxicity of R79406

Species	Strain	Sex	Route	Purity (%)	LD_{50}	Reference
Rat	Alderley Park SPF albino	M + F[a]	Oral	nr	> 500 mg/kg bw[b]	Parkinson (1976)[c]
Rat	Alderley Park SPF albino	M + F	Dermal	nr	> 4 mL/kg bw[d]	Parkinson (1976)[c]

bw: body weight; F: females; GLP: good laboratory practice; LD_{50}: median lethal dose; M: males; nr: not reported

[a] Two male and two female rats.

[b] No noticeable adverse systemic effects. However, no individual animal data were reported.

[c] Study was not carried out under GLP and was of low quality, with only limited information provided.

[d] No daily examination was reported; application area was not reported.

Table 12. Studies of acute dermal and eye irritation potential of R79406

Species	Strain	End-point	Purity (%)	Result	Reference
Rabbit	New Zealand white	Skin irritation	nr	Non-irritating[a]	Parkinson (1976)[b]
Rabbit	New Zealand white	Eye irritation	nr	Moderately irritating[c]	Parkinson (1976)[b]

GLP: good laboratory practice; nr: not reported

[a] Occlusive dressing (aluminium foil) was used, and exposure lasted 24 hours. No individual animal data were reported.

[b] Study was not carried out under GLP and was of low quality, with only limited information provided.

[c] Two animals were treated with 0.05 mL. Ocular reaction was reported at 1–2 hours and 1, 2, 4 and 7 days after application.

Impurity and plant metabolite R110649

A study of the acute toxicity of impurity and plant metabolite R110649 is summarized in Table 13.

Table 13. Study of acute toxicity of R110649

Species	Strain	Sex	Route	Purity (%)	LD_{50}	Reference
Rat	Alderley Park SPF albino	M + F	Oral	nr	M: 1 889 mg/kg bw[a] F: 2 316 mg/kg bw[a]	Oliver (1982)

bw: body weight; F: females; LD_{50}: median lethal dose; M: males; nr: not reported

[a] Clinical signs consisted of subdued behaviour, signs of dehydration, urinary incontinence, excessive salvation, piloerection, flaccidity, hypothermia, shaking, stains around snout, sides pinched and upward curvature of the spine.

Impurity R290076

A study of the genotoxicity of impurity R290076 is summarized in Table 14.

Table 14. Study of genotoxicity of R290076

End-point	Test object	Concentration[a]	Purity (%)	Results	Reference
In vitro					
Reverse mutation	*S. typhimurium* strains TA98, TA100, TA1535 and TA1537 and *E. coli* WP2 *uvr*A and WP2 *uvr*A (pKM101)	3–5 000 µg/plate (±S9)	97	Negative	Sokolowski (2009)

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

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

3. Observations in humans

Between 2003 and 2015, 2252 cases were reported relating to the use of lambda-cyhalothrin. Of these cases, 147 (7%) were related to intentional misuse. The other incidents were caused by occupational (1375 cases, 61%), accidental (543 cases, 24%) or uncertain (187 cases, 8%) exposure. The majority of reported incidents were of very low severity grade (mild, transient and spontaneously resolving symptoms), representing 82% of all reported incidents. The higher severity grade cases (severe or life-threatening symptoms) were predominantly caused by intentional self-harm or

uncertain reasons (in total, 68% of all severe cases). Fatality occurred in six cases due to intentional misuse.

In general, occupational and accidental exposure, predominantly occurring via the dermal route, mainly caused temporary health effects of minor severity grade. Most of the incidents were related to paraesthesia, a phenomenon that is known to occur with pyrethroids (Syngenta, unpublished data, 2018).

Comments

Biochemical aspects

In the biliary elimination and biotransformation study in rats, oral absorption was 19–24% following administration of [phenoxy-^{14}C]lambda-cyhalothrin at the low dose of 1 mg/kg bw and 11–12% at the high dose of 12.5 mg/kg bw, based on the radioactivity in urine, bile, cage wash and carcass. The majority of the administered dose was found in faeces (83–84% at the low dose and 88–90% at the high dose), largely as lambda-cyhalothrin, and the majority of the radioactivity (>90%) was excreted by 48 hours post-dosing. There was 1.2% or less radioactivity remaining in the carcass or gastrointestinal tract in all treatment groups, indicating that excretion was essentially complete by 96 hours post-dosing.

No individual unconjugated metabolite accounted for more than 1% of the administered dose in urine or bile samples. The most abundant metabolites of lambda-cyhalothrin in samples hydrolysed with glucuronidase and sulfatase were R119890 (up to 2.1% of the administered dose in bile), R175447 (up to 9.3% of the administered dose in urine and bile) and R211133 (up to 3.2% of the administered dose in bile) (Tomlinson, 2011).

Toxicological data

Two additional bacterial gene mutation studies were provided (Callander & Priestley, 1989; Sokolowski, 2012), which were both negative.

A preliminary developmental toxicity study in rats, which was the basis for the doses selected for the main study that was previously evaluated by the 2007 Meeting, was submitted (Williams, 2001). The Meeting noted that the effects seen in this preliminary study were consistent with those seen in the main study.

Toxicological data on metabolites and/or degradates

In rats, R119890 (plant metabolite) was not acutely toxic after oral (LD_{50} > 4990 mg/kg bw; Oliver, 1984), dermal (LD_{50} > 2000 mg/kg bw; Oliver 1984) or inhalation (LC_{50} > 1.1 mg/L; Hext, 1987) exposure. Metabolite R119890 was slightly irritating to the skin of rabbits, moderately irritating to the eyes of rabbits and not sensitizing to the skin of guinea-pigs (Chester, 1984). The compound was negative in an Ames test (Callander, 1984).

R41207 (plant metabolite) was not acutely toxic in rats after oral exposure (LD_{50} > 3000 mg/kg bw; Smith, 1981).

R79406 (plant metabolite) was tested in a study that evaluated its acute oral toxicity in rats, acute dermal toxicity in rats, and skin and eye irritation potential in rabbits (Parkinson, 1976). The study was considered to be of unacceptable quality, with only limited information provided on the conduct of the study and on the study results.

The acute oral LD_{50} of R110649 (plant metabolite) in rats was 1889 mg/kg bw (Oliver, 1982).

The Meeting concluded that the metabolites for which reliable acute oral toxicity data are available (R119890, R41207, R110649) are of lower acute oral toxicity than the parent compound (oral LD_{50} of 56 mg/kg bw; Southwood, 1985).

Human data

In total, 2252 cases were reported describing adverse effects relating to the use of lambda-cyhalothrin. In general, occupational and accidental exposure mainly caused temporary health effects of minor severity. The severe cases were related to intentional misuse. There is no indication of concern for dietary risk assessment (Syngenta, unpublished data, 2018).

Toxicological evaluation

The Meeting concluded that the new studies do not have any impact on the ADI of 0–0.02 mg/kg bw or the ARfD of 0.02 mg/kg bw established in 2007.

The Meeting also concluded that the metabolites for which reliable acute oral toxicity data are available appear to be less acutely toxic orally than the parent compound.

References

Callander R (1984). PP890: an evaluation in the *Salmonella* mutagenicity assay. Unpublished report no. CTL/P/999. Imperial Chemical Industries PLC, Macclesfield, United Kingdom. Syngenta File No. PP890/0005. Submitted to WHO by Syngenta Crop Protection AG.

Callander R, Priestley K (1989). Lambda-cyhalothrin production material: an evaluation in the *Salmonella* mutation assay. Unpublished report no. CTL/P/2590. ICI Central Toxicology Laboratory, Macclesfield, United Kingdom. Syngenta File No. PP321/2164. Submitted to WHO by Syngenta Crop Protection AG.

Chester G (1984). PP890: skin irritation, eye irritation and skin sensitisation studies. Unpublished report no. CTL/P/1016. Imperial Chemical Industries PLC, Macclesfield, United Kingdom. Syngenta File No. PP890/0004. Submitted to WHO by Syngenta Crop Protection AG.

Cross M (1985). PP321: assessment of mutagenic potential using L5178Y mouse lymphoma cells. Unpublished report no. CTL/P/1340. Imperial Chemical Industries PLC Central Toxicology Laboratory, Macclesfield, United Kingdom. Submitted to WHO by Syngenta Crop Protection AG.

Hext P (1987). PP890: 4-hour acute inhalation toxicity study in the rat. Unpublished report no. CTL/T/2553. Imperial Chemical Industries PLC, Macclesfield, United Kingdom. Syngenta File No. PP890/0002. Submitted to WHO by Syngenta Crop Protection AG.

Hext P (1990). Lambda-cyhalothrin production material: 21 days sub-acute inhalation toxicity study in the rat. Unpublished report no. CTL/P/2772. ICI Central Toxicology Laboratory, Macclesfield, United Kingdom. Syngenta File No. PP321/1739. Submitted to WHO by Syngenta Crop Protection AG.

Leah A (1989). Lambda-cyhalothrin: 21-day dermal toxicity to the rat. Unpublished report no. CTL/P/2532. ICI Central Toxicology Laboratory, Macclesfield, United Kingdom. Syngenta File No. PP321/1582. Submitted to WHO by Syngenta Crop Protection AG.

Milburn GM (2004). Lambda-cyhalothrin: developmental neurotoxicity study in rats. Unpublished report no. CTL/RR0969. Central Toxicology Laboratory, Macclesfield, United Kingdom. Submitted to WHO by Syngenta Crop Protection AG.

Oliver G (1982). R110649 (3-phenoxybenzaldehyde): acute oral toxicity to rats. Unpublished report no. CTL/P/681. Imperial Chemical Industries PLC, Macclesfield, United Kingdom. Syngenta File No. CGA55186/0461. Submitted to WHO by Syngenta Crop Protection AG.

Oliver G (1984). PP890: acute oral toxicity and acute dermal toxicity studies. Unpublished report no. CTL/P/1034. Imperial Chemical Industries PLC, Macclesfield, United Kingdom. Syngenta File No. PP890/0003. Submitted to WHO by Syngenta Crop Protection AG.

Parkinson G (1976). R79406: acute toxicity and local irritancy. Unpublished report no. CTL/P/234. Imperial Chemical Industries PLC Central Toxicology Laboratory, Macclesfield, United Kingdom. Syngenta File No. PP557/0659. Submitted to WHO by Syngenta Crop Protection AG.

Sheldon T, Howard CA, Richardson CR (1985). PP321: a cytogenetic study in human lymphocytes in vitro. Unpublished report no. CTL/P/1333. Imperial Chemical Industries PLC Central Toxicology Laboratory, Macclesfield, United Kingdom. Submitted to WHO by Syngenta Crop Protection AG.

Sheldon T, Richardson CR, Shaw J, Barber G (1984). An evaluation of PP321 in the mouse micronucleus test. Unpublished report no. CTL/P/1090. Imperial Chemical Industries PLC Central Toxicology Laboratory, Macclesfield, United Kingdom. Submitted to WHO by Syngenta Crop Protection AG.

Smith I (1981). R41207 (3-phenoxybenzoic acid): acute oral toxicity to rats. Unpublished report no. CTL/P/627. Central Toxicology Laboratory, Macclesfield, United Kingdom. Syngenta File No. CGA55186/0460. Submitted to WHO by Syngenta Crop Protection AG.

Sokolowski A (2009). R290076 – *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay. Unpublished report no. 1286800. Harlan Cytotest Cell Research GmbH, Rossdorf, Germany. Syngenta File No. R290076_10000. Submitted to WHO by Syngenta Crop Protection AG.

Sokolowski A (2012). Lambda-cyhalothrin technical – *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay. Unpublished report no. 1458600. Harlan Cytotest Cell Research GmbH, Rossdorf, Germany. Syngenta File No. PP321_11497. Submitted to WHO by Syngenta Crop Protection AG.

Southwood J (1985). PP321: acute oral toxicity studies. Unpublished report no. CTL/P/1102. Imperial Chemical Industries PLC Central Toxicology Laboratory, Macclesfield, United Kingdom. Submitted to WHO by Syngenta Crop Protection AG.

Tomlinson J (2011). Lambda-cyhalothrin – biliary elimination and biotransformation in the rat following single oral administration of [^{14}C]-lambda-cyhalothrin. Unpublished report no. 31827. Charles River, Tranent, United Kingdom. Syngenta File No. PP321_11457. Submitted to WHO by Syngenta Crop Protection AG.

Trueman RW (1989). Lambda-cyhalothrin: assessment for the induction of unscheduled DNA synthesis in primary rat hepatocyte cultures. Unpublished report no. CTL/P/2707. ICI Central Toxicology Laboratory, Macclesfield, United Kingdom. Submitted to WHO by Syngenta Crop Protection AG.

Williams J (2001). Second preliminary developmental neurotoxicity study in rats. Unpublished report no. CTL/RR0812. Central Toxicology Laboratory, Macclesfield, United Kingdom. Syngenta File No. PP321/1537. Submitted to WHO by Syngenta Crop Protection AG.

MANDESTROBIN

First draft prepared by
Luca Tosti,[1] David Eastmond[2] and Carl Cerniglia[3]

[1] Department of Biomedical and Clinical Sciences, University of Milan, and International Centre for Pesticides and Health Risk Prevention, ASST Fatebenefratelli Sacco, Milan, Italy
[2] Department of Molecular, Cell and Systems Biology, University of California, Riverside, California, United States of America (USA)
[3] Division of Microbiology, National Center for Toxicological Research, Food and Drug Administration, Jefferson, Arkansas, USA

Explanation....295
Evaluation for acceptable intake....296
1. Biochemical aspects....296
1.1 Absorption, distribution and excretion....297
1.2 Biotransformation....301
2. Toxicological studies....311
2.1 Acute toxicity....311
(a) Lethal doses....311
(b) Dermal irritation....313
(c) Ocular irritation....313
(d) Dermal sensitization....313
2.2 Short-term studies of toxicity....313
(a) Oral administration....314
(b) Dermal application....337
2.3 Long-term studies of toxicity and carcinogenicity....337
2.4 Genotoxicity....351
(a) In vitro....352
(b) In vivo....353
2.5 Reproductive toxicity....353
(a) Multigeneration studies....353
(b) Developmental toxicity....368
2.6 Special studies....374
(a) Neurotoxicity....374
(b) Immunotoxicity....378
(c) Mechanistic studies....380
(d) Toxicity studies on metabolites and impurities....387
(e) Microbiological effects....394
3. Observations in humans....395
Comments....395
Toxicological evaluation....400
References....403

Explanation

Mandestrobin is the common name approved by the International Organization for Standardization for 2-[2-[(2,5-dimethylphenoxy)methyl]phenyl]-2-methoxy-*N*-methylacetamide (International Union of Pure and Applied Chemistry), with the Chemical Abstracts Service number 173662-97-0. It is a fungicide that is marketed as a racemic mixture (50:50) consisting of the *R*-isomer and the *S*-isomer (Fig. 1). The fungicidal mode of action is inhibition of mitochondrial respiration via binding to complex III.

Fig. 1. Structures of R- and S-isomers of mandestrobin

***R*-Mandestrobin** **S-Mandestrobin**

Mandestrobin 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) and were conducted in accordance with relevant national or international test guidelines, unless otherwise specified. No additional information from a literature search was identified that complemented the toxicological information submitted for the current assessment.

Evaluation for acceptable intake

1. Biochemical aspects

The absorption, distribution, metabolism and excretion (ADME) of mandestrobin (code name S-2200) were investigated in rats following the administration of mandestrobin labelled with ^{14}C at either the benzyl or the phenoxy ring in a single oral low dose or a single oral high dose or the administration of radiolabelled mandestrobin in a single oral low dose repeated for up to 14 consecutive days. The rates and routes of elimination were also determined in bile duct–cannulated animals after administration of a single oral low dose. Possible preferential metabolism was investigated by administering single low doses of the *R*-isomer and the *S*-isomer of mandestrobin, labelled uniformly with ^{14}C at the benzyl position.

The positions of the radiolabels in the mandestrobin used in the ADME studies are shown in Fig. 2.

Fig. 2. Position of radiolabels in mandestrobin used in ADME studies

[benzyl-^{14}C]Mandestrobin **[phenoxy-^{14}C]Mandestrobin**

* Denotes radiolabelled ring

Source: Kendrick (2012a)

1.1 Absorption, distribution and excretion

In an absorption, distribution and excretion study, Hannover Wistar (Crl) rats (four of each sex) were administered [benzyl-^{14}C]mandestrobin (purity 98.3%) or [phenoxy-^{14}C]mandestrobin (purity 98.9%) in a single oral gavage (low) dose of 5 mg/kg body weight (bw) (groups A and B) or [benzyl-^{14}C]mandestrobin in a single oral gavage (high) dose of 1000 mg/kg bw (group C) for the determination of rates and routes of excretion at various intervals up to 7 days. A further four male and four female rats were given [benzyl-^{14}C]mandestrobin in a single oral gavage dose of 5 mg/kg bw (group D) or 1000 mg/kg bw (group E) for the determination of plasma concentrations at various intervals up to 120 hours. Biliary excretion was investigated in seven male and seven female rats administered [benzyl-^{14}C]mandestrobin in a single oral gavage dose of 5 mg/kg bw (group F). An additional group of 16 male and 16 female rats was given [benzyl-^{14}C]mandestrobin in a single oral gavage dose of 5 mg/kg bw (group G) or 1000 mg/kg bw (group H) for the determination of tissue distribution at various intervals up to 7 days (Table 1). The dosing vehicle was 0.5% aqueous methylcellulose.

Table 1. Animal assignments in ADME studies

Dose group	Label position	Parameter investigated	Dose		Number of animals	
			mg/kg bw	MBq/kg bw	Males	Females
A	Benzyl ring	Excretion balance	5	5	4	4
B	Phenoxy ring		5	5	4	4
C	Benzyl ring		1 000	5	4	4
D	Benzyl ring	Pharmacokinetics	5	10	4	4
E	Benzyl ring		1 000	10	4	4
F	Benzyl ring	Biliary excretion	5	5	7[a]	7[a]
G	Benzyl ring	Tissue distribution	5	5	16	16
H	Benzyl ring		1 000	5	16	16

bw: body weight

[a] Additional animals were included to ensure that four animals of each sex completed the experimental regimen.

Source: Kendrick, Farrell & Murphy (2012)

Following administration of a single high or low oral dose of racemic mandestrobin labelled with ^{14}C in either the benzyl or phenoxy ring, the radioactivity was rapidly absorbed. Faecal excretion was the primary route of elimination of the radioactivity. More than 70% of the total excreted radioactivity was eliminated within 48 hours of administration. There did not appear to be any sex-, dose- or radiolabel-related differences in either the rates or routes of excretion (Table 2). In bile duct–cannulated rats, elimination of radioactivity was rapid, with more than 95% of the dose eliminated within the first 24 hours. About 80% was eliminated via the bile, and radioactivity in faeces accounted for less than 2% of the total radioactivity. Elimination via urine was comparable to that of intact animals. Urinary elimination in bile duct–cannulated rats was substantially more rapid than in intact animals, where it was apparently slowed by enterohepatic recirculation.

Absorption of mandestrobin was greater than 90% of the administered dose at 5 mg/kg bw, based on radioactivity recovered in urine and bile. Recoveries of radioactivity were complete at 168 hours (95.6–101%) in all dose groups. After 168 hours, only 0.7–2.1% of the radioactivity remained in carcass and tissues.

For both low and high doses, there were no significant sex differences in the pharmacokinetic parameters. Mean plasma elimination half-lives were approximately 20 hours (low dose) and 27 hours (high dose). The maximum plasma concentration (C_{max}) was observed within 3 and 10 hours post-dosing for the low and high doses, respectively (Table 3).

Table 2. Cumulative recovery of radioactivity in excreta following administration of a single oral low dose of [benzyl-^{14}C]mandestrobin or [phenoxy-^{14}C]mandestrobin or a single oral high dose of [benzyl-^{14}C]mandestrobin to rats at 168 hours or a single oral low dose of [benzyl-^{14}C]mandestrobin to bile duct–cannulated rats at 72 hours

	% of administered dose							
	5 mg/kg bw				1 000 mg/kg bw		5 mg/kg bw	
	[benzyl-^{14}C]		[phenoxy-^{14}C]		[benzyl-^{14}C]		[benzyl-^{14}C], bile duct–cannulated rats	
Matrix	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
Urine	18.0	20.6	15.8	21.5	17.2	17.2	18.0	13.7
Faeces	72.9	71.3	73.9	66.5	74.8	72.0	1.43	1.92
Bile	ND	ND	ND	ND	ND	ND	79.7	81.9
Cage wash	4.88	5.99	3.98	6.51	3.65	6.74	1.39	1.35
Cage debris	0.282	0.069	0.172	0.038	0.048	0.117	0.036	0.068
Final cage wash	0.15	0.108	0.071	0.087	0.082	0.357	0.157	0.264
Tissue carcass	2.13	0.974	1.69	1.31	1.22	0.702	0.262	0.388
Total	98.3	99.0	95.6	95.9	97.0	97.1	101	99.6

bw: body weight; ND: not determined
Source: Kendrick, Farrell & Murphy (2012)

Table 3. Mean pharmacokinetic parameters in plasma following oral administration of radiolabelled (benzyl-^{14}C) mandestrobin to rats

	5 mg/kg bw		1 000 mg/kg bw	
Pharmacokinetic parameters	**Males**	**Females**	**Males**	**Females**
$t_{½}$ (h)	22.52	18.32	24.51	29.4
T_{max} (h)	2.625	1.25	7	9.125
C_{max} (µg eq/g)	0.842	0.829	690	49.2
$AUC_{0–120}$ (h·µg eq/g)	15.64	13.92	1 541	1 262
$AUC_{0–\infty}$ (h·µg eq/g)	16.02	14.08	1 577	1 303
$AUC_{0–120}$ as a % of $AUC_{0–\infty}$	98	99	98	97

$AUC_{0–120}$: area under the plasma concentration–time curve from 0 to 120 hours; $AUC_{0–\infty}$: area under the plasma concentration–time curve from time 0 to infinity; bw: body weight; C_{max}: maximum concentration; eq: equivalents; $t_{½}$: elimination half-life; T_{max}: time taken to reach C_{max}
Source: Kendrick, Farrell & Murphy (2012)

At 1000 mg/kg bw, after T_{max} was achieved, concentrations of radioactivity did not decrease steadily, as was observed following administration of the 5 mg/kg bw dose. Following dose administration at 1000 mg/kg bw in both male and female animals, additional absorption maxima were observed, which were particularly prominent during the first 12 hours post-dosing. The multiple maxima could possibly reflect enterohepatic recirculation, which is supported by the enhanced rapidity of elimination in bile duct–cannulated animals. Systemic exposure (area under the concentration–time curve [AUC]) to mandestrobin-derived radioactivity was about 90- to 100-fold higher in male and female animals receiving the 1000 mg/kg bw dose compared with animals receiving the 5 mg/kg bw dose. As such, exposure was sub-dose-proportional, indicating that saturation of absorption processes

may also have occurred (Table 3). At the terminal time point, plasma radioactivity concentrations were less than or equal to 2% of the concentrations observed at the C_{max}.

Tissue distribution of radioactivity was similar at the high and low doses (Table 4). At the first two time points (0.5 and 2 hours post-dosing for the low-dose groups and 2 and 8 hours post-dosing for the high-dose groups), the majority of radioactivity was detected in the upper half of the gastrointestinal tract. Transit through the gastrointestinal tract was evident over time; at the terminal time point (168 hours), only about 1% of the total radioactivity was associated with tissue and carcass. Excluding the components of the gastrointestinal tract, the highest concentrations of radioactivity were measured in liver and kidneys (up to 9% and 0.4% of the administered radioactivity, respectively). Notable amounts of radioactivity were also detected in fat, pancreas, spleen, ovaries and uterus. Plasma:tissue radioactivity ratios were generally below 1, except for the above-noted organs (Kendrick, Farrell & Murphy, 2012).

Table 4. Tissue distribution of radioactivity in low-dose and high-dose groups at 168 hours after administration

	Tissue distribution of radioactivity (mean ng equivalents/g)					
	5 mg/kg bw [benzyl-^{14}C]		**5 mg/kg bw [phenoxy-^{14}C]**		**1 000 mg/kg bw [benzyl-^{14}C]**	
Tissue	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
Adrenals	21	7	8	ND	ND	ND
Blood	10	3	9	3	732	1 352
Bone	ND	3	ND	ND	ND	ND
Bone marrow	ND	ND	ND	ND	ND	ND
Brain	ND	ND	ND	ND	ND	ND
Eyes	3	1	1	10	ND	ND
Fat	50	19	21	31	593	ND
Hair and skin	8	3	14	8	522	ND
Heart	2	ND	2	2	762	ND
Kidney	34	14	35	19	2 627	1 458
Liver	147	98	130	92	9 033	7 144
Lung	9	ND	6	4	644	ND
Mandibular gland	2	ND	3	2	ND	ND
Muscle (quadriceps)	ND	ND	2	ND	ND	ND
Ovaries	NA	32	NA	46	NA	874
Pancreas	61	20	7	30	1 561	1 225
Pituitary	ND	ND	ND	ND	ND	ND
Plasma	11	2	9	4	170	ND
Red blood cells	9	6	8	2	1 910	2 712
Sciatic nerve	ND	ND	ND	ND	ND	ND
Spinal cord	ND	ND	ND	ND	ND	ND
Spleen	19	5	7	44	607	ND
Testes	ND	NA	ND	NA	ND	NA

Tissue	Tissue distribution of radioactivity (mean ng equivalents/g)					
	5 mg/kg bw [benzyl-^{14}C]		5 mg/kg bw [phenoxy-^{14}C]		1 000 mg/kg bw [benzyl-^{14}C]	
	Males	Females	Males	Females	Males	Females
Thymus	ND	ND	2	6	ND	ND
Thyroid	23	ND	ND	ND	ND	NA
Uterus	NA	62	NA	26	NA	982
Gastrointestinal tract						
Caecum	247	103	300	177	5 547	5 494
Caecum contents	358	132	456	146	8 387	8 775
Large intestine	198	62	162	110	4 179	2 994
Large intestine contents	295	105	312	190	7 380	6 664
Small intestine	775	624	638	526	3 194	655
Small intestine contents	2 050	12	1 241	1 134	1 093	1 780
Stomach	55	41	55	58	17 840	8 373
Stomach contents	118	98	41	114	39 380	23 880

bw: body weight; ND: not detected; NA: not available
Source: Kendrick, Farrell & Murphy (2012)

In an absorption, distribution and excretion study, six groups of Hannover Wistar (Crl) rats (four of each sex per group) were administered [benzyl-^{14}C]mandestrobin (purity 98.9%) orally by gavage in 0.5% aqueous methylcellulose at 5 mg/kg bw per day for up to 14 consecutive days. The rates and routes of excretion were determined by collecting excreta at 24-hour intervals 1) until 24 hours after the 14th dose and 2) until 336 hours after the 14th dose. Tissue distribution of radioactivity was determined over a time course of up to 336 hours after the 14th daily dose.

During the first 14 days, radioactivity was eliminated primarily in the faeces, with approximately 57.3% and 49.5% of the administered dose eliminated in males and females, respectively. Renal elimination accounted for about 13% and 15% of the administered dose in male and female animals, respectively. Radioactivity recovered from the cage washes accounted for about 6% and 8% of the administered dose in males and females, respectively (Table 5).

Table 5. Recovery of radioactivity in excreta following repeated administration of [benzyl-^{14}C]mandestrobin at 5 mg/kg bw

Matrix	% of administered dose[a]			
	Days 1–14		Days 14–28	
	Males	Females	Males	Females
Urine	13.25	14.81	1.89	2.07
Faeces	57.25	49.45	9.96	10.56
Cage wash[b]	6.11	7.99	0.56	0.83
Total	76.61	72.25	12.42	13.47

[a] Calculated from the sum of 14 administered doses.
[b] Includes cage debris, final cage wash and tissues.
Source: Kendrick & Farrell (2012)

During the 14 days after the last dose, concentrations of radioactivity in excreta were generally much lower, given that the vast majority of the administered dose (14 daily doses) had already been eliminated prior to confinement in metabolism cages. Radioactivity was eliminated primarily in the faeces, with about 10% and 10.6% of the administered dose eliminated in males and females, respectively. Renal elimination accounted for approximately 1.9% and 2.1% of the administered dose in male and female animals, respectively (Table 5).

Following the repeated administration of [benzyl-^{14}C]mandestrobin at 5 mg/kg bw per day to male and female rats for up to 14 days, radioactivity was widely distributed at all of the time points examined (2 hours after one, six, 10 and 14 doses and 168 and 336 hours after the 14th daily dose) (Table 6). Radioactivity was detected in all of the tissues examined on at least one occasion. At 648 hours after dosing (i.e. 336 hours after the final dose), radioactivity was detected in around half of the tissues examined. Excluding the components of the gastrointestinal tract, the highest mean concentrations of radioactivity were measured in the tissues involved in the metabolism and excretion of xenobiotics (i.e. liver and kidney).

As the number of doses progressed, the pancreas, spleen, thyroid, fat, uterus and ovaries became prominent depots for radioactivity. Plasma concentrations, together with all tissue concentrations, declined between 168 and 336 hours after the 14th daily dose. At this time, plasma concentrations in both male and female animals were below the limit of detection, and approximately half or more of the tissues did not contain detectable radioactivity. The observable decline of residues in all tissues indicated no potential for long-term accumulation of radioactivity with repeated daily administration of [benzyl-^{14}C]mandestrobin at a dose of 5 mg/kg bw per day (Table 6) (Kendrick & Farrell, 2012).

In an absorption, distribution and excretion study, two groups of Hannover Wistar (Crl) rats (four of each sex per group) were administered [benzyl-^{14}C]mandestrobin, *R*-isomer (purity 99.2%) or *S*-isomer (purity 99.7%), in a single oral gavage dose (in 0.5% aqueous methylcellulose) of 5 mg/kg bw. Rates and routes of excretion were determined at various intervals up to 7 days. Distribution of radioactivity in tissues and organs was determined 7 days after administration.

After 7 days, both isomers were eliminated mainly via faeces: approximately 64–75% of the radioactivity for the *R*-isomer and about 73–80% of the radioactivity for the *S*-isomer. Both isomers were absorbed immediately after administration. The *R*-isomer was almost completely excreted (>95%) after 3 days, whereas excretion of the *S*-isomer was slower, with an almost complete excretion (>94%) after 5 days. Elimination via urine accounted for 22% and 32% of the radioactivity in males and females, respectively, for the *R*-isomer after 3 days and for 15% and 25% of the radioactivity in males and females, respectively, for the *S*-isomer after 5 days. The majority of radioactivity was excreted in faeces, accounting for 73% and 63% of the radioactivity in males and females, respectively, for the *R*-isomer after 3 days and for 77% and 70% of the radioactivity in males and females, respectively, for the *S*-isomer after 5 days. No significant sex differences in the routes of excretion of both isomers were observed. Excretion into expired air was negligible with both isomers and both sexes.

Tissue distribution was generally low (total radioactivity <1%). Residuals were slightly higher in animals given the *S*-isomer, reflecting the slower elimination (Mikata, 2011).

1.2 Biotransformation

The metabolic fate of mandestrobin was investigated in Hannover Wistar (Crl) rats. The test item was administered by gavage to six groups of rats as described in Table 1 of section 1.1. Samples from dose groups A, B, C, F, G and H were pooled per time point, sex and dose group for metabolite isolation and identification by radio-high-performance liquid chromatography (HPLC) and liquid chromatography with mass spectrometry (LC-MS), respectively. Metabolites were determined in urine, faeces, liver, kidney, plasma and bile.

Table 6. Concentrations of radioactivity in organs and tissues following repeated oral administration of ^{14}C-labelled mandestrobin to rats at 5 mg/kg bw per day

	Concentration of radioactivity (ng equivalents/g)											
	2 hours after 1st dose		**122 hours after 1st dose**		**218 hours after 1st dose**		**314 hours after 1st dose**		**480 hours after 1st dose**		**648 hours after 1st dose**	
	–		**–**		**–**		**2 hours after 14th daily dose**		**168 hours after 14th daily dose**		**336 hours after 14th daily dose**	
Tissue[a]	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Liver	4 724	2 025	7 762	4 687	11 760	6 618	10 150	8 868	415	686	115	150
Carcass	215	239	621	603	996	1 041	933	1 165	59	62	86	52
Skin + hair	167	67	301	145	452	302	450	272	93	44	81	40
Bone	66	22	94	55	151	92	134	115	20	10	ND	32
Kidney	1 157	446	1 730	923	3 057	1 094	2 459	1 574	109	91	40	27
Red blood cells	275	67	319	141	431	148	382	208	105	68	38	21
Ovaries	–	874	–	1 813	–	2 360	–	1 123	–	105	–	12
Spleen	169	168	504	724	476	1 319	707	840	29	36	21	11
Uterus	–	1 310	–	3 189	–	4 379	–	4 810	–	90	–	8
Pancreas	571	635	1 019	3 239	1 622	2 878	11 590	4 370	40	67	ND	ND
Plasma	773	227	1 052	513	1 582	582	1 197	868	14	8	ND	ND
Fat	339	270	603	1 124	997	1 151	1 956	803	7	36	ND	ND
Thyroid	322	246	492	249	908	1 201	1 122	686	28	ND	ND	ND
Blood	495	145	686	322	1 031	376	781	571	64	44	7	ND
Adrenals	315	90	462	298	734	427	673	522	13	17	ND	ND
Lung	288	137	473	215	737	417	632	426	32	33	ND	ND
Sciatic nerve	221	ND	301	95	476	223	433	336	38	ND	ND	ND
Pituitary	68	ND	94	91	751	ND	428	261	ND	ND	ND	ND
Muscle (quadriceps)	92	58	145	99	253	167	237	248	ND	6	ND	ND

Tissue[a]	Concentration of radioactivity (ng equivalents/g)											
	2 hours after 1st dose		122 hours after 1st dose		218 hours after 1st dose		314 hours after 1st dose		480 hours after 1st dose		648 hours after 1st dose	
	–		–		–		2 hours after 14th daily dose		168 hours after 14th daily dose		336 hours after 14th daily dose	
	M	F	M	F	M	F	M	F	M	F	M	F
Heart	209	81	301	128	484	185	336	244	8	ND	ND	ND
Mandibular gland	147	51	231	112	366	192	302	227	ND	ND	ND	ND
Testes	131	–	179	–	273	–	229	–	ND	–	ND	–
Thymus	94	50	197	98	280	149	250	171	7	ND	ND	ND
Eyes	76	41	131	59	227	125	212	119	8	ND	ND	ND
Bone marrow	57	ND	195	ND	146	ND	129	92	ND	ND	ND	ND
Spinal cord	33	27	80	35	159	97	102	75	ND	ND	ND	ND
Brain	39	12	71	39	108	45	78	61	ND	ND	ND	ND
Gastrointestinal tract												
Caecum	2 648	8 859	20 270	30 420	33 170	56 130	42 420	71 510	351	486	32	47
Caecum contents	3 234	12 290	34 520	41 870	55 550	78 190	53 210	51 580	465	551	47	65
Large intestine	644	1 466	6 706	10 630	16 680	14 570	20 320	16 980	168	271	9	14
Large intestine contents	160	498	15 550	14 710	34 300	44 160	27 340	36 900	316	622	40	33
Small intestine	21 980	17 450	43 020	37 680	62 130	57 840	95 130	67 350	381	814	61	65
Small intestine contents	44 260	45 220	132 700	132 000	176 400	167 000	228 300	100 600	1 240	1 508	100	98
Stomach	7 592	5 772	19 310	8 914	33 770	17 400	26 010	27 010	30	119	ND	6
Stomach contents	20 250	22 320	54 780	16 440	83 540	31 430	94 490	49 320	44	103	16	35

F: females; M: males; ND: not determined

[a] Descending order of magnitude of residues in female tissues sampled 14 days after 14th daily dose (i.e. 648 hours after first dose), excluding gastrointestinal tract.

Source: Kendrick & Farrell (2012)

Mandestrobin was extensively metabolized to numerous metabolites (unchanged parent was found in faeces at only <0.2% of the administered dose at the low dose and <6% at the high dose). No major sex or dose differences were observed, and no major differences in the profiles from intact and bile duct–cannulated animals were apparent. Metabolite profiles generated from the administration of differently labelled molecules (benzyl vs phenoxy) indicated that little cleavage occurred between the two rings. The primary routes of metabolism were by (1) oxidation and subsequent conjugation with glucuronic acid, (2) demethylation with subsequent oxidation or (3) oxidation with subsequent demethylation. The metabolite profiles in urine, faeces and bile are summarized in Table 7.

Table 7. Quantification of metabolites in pooled excreta following administration of a single low or high dose of mandestrobin to rats

	% of administered dose															
	[benzyl-^{14}C]												**[phenoxy-^{14}C]**			
	5 mg/kg bw								**1 000 mg/kg bw**				**5 mg/kg bw**			
	Urine		**Urine (cannulated)**		**Bile (cannulated)**		**Faeces**		**Urine**		**Faeces**		**Urine**		**Faeces**	
Metabolite identity[a]	**M 48 h**	**F 48 h**	**M 24 h**	**F 24 h**	**M 12 h**	**F 24 h**	**M 96 h**	**F 72 h**	**M 48 h**	**F 48 h**	**M 72 h**	**F 48 h**	**M 48 h**	**F 48 h**	**M 72 h**	**F 72 h**
Mandestrobin (S-2200)	–	–	–	–	–	–	0.09	0.139	–	–	3.92	5.49	–	–	0.08	–
5-CA-S-2200-NHM	3.58	5.70	4.76	4.93	6.86	3.43	19.87	16.57	4.15	4.35	11.84	8.56	2.94	5.48	18.11	15.28
4-OH-S-2200	–	0.03	–	–	–	–	9.47	18.21	–	–	10.45	12.59	–	–	7.09	16.47
4-OH-S-2200-GlucA	0.08	0.01	–	–	33.78	36.67	–	–	–	0.11	–	–	0.02	0.03	–	–
COOH-S-2200 glucuronides	–	–	–	–	20.62	23.76	–	–	–	–	–	–	–	–	–	–
5-COOH-S-2200	1.26	3.25	–	–	1.63	3.38	7.42	8.32	1.78	4.43	10.74	8.71	0.64	2.63	8.05	6.79
5-CA-2-HM-S-2200	0.85	0.65	0.92	0.73	0.10	1.40	4.86	3.97	1.58	0.60	4.74	5.04	0.90	0.68	4.74	4.44
5-CA-2-HM-S-2200-NHM	0.89	0.55	1.59	0.84	1.67	0.49	6.49	0.68	0.61	0.18	2.12	1.51	0.88	0.60	6.37	2.88
De-Xy-S-2200	0.12	0.19	–	–	–	–	1.73	2.75	0.18	0.15	3.29	4.44	–	–	–	0.01
2-COOH-S-2200	0.33	0.64	0.54	0.22	–	–	0.78	0.23	0.58	0.29	–	0.78	0.36	0.86	0.06	0.60
5-CH_2OH-S-2200	0.00	–	0.28	0.13	–	–	0.21	0.17	–	0.03	–	1.14	–	–	0.92	0.36
2-CH_2OH-S-2200	0.02	0.01	–	–	–	–	0.15	–	–	–	–	0.37	–	–	0.14	–
5-CA-MCBX-NDM	1.79	1.77	3.06	1.58	–	–	0.09	0.06	0.27	0.23	0.96	0.44	1.62	2.19	2.09	0.12
5-CA-S-2200-NDM	0.38	0.60	0.69	0.64	0.04	0.95	–	0.09	0.19	0.36	–	–	0.46	0.74	0.27	–

	% of administered dose															
	[benzyl-^{14}C]												[phenoxy-^{14}C]			
	5 mg/kg bw								1 000 mg/kg bw				5 mg/kg bw			
	Urine		Urine (cannulated)		Bile (cannulated)		Faeces		Urine		Faeces		Urine		Faeces	
Metabolite identity[a]	**M 48 h**	**F 48 h**	**M 24 h**	**F 24 h**	**M 12 h**	**F 24 h**	**M 96 h**	**F 72 h**	**M 48 h**	**F 48 h**	**M 72 h**	**F 48 h**	**M 48 h**	**F 48 h**	**M 72 h**	**F 72 h**
5-CA-2-HM-MCBX	0.87	0.33	0.63	0.14	–	–	0.04	–	0.87	0.28	0.53	–	1.16	0.34	0.29	0.14
DX-CA-S-2200	–	–	–	–	–	–	–	–	0.13	–	–	–	–	–	–	–
5-CA-2-HM-MCBX(-2H)	0.07	0.09	–	–	–	–	–	–	0.79	0.09	–	–	–	0.04	–	–
MCBX	–	–	–	–	–	–	–	–	–	–	0.04	–	–	–	–	–
% of dose identified	10.25	13.81	12.46	9.20	64.69	70.07	51.20	51.20	11.12	11.10	48.62	49.06	8.97	13.58	48.22	47.09
% of dose detected	15.20	18.57	17.42	13.30	79.24	81.41	67.19	64.46	15.68	15.36	70.39	65.43	13.94	19.25	66.74	59.75

–: not detected or below the limit of quantification; bw: body weight; F: females; LC-MS: liquid chromatography with mass spectrometry; M: males

[a] Confirmed by LC-MS.

Source: Kendrick, Farrell & Murphy (2012)

In the liver, three metabolites dominated the profiles at both doses: 5-CA-S-2200-NHM, 5-CA-S-2200-NDM and 5-COOH-S-2200. In the kidneys, 5-CA-S-2200-NHM, 5-CA-2-HM-S-2200, 5-CA-MCBX-NDM and 5-COOH-S-2200 were identified. In the plasma, 2-COOH-S-2200, 5-COOH-S-2200, 5-CA-S-2200-NHM and 5-CA-S-2200-NDM were detected (Kendrick, Farrell & Murphy, 2012).

In another biotransformation study, four groups of Hannover Wistar (Crl) rats (four of each sex per group) were administered [benzyl-^{14}C]mandestrobin (purity 98.9%) orally by gavage (in 0.5% aqueous methylcellulose) at 5 mg/kg bw per day for up to 14 consecutive days. Metabolites were determined by radio-HPLC and LC-MS in urine, faeces, liver, kidney and plasma, from samples pooled per time point, sex and dose group. Metabolites in urine and faeces were determined in pooled samples after a single (0–24 hours after dosing) or repeated administration (312–336 hours after the first dose) of radiolabelled mandestrobin. Metabolites in liver, kidney and plasma were determined at 2 hours after a single dose and repeated doses (314 hours).

In urine, the major metabolites following the administration of both single and repeated doses were 5-CA-S-2200-NHM and 5-CA-MCBX-NDM. Other metabolites following single dosing included 5-COOH-S-2200, 5-CA-2-HM-S-2200-NHM, 5-CA-2-HM-S-2200 and 4-OH-S-2200-GlucA. Following repeated administration, 2-COOH-S-2200, 5-COOH-S-2200 and 4-OH-S-2200-GlucA were especially prominent (Table 8).

In faeces, the major metabolites following both single and repeated daily doses of mandestrobin were 5-CA-S-2200-NHM, 5-CA-2-HM-S-2200-NHM, 5-CA-2-HM-S-2200, 5-CA-MCBX-NDM, 4-OH-S-2200 and 5-COOH-S-2200. Other metabolites identified following single dosing included 5-CA-2-HM-MCBX and 5-CA-S-2200-NDM. Following repeated administration, De-Xy-S-2200 was prominent, particularly in the female profile. Overall, although quantitative differences were apparent in the proportions of some metabolites between male and female profiles, no major qualitative differences were evident. Repeated administration did not significantly affect the profile of the metabolites observed (Table 8).

Table 8. Metabolites in pooled excreta following single or repeated oral administration of radiolabelled mandestrobin to rats at a dose of 5 mg/kg bw

	% of administered dose							
	Urine				**Faeces**			
	0–24 hours (single)		**312–336 hours (repeated)**		**0–24 hours (single)**		**312–336 hours (repeated)**	
Metabolite identity[a]	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Mandestrobin (S-2200)	ND	ND	ND	ND	0.148	0.097	0.009	0.012
2-COOH-S-2200	0.149	ND	0.047	0.102	0.189	0.046	0.106	0.040
4-OH-S-2200	ND	ND	ND	ND	0.695	0.896	0.547	1.164
4-OH-S-2200-GlucA	0.242	1.048	0.023	0.147	ND	ND	ND	ND
5-CA-2-HM-MCBX	0.105	ND	ND	0.016	0.669	0.139	0.214	0.029
5-CA-2-HM-MCBX(-2H)	0.115	ND	0.026	ND	ND	ND	ND	ND
5-CA-2-HM-S-2200	0.328	0.299	0.063	0.060	1.506	1.150	0.408	0.298
5-CA-2-HM-S-2200-NHM	0.365	0.170	0.060	0.039	2.403	0.521	0.458	0.114
5-CA-MCBX-NDM	0.666	0.475	0.169	0.099	1.157	0.314	0.360	0.128
5-CA-S-2200-NDM	0.141	0.167	0.043	0.044	0.497	0.275	0.179	0.154
5-CA-S-2200-NHM	1.713	1.946	0.209	0.403	6.747	3.693	1.471	1.266
5-COOH-S-2200	0.471	0.535	0.040	0.080	1.715	1.229	0.677	0.830
COOH-S-2200 glucuronide	ND	0.186	0.010	0.051	ND	ND	ND	ND
COOH-S-2200 glucuronide	ND	ND	0.010	ND	ND	ND	ND	ND
COOH-S-2200 glucuronide	0.090	0.412	0.011	0.059	ND	ND	ND	ND
De-Xy-S-2200	ND	ND	ND	ND	0.310	0.446	0.157	1.053
% of dose	6.881	7.741	1.352	1.361	17.64	9.594	5.784	5.529

F: female; LC-MS: liquid chromatography with mass spectrometry; M: male; ND: not detected
[a] Confirmed by LC-MS.
Source: Kendrick & Farrell (2012)

In plasma from males administered a single dose, almost double the number of metabolites were identified compared with females. In animals receiving 14 daily doses, a greater number of metabolites could be identified. Although quantitative differences were observed in the proportion of metabolites between males and females, there were no major qualitative differences between sexes or between single and repeated dosing (Table 9).

In the liver, 5-CA-S-2200-NDM and 5-COOH-S-2200 were the major metabolites following a single-dose administration in both aqueous and organic extracts from males and females. Other metabolites in both sexes were De-Xy-S-2200, 5-CA-MCBX-NDM, 5-CA-S-2200-NHM, 5-CA-2-HM-S-2200-NHM, 5-CA-2-HM-S-2200 and 2-COOH-S-2200. Following the repeated administration, a greater number of metabolites were prominent in aqueous liver extract profiles from male and female animals. However, the same known metabolites were the most prominent (De-Xy-S-2200, 5-CA-MCBX-NDM, 5-CA-S-2200-NHM, 5-CA-S-2200-NDM and 5-COOH-S-2200). The next most prominent metabolites included "Unknowns" and 5-CA-2-HM-S-2200-NHM, 5-CA-2-HM-S-2200 and 2-COOH-S-2200. In the organic extract, metabolites De-Xy-S-2200, 5-CA-S-2200-NHM, 5-CA-S-2200-NDM and 5-COOH-S-2200 were the most abundant in both males and females. An additional region of interest in the female metabolic profile was assigned the identity of 5-CA-MCBX-NDM. Therefore, although quantitative differences existed in the proportions of some metabolites, there were

no major qualitative differences in the liver between the sexes. Repeated administration did not significantly alter the metabolic profile (Table 10).

Table 9. Metabolites in plasma following single or repeated oral administration of radiolabelled mandestrobin to rats at a dose of 5 mg/kg bw

	Concentration in plasma (ng equivalents/g)			
	2 hours (single)		314 hours (repeated)	
Metabolite identity[a]	Males	Females	Males	Females
2-COOH-S-2200	22.65	ND	108.5	ND
4-OH-S-2200-GlucA	73.77	57.94	186.7	283.1
5-CA-S-2200-NDM	28.33	ND	25.64	27.14
5-CA-S-2200-NHM	54.86	ND	49.21	81.41
5-COOH-S-2200	116.0	65.43	131.1	194.1
COOH-S-2200 glucuronide	ND	ND	62.57	ND
COOH-S-2200 glucuronide	19.92	ND	14.22	ND
COOH-S-2200 glucuronide	66.54	ND	ND	44.53
UK (Rt 31.3)	ND	ND	66.58	ND
UK (Rt 33.9)	87.63	ND	94.28	91.50
UK (Rt 37.5–38.1)	138.2	<LOQ	147.4	67.50
UK (Rt 38.5–39.3)	81.01	55.04	81.04	57.75
Total concentration	778.2	229.7	1 215	869.8

LC-MS: liquid chromatography with mass spectrometry; LOQ: limit of quantification; ND: not detected; Rt: retention time; UK: unknown

[a] Confirmed by LC-MS.

Source: Kendrick & Farrell (2012)

Table 10. Metabolites in liver following single or repeated oral administration of radiolabelled mandestrobin to rats at a dose of 5 mg/kg bw

	Concentration in liver (ng equivalents/g)							
	2 hours				314 hours			
	Aqueous phase		Organic phase		Aqueous phase		Organic phase	
Metabolite identity[a]	Males	Females	Males	Females	Males	Females	Males	Females
2-COOH-S-2200	130.9	ND	ND	ND	172.2	32.15	ND	ND
5-CA-2-HM-S-2200	176.5	54.77	ND	ND	324.3	328.1	ND	ND
5-CA-2-HM-S-2200-NHM	88.25	30.20	ND	ND	320.2	176.4	ND	ND
5-CA-MCBX-NDM	250.7	105.7	ND	ND	1 275	812.0	1 500	580.4
5-CA-S-2200-NDM	1 393	651.2	2 710	1 016	1 331	779.1	3 885	1 787
5-CA-S-2200-NHM	303.3	163.9	548.0	ND	412.9	826.0	1 135	1 790
5-COOH-S-2200	1 127	745.4	1 221	1 238	1 686	1 644	2 114	2 912
De-Xy-S-2200	441.7	332.5	534.5	ND	1 286	2 081	1 073	1 174

Metabolite identity[a]	Concentration in liver (ng equivalents/g)							
	2 hours				314 hours			
	Aqueous phase		Organic phase		Aqueous phase		Organic phase	
	Males	Females	Males	Females	Males	Females	Males	Females
UK (Rt 16.7–16.9)	ND	ND	ND	ND	359.5	212.7	ND	ND
UK (Rt 20.9–21.1)	ND	ND	ND	ND	67.47	136.0	ND	ND
UK (Rt 24.5)	ND	ND	ND	ND	110.8	96.45	ND	ND
UK (Rt 25.3–25.7)	ND	ND	ND	ND	95.67	117.9	ND	ND
UK (Rt 26.7–27.1)	ND	ND	ND	ND	129.9	53.59	ND	ND
UK (Rt 27.9)	ND	ND	ND	ND	138.0	79.14	ND	ND
UK (Rt 29.1–29.3)	274.8	54.77	ND	ND	677.7	158.3	ND	ND
UK (Rt 35.9–36.5)	144.4	84.53	ND	ND	272.9	153.3	ND	ND
UK (Rt 48.1–48.5)	483.9	ND	ND	ND	668.6	ND	ND	ND
Total concentration	4 814	2 223	5 014	2 254	10 070	8 244	10 070	8 244

LC-MS: liquid chromatography–mass spectrometry; ND: not detected; Rt: retention time; UK: unknown

[a] Confirmed by LC-MS.

Source: Kendrick & Farrell (2012)

In the kidney following the single oral administration of mandestrobin, males showed a greater number of metabolites compared with females. The major metabolites in the males were 5-CA-2-HM-S-2200-NHM, an unknown region of interest, 5-CA-S-2200-NHM and 5-COOH-S-2200. In females, 5-CA-S-2200-NHM and 5-COOH-S-2200 dominated.

Following repeated oral administration for 14 days, a greater number of metabolites in both male and female profiles were observed. In females, an unknown region of interest and 5-CA-MCBX-NDM were considered of note, in addition to those observed following single dosing (5-CA-S-2200-NHM and 5-COOH-S-2200).

Chromatographic resolution of the kidney metabolic profiles from male animals was much greater. The four most prominent metabolites observed in the female kidney were also prominent in the male profiles.

However, seven additional metabolites were also observed in the males. These were similar to those observed following single administration and were identified as De-Xy-S-2200, 5-CA-2-HM-S-2200-NHM and 5-CA-2-HM-S-2200. Four additional unidentified metabolites were present at concentrations above 100 ng equivalents/g. Overall, in the kidney, there were quantitative and qualitative differences in the metabolites observed between male and female animals and following both single and repeated administration of mandestrobin (Table 11) (Kendrick & Farrell, 2012).

In another study aimed at evaluating the metabolic reactions of (benzyl-^{14}C)-labelled *S*- and *R*-isomers of mandestrobin after a single application, two groups of Hannover Wistar (Crl) rats (four of each sex per group) were administered [benzyl-^{14}C]mandestrobin, *R*-isomer (purity 99.2%) or *S*-isomer (purity 99.7%), in a single oral gavage dose (in 0.5% aqueous methylcellulose) of 5 mg/kg bw. Identification of metabolites was performed by co-HPLC with known standards (ultraviolet absorption retention time compared with that from the refractive index chromatogram) and confirmed using LC-MS. Metabolites were determined in urine and faecal samples collected up to 3 and 4 days after administration, respectively. For both matrices, samples were pooled per time point and sex.

Twelve metabolites were identified and quantified.

Table 11. Metabolites in kidney following single or repeated oral administration of radiolabelled mandestrobin to rats at a dose of 5 mg/kg bw

	Concentration in kidney (ng equivalents/g)			
	2 hours (single)		314 hours (repeated)	
Metabolite identity[a]	**Males**	**Females**	**Males**	**Females**
2-COOH-S-2200	66.50	ND	72.79	ND
5-CA-2-HM-S-2200	85.22	ND	104.3	ND
5-CA-2-HM-S-2200-NHM	146.9	ND	135.9	ND
5-CA-MCBX-NDM	ND	ND	365.8	207.6
5-CA-S-2200-NDM	ND	ND	64.02	ND
5-CA-S-2200-NHM	310.6	158.3	372.2	719.9
5-COOH-S-2200	179.2	108.3	216.0	417.6
De-Xy-S-2200	83.35	ND	111.0	<LOQ
UK (Rt 24.9)	ND	ND	134.9	ND
UK (Rt 27.1)	ND	ND	157.4	ND
UK (Rt 28.1)	ND	ND	127.1	ND
UK (Rt 32.1–32.5)	ND	ND	130.4	171.4
UK (Rt 34.7–35.5)	105.1	ND	104.3	ND
Total concentration	1 101	402.5	2 371	1 597

LC-MS: liquid chromatography–mass spectrometry; LOQ: limit of quantification; ND: not detected; Rt: retention time; UK: unknown

[a] Confirmed by LC-MS.

Source: Kendrick & Farrell (2012)

In the group administered the *R*-isomer, 5-CA-S-2200-NHM was the predominant metabolite in excreta, accounting for 38.6% and 41.2% of the dose in males and females, respectively. The other major metabolites, 5-CA-MCBX-NDM, 4-OH-S-2200, 5-CA-S-2200-NDM and 5-CA-2-HM-S-2200-NHM, were present at 11.8% and 9.5%, 5.5% and 5.0%, 5.8% and 5.6%, and 6.4% and 4.3% of the dose in males and females, respectively. The results showed that the amounts of metabolites in urine and faecal samples between males and females were slightly different, depending on the metabolite (Table 12).

Table 12. Metabolites identified in excreta 3 days (R-isomer) or 4 days (S-isomer) after administration of radiolabelled mandestrobin to rats at a dose of 5 mg/kg bw[a]

	% of administered dose			
	***R*-isomer**		***S*-isomer**	
Metabolite	**Males**	**Females**	**Males**	**Females**
Urine				
De-Xy-S-2200 (UM-7)	0.6	0.7	0.6	0.7
DX-CA-S-2200 (UM-8)	0.5	0.4	ND	ND
5-CA-2-HM-MCBX (UM-12)	1.0	0.6	1.8	1.8
5-CA-2-HM-S-2200-NHM (UM-14)	1.3	1.2	0.6	0.6
5-CA-2-HM-S-2200 (UM-16)	0.7	0.5	1.1	1.6

Metabolite	% of administered dose			
	R-isomer		*S*-isomer	
	Males	Females	Males	Females
4-OH-S-2200-GlucA (UM-18)	0.3	4.3	1.3	3.4
5-CA-MCBX-NDM (UM-21)	3.9	4.3	0.6	2.6
2-COOH-S-2200 (UM-23)	0.1	0.1	0.2	0.1
4-OH-S-2200 (UM-25)	0.0	0.6	0.0	0.0
5-CA-S-2200-NHM (UM-26)	5.7	11.7	1.3	1.7
5-CA-S-2200-NDM (UM-27)	0.5	1.1	ND	0.3
5-COOH-S-2200 (UM-28)	0.1	0.5	1.2	3.8
Others[b]	6.9	6.1	6.6	8.2
Total urine	21.6	32.1	15.3	24.8
Faeces				
De-Xy-S-2200 (FM-7)	1.2	1.1	3.1	3.6
DX-CA-S-2200 (FM-8)	ND	0.3	ND	ND
5-CA-2-HM-MCBX (FM-12)	1.1	0.4	1.8	0.9
5-CA-2-HM-S-2200-NHM (FM-14)	5.1	3.1	1.8	0.8
5-CA-2-HM-S-2200 (FM-16)	1.3	1.8	4.7	4.9
4-OH-S-2200-GlucA (FM-18)	0.1	0.2	1.8	0.5
5-CA-MCBX-NDM (FM-21)	7.9	5.2	1.2	0.8
2-COOH-S-2200 (FM-23)	0.7	0.7	1.8	1.3
4-OH-S-2200 (FM-25)	5.5	4.4	23.1	28.4
5-CA-S-2200-NHM (FM-26)	32.9	29.5	7.0	5.7
5-CA-S-2200-NDM (FM-27)	5.3	4.5	1.5	1.7
5-COOH-S-2200 (FM-28)	1.9	3.0	18.0	11.6
Others[c]	6.5	4.5	6.9	4.5
Subtotal	69.5	58.7	72.7	64.7
Unextractable	3.9	4.1	3.8	5.1
Total faeces	73.4	62.8	76.5	69.8

bw: body weight; FM: faecal metabolite; ND: not detected; UM: urinary metabolite

[a] Data were obtained from the pooled sample of four rats.

[b] Sum of the 16 unidentified metabolites, which were below 3.6% of the dose.

[c] Sum of the nine unidentified metabolites, which were below 2.7% of the dose.

Source: Mikata (2011)

In the group administered the *S*-isomer, 4-OH-S-2200 was the predominant metabolite in excreta (faeces), accounting for 23.1% and 28.4% of the dose in males and females, respectively. The other metabolites, 5-COOH-S-2200, 5-CA-S-2200-NHM, 5-CA-2-HM-S-2200 and 4-OH-S-2200-GlucA, occurred in excreta at 19.2% and 15.4%, 8.3% and 7.4%, 5.8% and 6.5%, and 3.1% and 3.9% of the dose in males and females, respectively (Table 12).

The *R*-isomer was absorbed into the body and metabolized to the predominant metabolite, 5-CA-S-2200-NHM, via 5-COOH-S-2200. Thereafter, 5-CA-S-2200-NHM and its metabolites, 5-CA-2-HM-S-2200-NHM, 5-CA-S-2200-NDM and 5-CA-MCBX-NDM, were rapidly and almost completely

eliminated from the body in urine and faeces. No potential for accumulation in tissues was demonstrated.

The rate of excretion of the *S*-isomer was slower than that of the *R*-isomer. The major metabolite in the *S*-isomer dose group was 4-OH-S-2200, and its glucuronide conjugate (4-OH-S-2200-GlucA) was detected after enzyme hydrolysis. It is likely that 4-OH-S-2200-GlucA is hydrolysed in the small intestine, subjected to enterohepatic circulation and finally excreted as 4-OH-S-2200 in faeces. The enterohepatic recirculation slowed excretion of the *S*-isomer-derived radioactivity (Mikata, 2011).

The proposed metabolic pathway in rats is presented in Fig. 3.

Fig. 3. Proposed metabolic pathway of mandestrobin (S-2200) in rats

5-CA-2-HM-MCBX - 2H
5-CA-2-HM-MCBX
MCBX
4-OH-S-2200
4-OH-S-2200-GlucA
De-Xy-S-2200
DX-CA-S-2200
5-COOH-S-2200
5-CH$_2$OH-S-2200
S-2200
2-CH$_2$OH-S-2200
2-COOH-S-2200
5-CA-2-HM-S-2200-NHM
5-CA-2-HM-S-2200
5-CA-S-2200-NHM
5-CA-S-2200-NDM
5-CA-MCBX-NDM
5-COOH-S-2200-GlucA
and/or
2-COOH-S-2200-GlucA
5-COOH-S-2200-methylated
and/or
2-COOH-S-2200-methylated

Source: Kendrick, Farrell & Murphy (2012)

2. Toxicological studies

2.1 Acute toxicity

The results of acute oral, dermal and inhalation toxicity studies with mandestrobin, along with the results of dermal and eye irritation and skin sensitization studies, are summarized in Table 13, and the studies are described in more detail below.

(a) Lethal doses

In a limit test, mandestrobin (purity 93.4%) was administered sequentially to two groups of female Wistar rats (three rats per group) via gavage at 2000 mg/kg bw (in 0.5% weight per volume [w/v] aqueous methylcellulose; dosing volume 10 mL/kg bw). Mortality and clinical signs were recorded at 10 and 30 minutes and 1, 2 and 4 hours after treatment and once daily for 14 days thereafter. Body weights were determined on the day of dosing (day 0), day 7 and day 14.

Table 13. Acute toxicity of mandestrobin

Species	Strain	Sex	Route	Purity (%)	Result	Reference
Rat	Wistar	Female	Oral	93.4	LD_{50} > 2 000 mg/kg bw	Asano (2010a)
Rat	Wistar	Male and female	Dermal	93.4	LD_{50} > 2 000 mg/kg bw	Asano (2010b)
Rat	Wistar	Male and female	Inhalation	93.4	LC_{50} > 4.96 mg/L (maximum attainable concentration)	Deguchi (2010)
Rabbit	New Zealand white	Male	Skin irritation	93.4	Not irritating	Ota (2010a)
Rabbit	New Zealand white	Male	Eye irritation	93.4	Mildly irritating	Ota (2010b)
Guinea-pig	Hartley	Female	Skin sensitization	93.4	Not sensitizing (Magnusson & Kligman)	Ota (2010c)

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

No mortality was noted during the study. Clinical signs appeared from 4 hours after administration and included stains around the anus and liquid stools containing a white compound; the signs disappeared on day 1. Body weight was not affected, and no gross abnormalities at necropsy were found. The acute oral median lethal dose (LD_{50}) of mandestrobin in female rats was greater than 2000 mg/kg bw (Asano, 2010a).

Five male and five female Wistar rats were treated dermally with mandestrobin (purity 93.4%) spread on a gauze pad moistened with water at a dose of 2000 mg/kg bw. The gauze pad was applied to the shaved skin in an area of approximately 4 $cm^2 \times 5$ cm^2, covered with an impermeable sheet and held in place with surgical tape for 24 hours, after which it was removed. Mortality and clinical signs were recorded at 10 and 30 minutes and 1, 2 and 4 hours after initiation of treatment and then daily for 14 days. Body weights were recorded on the day of dosing and again at 7 and 14 days post-dosing.

No mortality and no clinical signs were observed. Body weights of the rats were unaffected. No abnormalities were detected at necropsy. The acute dermal LD_{50} of mandestrobin in rats was greater than 2000 mg/kg bw for both sexes (Asano, 2010b).

Five male and five female Wistar rats were exposed to a 4-hour nose-only dust aerosol atmosphere of mandestrobin (purity 93.4%) at a concentration of 4.96 mg/L (maximum attainable concentration, analytically and gravimetrically determined). The mass median aerodynamic diameter was 3.80 µm, and the geometric standard deviation was 2.65. Mortality and clinical signs were recorded at 0.5, 1, 2, 3 and 4 hours after initiation of exposure, immediately and hourly for up to 2 hours after termination of exposure, and daily for 14 days thereafter. Body weights were recorded on days 0 (before exposure), 1, 3, 7 and 14 during the observation period.

No mortality was noted. Clinical signs included wet fur and stains around the nose in both sexes, which disappeared within 2 hours post-dosing. This finding is considered to be a result of the restraint procedure and not of toxicity related to treatment. Body weight was unaffected by treatment. No abnormalities were detected at necropsy. Therefore, the acute inhalation median lethal concentration

(LC_{50}) of mandestrobin in rats was greater than 4.96 mg/L (the maximum attainable concentration) for both sexes (Deguchi, 2010).

(b) Dermal irritation

Mandestrobin (purity 93.4%) was applied on 6.25 cm^2 of clipped skin of three male New Zealand white rabbits at a dose of 0.5 g (moistened with 0.4 mL of corn oil) under a semi-occlusive bandage for 4 hours. The animals were monitored for skin reactions of erythema and oedema using the Draize method at 1, 24, 48 and 72 hours after patch removal.

No clinical signs or skin irritation reactions were observed in any animal during the observation period of 72 hours after the removal of the patches (Ota, 2010a).

(c) Ocular irritation

Mandestrobin (purity 93.4%) was tested in six male albino New Zealand white rabbits for eye irritation. Three animals were used for the unwashed group, and the other three for the washed group. A volume of 0.1 mL (0.062 g by weight) was instilled into the conjunctival sac of the right eye of each animal. The treated eye of each of the three animals in the washed group was flushed after 30 seconds to remove the test material, whereas the treated eye of each of the three animals in the unwashed group was not flushed. The animals were monitored for ocular lesions using the Draize scale at 1, 24, 48 and 72 hours after application.

No clinical signs were noted in any of the animals during the experimental period. In the unwashed group, mandestrobin induced very slight conjunctival redness (grade 1), chemosis (grade 1), discharge (grades 1–2) and corneal opacity (grade: intensity 1, area 1) in all three animals. Additionally, congestion in the iris (grade 1) was observed in two of the three animals. All ocular reactions had disappeared by 72 hours after application. The maximum mean total score of the irritant was 10.3 at 24 hours after application. In the washed group, all animals exhibited conjunctival redness (grade 1) and chemosis (grade 1). All ocular reactions had disappeared by 48 hours after application. Washing effect was observed in the eye irrigation test.

Mandestrobin is judged to be mildly irritating to the eye of the rabbit (Ota, 2010b).

(d) Dermal sensitization

In a dermal sensitization study conducted following the Magnusson and Kligman method, 5% mandestrobin (purity 93.4%) in corn oil was injected intradermally into 20 female Hartley guinea-pigs (with and without Freund's Complete Adjuvant). One week later, a topical induction of 25% mandestrobin in acetone was performed for 48 hours. Two weeks after the topical induction, the animals were challenged by topical application of 25% mandestrobin in acetone for 24 hours. The application site was assessed 24 and 48 hours after patch removal. α-Hexylcinnamaldehyde was used as the positive control.

No dermal response was observed in any animal in the treated group (sensitization rate: 0%). In the control group, no skin reactions were noted in any of the 10 animals, whereas in the positive control group (α-hexylcinnamaldehyde), a positive response was recorded.

Mandestrobin is not sensitizing in guinea-pigs (Ota, 2010c).

2.2 Short-term studies of toxicity

The short-term toxicity of mandestrobin was evaluated in mice, rats and dogs.

(a) Oral administration

Mice

In a dose selection study, mandestrobin (purity 99%) was administered to four groups of approximately 6-week-old CD-1 mice (main: 10 mice of each sex per group; satellite: five mice of each sex per group) at a dietary concentration of 0, 350, 3500 or 7000 parts per million (ppm) (equal to 0, 49.2, 501.0 and 1036.8 mg/kg bw per day for males and 0, 63.5, 595.4 and 1306.9 mg/kg bw per day for females, respectively) for 14 days. Stability of the test article and achieved concentration were not evaluated. All animals were observed for viability/mortality and clinical signs daily. Feed consumption was recorded daily, and body weight was determined on days −7, 1, 4, 9 and 14. Blood samples were taken at termination of the study for haematology and clinical chemistry, including iron measurements. All animals were subjected to detailed postmortem gross examination. Organ weights were recorded, and an extensive list of tissues was examined histopathologically. The study did not follow a specific guideline and was not performed under GLP, but it was conducted in the spirit of GLP.

There were no decedents during the study, and clinical observations were generally minor findings considered unrelated to treatment with the test article, such as thinning fur and sores/lesions. Females from the main group given 7000 ppm showed a statistically significant increase in body weight gain relative to controls from day 9 to day 15 and from day 1 to day 15. This finding was considered to be of no toxicological relevance, as no effect on body weight was observed. Haematological investigation showed a statistically significantly higher platelet count (25%) and plasma sodium level (2%) relative to controls in males at 7000 ppm. The statistically significantly higher platelet count was mainly due to one animal (#35). This animal also had a high reticulocyte count, lower haemoglobin concentration, lower red blood cell count and lower packed cell volume in comparison with the rest of the group. These findings are likely associated with the clinical observations and pathological findings that indicate injury to the urogenital area of this animal. Microscopically increased haematopoiesis in the bone marrow and spleen were noted. The group mean platelet count for high-dose males was within the 95% reference range of the historical control data.

Overall, these isolated findings were not considered to be toxicologically relevant.

The unsaturated iron binding capacity in high-dose males was 2-fold lower than in controls, and a statistically significant dose–response relationship in males was indicated. However, none of the group mean differences was statistically significantly different from controls as a result of large standard deviations. This finding is not considered to be toxicologically relevant because of the absence of any similar changes in the iron and total iron binding capacity measurements.

A statistically significant increase in relative liver weight (i.e. adjusted to body weight) was observed in males and females given 7000 ppm (11% and 14%, respectively). In females at 3500 ppm, relative liver weight was statistically significantly increased by 14%. Given the absence of changes in biochemistry or microscopic findings indicative of liver damage, the increase in liver weight was considered to be an adaptive change.

Based on the above results, the no-observed-adverse-effect level (NOAEL) for mandestrobin in this study was 7000 ppm (equal to 1036.8 mg/kg bw per day), the highest dose tested (Shaw, 2009a).

In a 13-week study, mandestrobin (purity 93.4%) was administered to four groups of 7-week-old CD-1 mice (12 mice of each sex per group) at a dietary concentration of 0, 1750, 3500 or 7000 ppm (equal to 0, 204, 405 and 807 mg/kg bw per day for males and 0, 252, 529 and 1111 mg/kg bw per day for females, respectively). Achieved concentrations and homogeneity were verified by chemical analysis. All animals were observed for viability/mortality twice daily. Clinical signs were recorded once daily during the treatment. Feed consumption and body weights were recorded weekly. Blood samples were taken at termination of the study for haematology and clinical chemistry. All animals were subjected to detailed postmortem gross examination. Organ weights (adrenals, brain, heart, kidneys, liver, ovaries, spleen, testes and epididymides, thymus, uterus and cervix) were recorded, and an extensive list of tissues was examined histopathologically.

No mortality or clinical signs were noted during the study. No treatment-related effects on feed consumption were observed. No effects on body weight or body weight gain were noted in males. Small, not statistically significant reductions in body weight gain were recorded in females given 1750 and 3500 ppm; the reduction was statistically significant in females given 7000 ppm (Table 14).

Table 14. Key findings of the 13-week dietary toxicity study in mice

	Males					Females				
Parameter	**0 ppm**	**1 750 ppm**	**3 500 ppm**	**7 000 ppm**	**HCD[a] (mean; range) or DR**	**0 ppm**	**1 750 ppm**	**3 500 ppm**	**7 000 ppm**	**HCD[a] (mean; range) or DR**
Body weight gain, weeks 0–13 (g)	10.5	9.6	8.9	9.7	–	8.3	7.5	7.4	6.1*	–
Feed consumption, mean, weeks 0–13 (g/mouse per day)	5.2	5.0	4.9	5.0	–	4.6	4.5	4.8	4.9	–
Initial body weight (g)	33.1	34.1	33.8	34.7	–	25.2	24.8	24.7	24.6	–
Terminal body weight (g)	43.6	43.7	42.7	44.4	–	33.5	32.3	32.1	30.7	–
Haematology										
Hb (g/dL)	14.6	14.3	13.2**	14.1	13.4; 12.0–15.1	14.6	14.9	14.8	14.1	–
PCV (%)	44.8	44.6	41.6*	44.9	44.5; 38.0–50.4	45.1	45.8	45.8	44.3	–
PLT (10^9/L)	1 292	1 756**	1 450	1 666*	1 390; 941–1 721	1 555	1 351	1 390	1 526	–
MCHC (g/dL)	32.3	32.1	31.7	31.5	DR*	32.4	32.5	32.3	31.9	–
Clinical pathology										
GLUC (mmol/L)	8.9	9.7	9.3	9.1	–	10.8	10.3	9.3	9.1*	9.7; 7.9–12.0
Cl (mmol/L)	109	109	109	107	DR*	112	112	112	112	–
Urea (mmol/L)	6.4	6.3	6.7	7.3	DR*	7.1	6.8	6.6	6.6	–
T Bili (µmol/L)	2.5	1.8	2.1	1.8	DR*	1.7	1.7	1.7	1.9	–
Organ weights										
Absolute liver weight (g)	2.00	2.11	2.14	2.35***	–	1.58	1.74	1.64	1.81	–
% change	–	*5.5*	*6.9*	*17.4*	–	–	*10.1*	*3.9*	*14.5*	–
Relative liver weight (%)	4.55	4.73	4.95**	5.22***	–	4.65	5.20*	5.02	5.66***	–
% change	–	*4.0*	*8.8*	*14.7*	–	–	*11.8*	*8.0*	*21.7*	–
Histopathology of liver										
No. examined	*12*	*12*	*12*	*12*	–	*12*	*12*	*12*	*12*	–
Focal necrosis	2	1	4	5	–	3	2	6	3	–

Parameter	Males 0 ppm	Males 1 750 ppm	Males 3 500 ppm	Males 7 000 ppm	Males HCD[a] (mean; range) or DR	Females 0 ppm	Females 1 750 ppm	Females 3 500 ppm	Females 7 000 ppm	Females HCD[a] (mean; range) or DR
Inflammatory cell foci	11	11	11	11	–	12	12	12	12	–
Mitotic figures	0	1	0	0	–	0	0	0	2	–
Hepatocyte vacuolation	0	0	0	0	–	0	1	0	0	–
Glycogen vacuolation	8	8	7	9	–	12	10	11	12	–
Pigmented histiocytes	0	2	0	0	–	0	1	0	1	–
Agonal congestion/ haemorrhage	0	0	1	0	–	0	2	0	0	–
Haematopoiesis	0	1	0	0	–	0	0	0	0	–

Cl: chloride; DR: dose–response test; GLUC: glucose; Hb: haemoglobin; HCD: historical control data; MCHC: mean cell haemoglobin concentration; PCV: packed cell volume; PLT: platelets; ppm: parts per million; T Bili: total bilirubin; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ (analysis of variance, dose–response and Dunnett's)
[a] Historical control data: five studies, 34 animals of same strain and age housed at this laboratory, from 2003.
Source: Beck (2011a)

Mandestrobin administration caused no obvious or adverse effects on haematology or clinical chemistry parameters. Statistical analysis revealed the following statistically significant changes: decreases in haemoglobin concentration and packed cell volume in males given 3500 ppm, and increased platelets in males given 1750 and 7000 ppm. However, these effects were not dose related and in general were within or close to historical control ranges, and therefore they were not considered to be treatment related. The statistically significant dose-responsive reduction in mean cell haemoglobin concentration noted in males was also considered not to be treatment related, as the differences observed were small and not statistically significant for pairwise comparison. In clinical chemistry, a significant decrease in mean glucose was noted in 7000 ppm females, but it was not considered to be of toxicological relevance because, although noted at the highest dose, the value was within the historical control range. Dose-responsive changes were noted for chloride, urea and total bilirubin, but were considered not to be treatment related, as the changes were minor and not statistically significant for any of the individual doses (Table 14).

A statistically significant increase in relative spleen weight was noted in males given 3500 ppm; however, in the absence of a dose–response relationship, the change was not considered to be treatment related. Increases in absolute and relative liver weights were noted in both sexes of all treated groups; however, these increases were not accompanied by any clinical chemistry or histopathological findings. As a result, the observed increased liver weight is considered to be an adaptive change related to mandestrobin metabolism and not an adverse toxic effect.[1] No other treatment-related macroscopic or microscopic findings were noted.

The NOAEL was 3500 ppm (equal to 529 mg/kg bw per day), based on reduced body weight gain in females at 7000 ppm (equal to 1111 mg/kg bw per day) (Beck, 2011a).

[1] The liver weight increase is considered to be due to liver enzyme induction (cytochrome P450 2B [CYP2B]), via activation of the constitutive androstane receptor (CAR) by mandestrobin, as evidenced by mode of action work presented in section 2.6(c) below.

Rats

Mandestrobin (purity 93.4%) was administered to five groups of 7-week-old Wistar rats (12 rats of each sex per group) at a dietary concentration of 0, 800, 4000, 10 000 or 20 000 ppm (equal to 0, 54, 283, 743 and 1545 mg/kg bw per day for males and 0, 62, 320, 788 and 1886 mg/kg bw per day for females, respectively) for 90 days. Achieved concentrations and homogeneity were verified by analysis. All animals were checked for overt clinical signs by daily general cage-side observations. Detailed clinical observations were performed once weekly during the study. Ophthalmological examination was performed on all animals in the control (0 ppm) and high-dose (20 000 ppm) groups in week 12. Body weights and feed consumption were measured weekly during the study. Functional observational battery (FOB) tests were performed weekly on all animals during the study; motor activity assessment was performed in week 12. Blood samples were taken after fasting at termination of the study for assessment of blood biochemical and haematological parameters. Urine analyses were conducted in the first six numbered males and females in week 12. All animals were subjected to detailed postmortem gross examination, and all abnormalities were recorded. Organ weights (adrenals, brain, heart, kidneys, liver, ovaries, spleen, testes and epididymides, thymus and uterus) were recorded. Histological evaluation was performed on an extensive list of organs and tissues.

One female from the 4000 ppm group died; although the cause of death could not be established, microscopic findings suggested that the death was not treatment related. There were no treatment-related clinical signs during the study. There was no treatment-related effect on feed consumption at any dose. Reduced body weight (9% for males and 4% for females) and body weight gain (15% for males and 11% for females) were recorded at the end of treatment for both sexes given 20 000 ppm (Table 15). No treatment-related differences between the controls and any treated group were noted in urine analysis, motor activity or ophthalmological examination. Forelimb grip strength was statistically significantly reduced in male animals in the highest-dose group. In the absence of any other effect, this isolated finding in one sex was considered incidental. Some changes in haematological parameters attained statistical significance at the highest dose; however, the changes were minor and within the range of historical control data.

Table 15. Key body weight, FOB, clinical chemistry and haematological findings of the 90-day dietary toxicity study in rats

	Males						Females					
Finding	**0 ppm**	**800 ppm**	**4 000 ppm**	**10 000 ppm**	**20 000 ppm**	**HCR[a] or DR**	**0 ppm**	**800 ppm**	**4 000 ppm**	**10 000 ppm**	**20 000 ppm**	**HCR[a] or DR**
Body weight (g)												
Week 0	195.3	193.3	188.2	189.9	189.6	–	150.5	145.7	149.7	146.2	149.3	–
Week 13	383.4	397.4	381.2	373.8	349.9	DR**	233.7	225.0	225.6	231.6	223.5	–
Body weight gain (g)												
Weeks 0–13	188.1	204.1	193.0	183.9	160.2	DR**	83.1	79.3	78.6	85.5	74.2	–
Functional observational battery												
Mean forelimb grip strength (kg)	1.023	1.008	1.052	1.070	0.760*	–	0.751	0.787	0.707	0.757	0.742	–
SD	0.270	0.253	0.292	0.149	0.235	–	0.207	0.178	0.205	0.264	0.233	–
Mean hindlimb grip	0.613	0.642	0.625	0.732	0.665	–	0.620	0.612	0.607	0.620	0.633	–

Finding	Males						Females					
	0 ppm	**800 ppm**	**4 000 ppm**	**10 000 ppm**	**20 000 ppm**	**HCR[a] or DR**	**0 ppm**	**800 ppm**	**4 000 ppm**	**10 000 ppm**	**20 000 ppm**	**HCR[a] or DR**
strength (kg)												
SD	0.126	0.191	0.178	0.132	0.118	–	0.124	0.157	0.161	0.117	0.373	–
Haematology												
Hb (g/dL)	16.7	16.5	16.5	16.6	16.2**	14.5–18.0	15.7	15.7	15.3	15.7	15.0*	14.2–17.0
MCH (pg)	18.2	18.1	18.0	17.9	17.5*	16.6–19.8	18.8	18.4	18.6	18.8	18.4	–
MCHC (g/dL)	35.5	35.4	34.9	35.1	34.6*	31.5–36.2	34.9	34.3	34.5	34.0	34.2	–
Neutrophils (10^9/L)	1.3	1.0	1.1	1.2	0.9**	0.5–3.0	0.6	0.5	0.6	0.6	0.6	–
Monocytes (10^9/L)	0.2	0.2	0.2	0.1	0.1*	0.0–0.3	0.1	0.1	0.1	0.1	0.1	–
Clinical chemistry												
AST (IU/L)	75	65*	66	69	62**	50–87	68	65	64	62	56**	49–103
ALT (IU/L)	46	33*	32*	39	32*	26–69	29	29	32	30	28	–
GGT (IU/L)	2	2	2	2	5**	–	2	2	2	3	4**	–
Na (mmol/L)	145	144*	144	144*	145	132–148	144	143	143	143	144	–
K (mmol/L)	4.7	4.8	4.7	4.7	4.9	–	4.1	4.1	4.1	4.7**	4.7**	3.5–5.0
Ca (mmol/L)	2.71	2.67	2.66	2.71	2.74	–	2.73	2.71	2.71	2.85**	2.88***	2.54–2.91
Total protein (g/L)	69	70	70	71	71	–	71	70	70	74	77**	63–78
Albumin (g/L)	45	45	46	47	47	DR**	48	49	47	50	51*	34–56
Total bilirubin (µmol/L)	2.1	2.0	1.5*	1.4*	1.4**	0.5–2.9	2.1	1.8	1.4**	2.0	2.2	1.0–3.5
Total cholesterol (mmol/L)	2.0	2.2	2.3	2.6**	2.8***	–	1.5	1.7	1.8	2.5***	2.6***	–
Creatinine (µmol/L)	37	35	35	36	36	–	40	40	38	40	34**	26–51

ALT: alanine aminotransferase; AST: aspartate aminotransferase; DR: significant dose–response test; GGT: gamma-glutamyl transferase; Hb: haemoglobin; HCR: historical control range; IU: international units; MCH: mean cell haemoglobin; MCHC: mean cell haemoglobin concentration; ppm: parts per million; SD: standard deviation; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$

[a] Historical control data from studies performed between March 2005 and January 2008. For haematology and clinical chemistry, 15 different studies were available (203 and 223 animals, respectively).

Source: Beck (2011b)

Total cholesterol was increased (up to 73%) compared with controls, and the increase was statistically significant in both sexes receiving 10 000 and 20 000 ppm. Gamma-glutamyl transferase (GGT) activity was increased by 2–2.5 times compared with controls, and the increase was statistically significant in both sexes given 20 000 ppm. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were statistically significantly decreased in some male treated groups (approximately 18% or less), but there was no dose–response relationship, and the decreased values were within the historical control range. Moreover, the apparent differences may be due to high control values, although they are also within the historical control range. Values for AST and ALT activities in treated males were similar to control female values. Therefore, in this context, the decreases in AST and ALT activities noted in males were not considered to be of any toxicological relevance. A statistically significant decrease in AST activity (18%) was noted in 20 000 ppm females; however, this was also within the historical control range and therefore was not considered to be toxicologically relevant. All other changes in clinical chemistry parameters were mild, within the range of historical control data, inconsistent between sexes and/or with no clear dose–response relationship.

Organ weight effects were noted only in the liver, for which increased absolute and/or relative weights were recorded in males of the 4000, 10 000 and 20 000 ppm groups and females of the 10 000 and 20 000 ppm groups. Mottled liver was also noted in males given 4000, 10 000 and 20 000 ppm. These changes were accompanied by hepatocyte hypertrophy in both sexes at 4000, 10 000 and 20 000 ppm. Incidence and severity increased with dose and correlated with large and/or mottled liver. Hepatocyte hypertrophy was characterized by hepatocytes with increased levels of pale, slightly granular cytoplasm. In the most severely affected livers, hypertrophy was recorded in hepatocytes in all zones of the liver. In less severely affected livers, there was no clear zonal distribution of the enlarged hepatocytes. Agonal congestion/haemorrhage was recorded for some treated males and was considered to be a consequence of the liver hypertrophy by the study author (Table 16).

Table 16. Key macroscopic and microscopic findings of the 90-day dietary toxicity study in rats

	Males					**Females**				
Finding	**0 ppm**	**800 ppm**	**4 000 ppm**	**10 000 ppm**	**20 000 ppm**	**0 ppm**	**800 ppm**	**4 000 ppm**	**10 000 ppm**	**20 000 ppm**
Body weight (g)	370.0	381.5	364.5	354.1	339.2	227.1	219.8	220.6	22.9	218.5
Absolute liver weight (g)	8.36	9.03	9.50***	10.18***	12.02***	5.80	5.53	6.08	6.79	8.34***
% change	–	*7.9*	*13.6*	*21.8*	*43.8*	–	*−4.7*	*4.8*	*17.0*	*43.8*
Relative liver weight (%)	2.26	2.37	2.61***	2.89***	3.54***	2.55	2.51	2.76	3.05***	3.81***
% change	–	*4.9*	*15.5*	*27.9*	*56.6*	–	*−1.6*	*8.2*	*19.6*	*49.4*
Macroscopic pathology: liver										
No. examined	*12*	*12*	*12*	*12*	*12*	*12*	*12*	*11*	*12*	*12*
Large	0	0	2	7	10	0	0	0	0	4
Mottled	0	0	1	3	5	0	0	0	0	0
Microscopic pathology: liver										
No. examined	*12*	*12*	*12*	*12*	*12*	*12*	*12*	*11*	*12*	*12*
Hepatocyte hypertrophy[a]										
Grade −	12	12	0	0	0	12	12	7	3	0
Grade 1	0	0	5	0	0	0	0	4	6	0
Grade 2	0	0	7	0	0	0	0	0	3	0

Finding	Males					Females				
	0 ppm	800 ppm	4 000 ppm	10 000 ppm	20 000 ppm	0 ppm	800 ppm	4 000 ppm	10 000 ppm	20 000 ppm
Grade 3	0	0	0	7	0	0	0	0	0	0
Grade 4	0	0	0	5	1	0	0	0	0	3
Grade 5	0	0	0	0	11	0	0	0	0	9
Agonal congestion/haemorrhage										
P[b]	0	0	1	3	5	2	0	0	0	0
Microscopic pathology: thyroid										
Follicular cell hypertrophy[a]										
No. examined	*12*	*12*	*12*	*12*	*12*	*12*	*12*	*11*	*12*	*12*
Grade −	10	10	6	3	5	10	11	7	7	6
Grade 1	2	2	6	8	5	2	1	4	5	6
Grade 2	0	0	0	1	2	0	0	0	0	0
Microscopic pathology: kidney										
Hyaline droplets[a]										
No. examined	*12*	*12*	*12*	*12*	*12*	*12*	*0*	*0*	*0*	*12*
Grade −	2	3	1	1	0	12	0	0	0	12
Grade 1	6	6	6	3	2	0	0	0	0	0
Grade 2	4	3	4	7	8	0	0	0	0	0
Grade 3	0	0	1	1	2	0	0	0	0	0
Kidney α_{2u}-globulin-positive reaction by immunohistochemistry[a]										
No. examined	*2*	*0*	*0*	*0*	*2*	*0*	*0*	*0*	*0*	*0*
Grade −	0	0	0	0	0	0	0	0	0	0
Grade 1	1	0	0	0	0	0	0	0	0	0
Grade 2	1	0	0	0	0	0	0	0	0	0
Grade 3	0	0	0	0	2	0	0	0	0	0

ppm: parts per million; ***: $P < 0.001$ (analysis of variance, dose–response and Dunnett's, Kruskal-Wallis, Jonckheere-Terpstra or Wilcoxon)

[a] "–": finding not present, 1 = minimal, 2 = slight, 3 = moderate, 4 = moderately severe, 5 = severe.

[b] P = finding recorded as present (not graded).

Source: Beck (2011b)

The increased liver weights with hepatocyte hypertrophy induced by mandestrobin treatment at and above 4000 ppm are likely a result of the induction of liver enzymes.[2] Nevertheless, in animals given 10 000 and 20 000 ppm, several clear alterations in blood biochemistry (i.e. increased total cholesterol levels at and above 10 000 ppm and GGT activity at 20 000 ppm) were noted and considered to be evidence of an adverse effect on the liver. Consequently, the liver alterations observed at 10 000 and 20 000 ppm were considered to be toxicologically relevant for humans, but alterations at 4000 ppm were not.

[2] Mandestrobin increased the activities of 7-pentoxyresorufin *O*-depentylase (PROD, a CYP2B marker) and thyroxine (T_4)-uridine diphosphate glucuronosyltransferase (UGT) in rat liver, with a mode of action identified as being due, at least in part, to CAR activation, similar to phenobarbital (see section 2.6(c) below).

In the thyroid, an increased incidence of follicular cell hypertrophy was noted in animals given 4000, 10 000 and 20 000 ppm. The incidence was higher and the grade was more severe in the males. Follicular cell hypertrophy was characterized by a diffuse increase in the height of the follicular epithelium, generally with a minor reduction in the level of colloid present. However, follicular cell hypertrophy in the rat is generally considered to be an adaptive response to increased thyroid hormone metabolism by the liver and is commonly associated with liver cell hypertrophy. The thyroid follicular cell hypertrophy observed at and above 4000 ppm is consistent with the observed liver alterations, including increased liver weights and hepatocyte hypertrophy. Furthermore, there is evidence in the general literature (Capen et al., 1999; Meek et al., 2003; Cohen et al., 2004) on the biochemical and physiological differences in thyroid function that indicate differences in susceptibility to alteration in thyroid hormone metabolism between rats and humans. Taken together, the thyroid changes induced by mandestrobin are not considered to be toxicologically relevant for humans.[3]

In the kidney, the severity of hyaline droplets was higher in males given 10 000 or 20 000 ppm; the lesion was characterized as densely eosinophilic, variably sized droplets in the cytoplasm of proximal tubular cells. Immunohistochemistry was performed on two control males and two males given 20 000 ppm, and all were positive for α_{2u}-globulin. The positive intensity for the α_{2u}-globulin was higher in the two males given 20 000 ppm compared with the controls. Hyaline droplets are a common background finding in the kidney of male rats. They generally represent accumulations of α_{2u}-globulin, a naturally occurring male rat protein. This male rat–specific hyaline droplet nephropathy is of little relevance for humans. Therefore, this finding was excluded from consideration for identifying the NOAEL in this study.

The NOAEL was 4000 ppm (equal to 283 mg/kg bw per day), based on increased liver weights accompanied by hepatocyte hypertrophy, increased total cholesterol and thyroid follicular hypertrophy in both sexes at 10 000 ppm (equal to 743 mg/kg bw per day) (Beck, 2011b).

Dogs

In a palatability study, mandestrobin (purity unknown) was administered to two groups of beagle dogs (one of each sex per group) at a dietary concentration of 12 500 or 25 000 ppm (equal to 448 and 1088 mg/kg bw per day for males and 451 and 970 mg/kg bw per day for females, respectively) for 7 days. Animals were observed daily for clinical signs, feed consumption and body weight. No statistical evaluation was performed.

Clinical observations were limited to soft faeces in two animals on day 1. Males were observed to be thin during week 1. These signs are unlikely to be due to treatment and are more consistent with eating and adapting to moistened diet.

Body weights showed no evidence of any adverse treatment-related effect. Three out of four dogs failed to maintain their starting body weight, but this appeared to be due to the addition of water to the diet. The same dogs lost weight during the acclimatization period (week −1) while on moistened control diet. Three out of four animals ate their entire allowance of food throughout the acclimatization and study periods (Shaw, 2008).

In a dose range–finding study, mandestrobin (purity 99%) was administered to four groups of approximately 5-month-old beagle dogs (one of each sex per group) at a dietary concentration of 0, 1000, 10 000 or 20 000 ppm (equal to 0, 45.2, 440.3 and 915.8 mg/kg bw per day for males and 0, 46.7, 478.6 and 992.2 mg/kg bw per day for females, respectively) for 4 weeks. Detailed clinical observations and feed consumption were recorded daily, and body weight was measured twice weekly and on the

[3] In the studies by Asano (2012e) and Yamada (2012a) in section 2.6(c) below, the rat-specific mode of action for the thyroid response was identified as being due, at least in part, to hepatic CAR activation and subsequent enhancement of serum T_4 clearance by induced T_4-UGT. Reduced serum T_4 and increased thyroid stimulating hormone (TSH) were measured in the mode of action study in rats (Asano, 2012e) and are the most likely mode of action for the thyroid follicular cell hyperplasia seen in the study by Beck (2011b).

day of necropsy. Blood collection after fasting or before dosing was performed on all animals before the start of treatment and at weeks 2 and 4. Urine analysis was performed on all animals before the start of treatment and at weeks 2 and 4. Ophthalmoscopy was not performed. All animals were subjected to necropsy, and the following organs were weighed: adrenals, brain, epididymides, heart, kidneys, liver, ovaries, pituitary, prostate, spleen, testes, thymus, thyroid and parathyroids, and uterus including cervix. An extensive list of tissues was examined histologically. The study did not follow a specific guideline and was not performed under GLP, although it was conducted in the spirit of GLP. No statistical evaluation was performed.

All animals survived, and no treatment-related clinical signs were observed. Post-dosing observations were limited to soft/liquid faeces and vomiting. These observations were limited to four animals, including two controls and two treated animals. The treated animals were observed with soft faeces on only two occasions (on day 11, 4 hours after feeding, and on day 27, 1 hour after feeding). Body weight was not affected by treatment. Body weight gain was reduced in males fed diets containing 10 000 or 20 000 ppm mandestrobin. No treatment-related effects on feed consumption were observed. No treatment-related effects on haematological parameters were apparent in either week 2 or week 4. Animal 3M had noticeably higher ALT levels relative to controls in weeks 2 and 4. All other treated animals had ALT levels similar to those of controls in weeks 2 and 4. Animal 3M also had higher levels of AST and alkaline phosphatase (ALP) relative to controls in weeks 2 and 4. Both high-dose animals (4M and 8F) had higher levels of ALP in week 4 compared with controls, and 4M also had higher levels of ALP in week 2.

Increased iron in males given 10 000 or 20 000 ppm mandestrobin in the diet in week 4 and decreased unsaturated iron binding capacity in the high-dose male in weeks 2 and 4 and in the high-dose female in week 4 were observed. However, the toxicological relevance of these changes was unclear.

No treatment-related effects on urinary parameters were apparent.

In males, a dose-related increase in organ weights was observed for adrenals, kidney, liver, brain and testes. Pituitary and thyroid/parathyroid weights were also higher than in controls from 10 000 ppm. In females, liver weights were higher than those of controls at 10 000 ppm and above, and kidney and spleen weights were lower than those of controls, in a dose-dependent manner. Uterus, ovaries, adrenals and thymus weights were lower than those of controls at 20 000 ppm. Besides liver, no correlated pathological findings were observed for any of the above-mentioned organs; therefore, the changes in organ weights were considered to be of no toxicological relevance. Macroscopically large liver was recorded in males given 20 000 ppm. The microscopic investigation showed increased hypertrophy (characterized by a ground glass appearance in the midzonal and centrilobular areas) in both sexes fed 20 000 ppm. Given the shortcomings of the study (e.g. low number of animals per group), the study is not considered suitable for the identification of a NOAEL (Shaw, 2009b).

Mandestrobin (purity 93.4%) was administered to four groups of four male and four female beagle dogs (aged 5–6 months) at a dietary concentration of 0, 4000, 12 000 or 40 000 ppm (equal to 0, 90.9, 267.8 and 933.1 mg/kg bw per day for males and 0, 102.7, 304.4 and 820.4 mg/kg bw per day for females, respectively) for 13 weeks. Achieved concentrations and homogeneity were verified by analysis. Detailed clinical observations and feed consumption were recorded daily, whereas body weight was measured weekly. Ophthalmoscopy was performed once pretest and at week 12. A battery of behavioural tests and observations was performed on all animals before initiation of treatment and once weekly thereafter. Blood collection after fasting was performed on all animals before the start of treatment and at weeks 4, 8 and 13 of the administration period. Urine analysis was performed on all animals before the start of treatment and at weeks 3, 7 and 12 of the administration period. Detailed necropsy was performed on all animals after scheduled termination, and the following organs were weighed: adrenals, brain, heart, kidneys, liver, ovaries, pituitary, prostate, spleen, testes, epididymides, thymus, thyroids (with parathyroids) and uterus (including cervix). An extensive list of tissues was examined histologically. Liver and kidneys were also evaluated by electron microscopy.

No mortality and no differences in ophthalmoscopy or urine analysis were noted. Thin appearance was noted in three males and one female given 40 000 ppm mandestrobin in the diet. Clinical signs included red eyes/ears/mouth, sores/lesions and earwax; however, they were considered incidental, as they were either infrequent or noted at a comparable rate in the concurrent control animals. Post-dosing observations were restricted to faecal abnormalities and emesis. Soft, mucoid or liquid faeces were noted sporadically over the dosing phase in males and females given 4000 ppm and above up to 8 hours following presentation of the diets. Emesis was seen in two males given 40 000 ppm in week 1, up to 8 hours following presentation of the diets. Based on their low and irregular incidence, faecal abnormalities and emesis were considered not to be treatment related.

Statistically significant decreases in body weight and body weight gain were recorded in both sexes at 40 000 ppm. Males gained notably less weight than controls over the 13-week period, with individual growth rates for treated animals being variable, ranging from a loss of 7.5% of body weight to a gain of 11.3%. Following 13 weeks of treatment, most females at 40 000 ppm had similar or slightly lower body weights compared with the initial values. Reduced feed consumption was noted only at 40 000 ppm in both sexes, from the fourth week of treatment (Table 17).

Occasional statistically significant differences in mean body temperature were recorded for both sexes at 12 000 ppm and for males at 40 000 ppm. Statistically significant differences in mean heart rate were recorded sporadically for 4000 ppm males and 12 000 ppm females; the effect was dose responsive for females in week 13. However, all these changes were transient, inconsistent and therefore not considered to be treatment related. Weekly neurological examinations revealed incidences of abnormal proprioceptive paw positioning, abnormal righting postural reactions, abnormal hemihopping postural reactions, abnormal wheelbarrowing postural reactions and decreased muscle tone in a few males or females at 40 000 ppm and protrusion of the nictitating membrane in males and females at and above 4000 ppm. In addition, there were single occurrences of abnormal hemihopping/wheelbarrowing postural reactions in one female at 4000 ppm in week 9 and abnormal proprioceptive paw positioning or abnormal hemihopping postural reactions in animals fed 12 000 ppm mandestrobin in week 4. These recorded observations were generally infrequent, occasionally observed in control animals and considered in some cases likely to be a result of the reduced body weight. Overall, no neurotoxicological effect related to mandestrobin was noted.

Increased platelet count was noted in 40 000 ppm males in week 4 and in both sexes at 40 000 ppm in weeks 8 and 13. A statistically significant increase in mean white blood cell count (1.34-fold) was seen in males given 40 000 ppm in week 13, mediated by increases in both neutrophils (1.55-fold) and monocytes (1.83-fold), compared with concurrent controls. It is noted that these changes were within historical control ranges and were comparable to pretest values. At 12 000 ppm and above, increased ALT, ALP and GGT activities were observed in both sexes. Increased AST activity and triglyceride levels and decreased albumin, albumin to globulin ratio, total cholesterol and glucose were observed at 40 000 ppm only, in both sexes (Table 17).

Treatment-related organ weight changes in kidney, brain, spleen, liver, thymus, heart, pituitary, prostate, testes/epididymides and uterus were observed, when compared with concurrent controls (Table 17). In males at 40 000 ppm, absolute and relative spleen weights were increased by 1.48 and 1.92 times, respectively, when compared with concurrent controls. No macroscopic or microscopic correlates were present, and mean values were generally within the historical control reference range (absolute: 21.440–81.380 g; relative: 0.1883–0.6848%; n = 103). Relative kidney weights were increased in both sexes (males: 1.30-fold; females: 1.33-fold) at 40 000 ppm, compared with controls. Relative brain weights (1.33-fold, both sexes) were increased at 40 000 ppm, compared with controls. Mean relative pituitary weights were increased in females offered 40 000 ppm. Changes in relative kidney weight, relative brain weight and relative pituitary weight were likely related to the body weight loss in these animals, as the absolute weights were not statistically significantly different, no changes were observed in related clinical chemistry parameters and there were no correlated macroscopic or microscopic findings. Absolute heart weight in males decreased (0.74-fold) at 40 000 ppm. Decreased heart weight was probably related to body weight loss in these animals, as the relative weights were not

Table 17. Key results of the 13-week dietary toxicity study in dogs: haematology, clinical chemistry and organ weights

	Males					Females				
	0 ppm	**4 000 ppm**	**12 000 ppm**	**40 000 ppm**	**HCD[a] (mean; range) or DR**	**0 ppm**	**4 000 ppm**	**12 000 ppm**	**40 000 ppm**	**HCD[a] (mean; range) or DR**
Terminal body weight (kg)	14.07	13.57	13.61	11.16**	–	12.36	11.94	11.93	9.03***	–
Body weight gain, weeks 0–13 (kg)	2.84	2.86	2.55	0.14***	–	3.01	2.38	2.47	−0.57***	–
Feed consumption (g/animal per week)	2 110	2 111	2 111	1 947***	–	2 100	2 109	2 058	1 430***	–
Haematology (week 13)										
WBC (10^9/L)	9.4	9.6	9.9	12.6*	11.5; 8.0–16.6	9.1	9.6	10.2	10.6	–
Neutrophils (10^9/L)	4.7	5.1	4.9	7.3**	6.6; 4.2–10.9	4.9	5.2	5.2	5.4	–
Monocytes (10^9/L)	0.6	0.6	0.6	1.1*	0.5; 0.0–1.1	0.6	0.5	0.5	0.6	–
Platelets (10^3/μL)	314	375	366	454	–	285	364	348	481*	–
Blood chemistry (week 13)										
AST (IU/L)	32	37	36	50***	–	30	31	39	47**	–
ALT (IU/L)	37	30	103	215***	–	37	44	121	198**	–
ALP (IU/L)	98	105	171*	288***	–	85	114	195***	297***	–
GGT (IU/L)	4	3	3	10*	–	3	4	5	8*	–
Triglycerides (mmol/L)	0.34	0.43	0.42	0.62**	–	0.38	0.39	0.42	0.67**	–
Albumin (g/L)	36	32	34	27***	–	37	37	35	29***	–

	Males					Females				
	0 ppm	**4 000 ppm**	**12 000 ppm**	**40 000 ppm**	**HCD[a] (mean; range) or DR**	**0 ppm**	**4 000 ppm**	**12 000 ppm**	**40 000 ppm**	**HCD[a] (mean; range) or DR**
A/G	1.8	1.6	1.7	1.0***	–	2.4	2.0	2.0	1.4***	–
Cholesterol (mmol/L)	6.7	5.5	5.6	3.2***	–	6.7	6.1	5.2	4.9*	–
Globulin (g/L)	20	22	20	29**	–	16	18	18	22	DR*
BIL (µmol/L)	2.2	2.2	1.8	2.9	–	2.0	2.0	1.4	2.9	–
GLU (mmol/L)	5.8	5.8	5.9	4.6***	–	5.7	5.9	5.9	5.0*	–
Absolute organ weights (g)										
Kidney	65.7	61.0	63.0	67.7	–	51.7	52.8	51.6	49.3	–
Liver	496.0	504.6	570.1	565.1	–	364.3	451.5***	453.2***	387.5**	–
Brain	82.8	84.3	81.8	85.2	–	78.9	75.8	75.8	71.8	–
Thymus	15.8	13.2	12.8	6.9	–	15.4	15.5	15.2	6.6	–
Spleen	47.2	44.4	46.2	69.9	37.3; 21.4–81.4	46.1	44.7	53.7	40.6	–
Heart	108.0	100.2	107.7	79.6**	–	90.2	96.3	93.9	71.4	–
Pituitary	0.094	0.097	0.084	0.077	–	0.070	0.077	0.076	0.065	–
Prostate	4.47	3.88	2.96	1.16*	–	–	–	–	–	–
Testes/ epididymides	28.86	21.88	24.51	20.53	–	–	–	–	–	–
Uterus	–	–	–	–	–	4.19	5.62	5.63	2.7	11.3; 1.6–51.7
Relative organ weights (%)										
Kidney	0.467	0.449	0.465	0.608*	–	0.416	0.447	0.432	0.552*	–
Liver	3.5	3.7	4.2	5.1***	–	2.9	3.8***	3.8***	4.3***	–

	Males					Females				
	0 ppm	**4 000 ppm**	**12 000 ppm**	**40 000 ppm**	**HCD[a] (mean; range) or DR**	**0 ppm**	**4 000 ppm**	**12 000 ppm**	**40 000 ppm**	**HCD[a] (mean; range) or DR**
Brain	0.6	0.6	0.6	0.8**	–	0.6	0.6	0.6	0.8*	–
Thymus	0.112	0.097	0.094	0.060*	–	0.122	0.132	0.128	0.071*	–
Spleen	0.334	0.327	0.336	0.640*	0.188–0.685	0.367	0.381	0.456	0.463	–
Heart	0.8	0.7	0.8	0.7	–	0.7	0.8	0.8	0.8	–
Pituitary	0.000 67	0.000 72	0.000 62	0.000 65	–	0.000 55	0.000 65	0.000 65	0.000 7**	–
Prostate	0.032	0.029	0.022	0.011*	–	–	–	–	–	–
Testes/ epididymides	0.20	0.16	0.18	0.18	–	–	–	–	–	–
Uterus	–	–	–	–	–	0.034	0.047	0.048	0.030	0.018–0.466

A/G: albumin/globulin; ALP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; BIL: bilirubin; DR: dose–response test; GGT: gamma-glutamyl transferase; GLU: glucose; HCD: historical control data; IU: international units; ppm: parts per million; WBC: white blood cells; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ (analysis of variance, dose–response and Dunnett’s)

[a] Historical control data from studies performed between January 1998 and March 2007. For haematology and clinical chemistry, data were obtained from 4–39 different studies and from 33–306 animals.

Source: Beck (2012a)

statistically significantly lower and there were no correlated macroscopic or microscopic findings. Absolute thymus weights (males: 0.44-fold; females: 0.43-fold) and relative thymus weights (males: 0.54-fold, females: 0.58-fold) were reduced for males (particularly animals 13M and 14M) and females given 40 000 ppm, compared with the concurrent controls. Thymus involution/atrophy was noted and characterized by a decreased cortex to medulla ratio, and an increase in tingible body macrophages was noted in some cases. As no microscopic findings were seen at 4000 ppm and no macroscopic or microscopic findings were seen at 12 000 ppm, the macroscopic change in the thymus at 4000 ppm is not considered to be biologically relevant. Increased mean liver weights were noted in males given 12 000 ppm and above and in females given 4000 ppm and above.

Absolute (0.26-fold) and relative (0.34-fold) prostate weights were reduced in males at 40 000 ppm. In addition, a decrease was recorded for two males (6M and 8M) given 4000 ppm and two males (9M and 12M) given 12 000 ppm. The prostate weight of one male (4M) was lower than those of the other animals in the control group. Lower absolute and/or relative testes/epididymides weights were recorded for males (6M, 8M, 10M, 13M, 14M and 16M) given 4000 ppm and above, compared with concurrent controls. A decrease in absolute and relative uterus weights was recorded for two females (29F and 32F) at 40 000 ppm, compared with concurrent controls.

Evidence of delayed sexual maturity in males (prostate, testes/epididymides) and females (ovaries) was noted. The prostate was immature in all males at 40 000 ppm and pubescent in one control male, two males at 4000 ppm and two males at 12 000 ppm. Immaturity was characterized by small, cuboidal acinar cells with no eosinophilic apical cytoplasm and little or no secretion. Pubescence was characterized by the development of a small amount of eosinophilic apical cytoplasm in some acini, with small amounts of secretion. The testes were immature in two males at 40 000 ppm. The testes were pubescent in one or two males from each group, including controls. Severe oligospermia was present in the epididymides of three males at 40 000 ppm, and minimal oligospermia, due to pubescence, was present in one male at 12 000 ppm. Immaturity was characterized by the absence of spermatids within seminiferous tubules and oligospermia in the epididymis. Pubescence was characterized by some spermatids in seminiferous tubules and epididymides. The incidence of pubescence was similar across control and treated groups. The ovaries of two females at 40 000 ppm were immature. The ovaries of all other animals in the study were pubescent. Immaturity was characterized by the absence of secondary or Graafian follicles or corpora lutea. The changes in prostate, testes/epididymides, ovaries and thymus generally correlated with organ weights and macroscopic observations. Decreased uterus weights in two females at 40 000 ppm were related to the body weight losses in these animals, as the absolute and relative weights were within historical control ranges and no gross or histopathological findings were observed; therefore, this finding was not considered to be toxicologically relevant (Table 18).

Table 18. Key results of the 13-week dietary toxicity study in dogs: macroscopic findings and histopathology

	Incidence of finding							
	Males				Females			
Finding	**0 ppm**	**4 000 ppm**	**12 000 ppm**	**40 000 ppm**	**0 ppm**	**4 000 ppm**	**12 000 ppm**	**40 000 ppm**
Macroscopic findings								
Animal thin	0/4	0/4	0/4	3/4	0/4	0/4	0/4	1/4
Liver – dark	0/4	0/4	2/4	4/4	0/4	0/4	2/4	4/4
Liver – large	0/4	0/4	3/4	3/4	0/4	0/4	1/4	0/4
Gall bladder – choleliths	0/4	0/4	0/4	4/4	0/4	0/4	0/4	3/4
Gall bladder – distention	0/4	0/4	0/4	2/4	0/4	1/4	0/4	1/4
Prostate – small	0/4	2/4	2/4	4/4	–	–	–	–

Finding	Incidence of finding							
	Males				Females			
	0 ppm	4 000 ppm	12 000 ppm	40 000 ppm	0 ppm	4 000 ppm	12 000 ppm	40 000 ppm
Thymus – small	0/4	1/4	0/4	2/4	0/4	0/4	0/4	1/4
Histopathology								
Liver								
Pigment	0/4	0/4	3/4	4/4	0/4	0/4	3/4	3/4
Periportal/centrilobular fibrosis	0/4	0/4	0/4	4/4	0/4	0/4	0/4	1/4
Centrilobular degeneration	0/4	0/4	2/4	3/4	0/4	0/4	4/4	4/4
Centrilobular hepatocellular swelling	0/4	0/4	3/4	1/4	0/4	3/4	3/4	0/4
Bridging fibrosis	0/4	0/4	0/4	1/4	0/4	0/4	0/4	0/4
Hepatocyte vacuolation	0/4	0/4	1/4	0/4	0/4	0/4	0/4	1/4
Gall bladder								
Choleliths	0/4	0/4	0/4	4/4	0/4	0/4	0/4	1/4
Adherent bile	2/4	0/4	1/4	4/4	0/4	1/4	1/4	2/4
Prostate								
Immature	0/4	0/4	0/4	4/4	–	–	–	–
Pubescent	1/4	2/4	2/4	0/4	–	–	–	–
Mature	3/4	2/4	2/4	0/4	–	–	–	–
Testes								
Immature	0/4	0/4	0/4	2/4	–	–	–	–
Pubescent	2/4	1/4	1/4	1/4	–	–	–	–
Mature	2/4	3/4	3/4	1/4	–	–	–	–
Segmental hypoplasia	1/4	3/4	0/4	1/4	–	–	–	–
Epididymides								
Oligospermia/spermatocytes	0/4	0/4	1/4	3/4	–	–	–	–
Ovaries								
Immature	–	–	–	–	0/4	0/4	0/4	2/4
Pubescent	–	–	–	–	4/4	4/4	4/4	2/4
Thymus								
Involution/atrophy	0/4	0/4	0/4	2/4	0/4	0/4	0/4	2/4
Agonal congestion/ haemorrhage	2/4	0/4	1/4	0/4	0/4	0/4	0/4	1/4

ppm: parts per million
Source: Beck (2012a)

Dogs used in 13-week toxicity studies are generally not fully sexually mature at termination, which makes interpretation of prostate, testes and ovary findings in these studies difficult (Hood, 2005). In this study, dogs were 20–24 weeks of age (5–6 months) at the beginning of mandestrobin

administration. At study termination, the dogs were 33–37 weeks old (about 8–9 months). Given that dogs achieve sexual maturity by about 7–10 months, termination in this study occurred approximately at the time dogs would be undergoing puberty, with delayed puberty at the high dose likely being secondary to retarded growth and detected as immaturity. It has been reported that immaturity or delayed development of prostate, testes and ovaries can be associated with stress, weight loss and nonspecific toxicity (Greaves, 2000).

Macroscopic evaluation indicated that three males and one female fed 40 000 ppm mandestrobin were thin. Dark and/or large livers were found in both sexes at 12 000 and/or 40 000 ppm. Abnormal contents were present in the gall bladder of both sexes at 40 000 ppm. Gall bladder was distended in two males and one female given 40 000 ppm and one female given 40 000 ppm (Table 18).

Microscopic evaluation of the liver showed centrilobular hepatocellular swelling in females at 4000 and 12 000 ppm and in males at 12 000 and 40 000 ppm. This correlated with increased liver weights at necropsy. Pigment and centrilobular degeneration were present in males and females fed 12 000 or 40 000 ppm. Pigment was characterized by accumulations of intracellular and intrasinusoidal gold/brown pigment. This pigment is considered to be porphyrin, as it was birefringent under polarized light. Fouchet's and Perls' Prussian Blue stains were negative. Centrilobular degeneration was characterized by decreased size of centrilobular hepatocytes, with a finely vacuolated appearance and often a finely granular gold/brown Schmorl's- and Long Ziehl-Neelsen–positive intracellular pigment (i.e. lipofuscin). Occasional single-cell necrosis of hepatocytes was present. Periportal/centrilobular fibrosis was present in males and females given 40 000 ppm. It was characterized by increased numbers of fibroblasts/fibrocytes in the portal and centrilobular areas. Bridging fibrosis was present in one male fed 40 000 ppm. It was characterized by replacement of centrilobular hepatocytes by fibroblasts/fibrocytes and confluence with periportal/centrilobular fibrosis. Hepatocyte vacuolation was present in one male fed 12 000 ppm and one female given 40 000 ppm (Table 18).

In the gall bladder, choleliths were seen in animals fed diets containing 40 000 ppm mandestrobin. Increased incidence and/or severity of adherent bile compared with controls were seen in males and females given 40 000 ppm (Table 18).

Microscopic findings in controls were generally infrequent, of a minor nature and consistent with the usual pattern of findings in dogs of this strain and age. Thin appearance noted in three males and one female offered 40 000 ppm generally correlated with minimal body weight gain or body weight loss, reduced feed consumption and decreased muscle tone recorded during the FOB. Pathological findings in the liver were consistent with increases in the weight of this organ in mandestrobin-treated males and females and with clinical chemistry changes. Therefore, the hepatic changes observed at 12 000 and 40 000 ppm were considered toxicologically relevant. The increased globulin and total bilirubin levels seen in one male offered 40 000 ppm were considered to be related to the morphological changes noted in the liver and gall bladder of this animal. On the basis that the only liver finding was centrilobular hepatocellular swelling in females and there were no associated changes in plasma enzyme levels, the changes in animals given 4000 ppm were considered not to be toxicologically relevant.

The NOAEL was 4000 ppm (equal to 90.9 mg/kg bw per day), based on increased liver weights accompanied by histopathological changes in the liver and increased ALP activity in blood in both sexes at 12 000 ppm (equal to 267.8 mg/kg bw per day) (Beck, 2012a).

Mandestrobin (purity 93.4%) was administered to beagle dogs (27–34 weeks old) distributed into five groups (four dogs of each sex per group) at a dietary concentration of 0, 200, 800, 4000 or 8000 ppm (equal to 0, 4.3, 19.2, 92.0 and 180.7 mg/kg bw per day for males and 0, 4.5, 20.4, 92.0 and 225.7 mg/kg bw per day for females, respectively) for 52 weeks. Achieved concentrations and homogeneity were verified by analysis. All animals were observed daily. Feed consumption was monitored daily, and body weight measurements were performed weekly. Ophthalmological examination was performed pretest and at week 51. A battery of behavioural tests and observations was performed on all animals before initiation of treatment and once weekly thereafter. Blood collection after fasting was performed on all animals before the start of treatment and at weeks 13, 26 and 51 of

the administration period. Urine analysis was performed on all animals before the start of treatment and at weeks 12, 25 and 51 of the administration period. Bone marrow smears were prepared for all animals but not examined, because no related findings were evident during haematology or histopathology. At necropsy, all animals were subjected to thorough gross examination, and the following organs were weighed: adrenals, brain, heart, kidneys, liver, ovaries, pituitary, prostate, spleen, testes, epididymides, thymus, thyroids (with parathyroids) and uterus (including cervix). Histopathology was performed on an extensive list of tissues.

No mortality and no effects on feed consumption (Table 19) or ophthalmology were noted. Thin appearance was observed in one female at 8000 ppm, and this was considered to correlate with body weight loss and occasional incidences of decreased muscle tone recorded during the FOB.

Lower body weight gain was recorded in females offered diets containing 8000 ppm mandestrobin at 52 weeks, which was due to just one female exhibiting an overall body weight loss of 6.0%. No other treatment-related clinical signs or body weight effects were noted (Table 19).

Overall, there were no findings from any assessment indicative of any neurotoxicological effect of mandestrobin. Occasional differences in body temperature were recorded for males fed at least 800 ppm and females fed at least 200 ppm, and these were dose responsive in males in weeks 15, 18, 28, 34 and 43 and in females in weeks 7, 8, 9, 11 and 15. Differences in heart rate were recorded sporadically for males given at least 200 ppm and females given 800 or 4000 ppm. These changes were transient, inconsistent and not considered to be treatment related. Neurological examinations showed one or more incidences of abnormal proprioceptive paw positioning, abnormal righting postural reactions, abnormal hemihopping postural reactions, abnormal wheelbarrowing postural reactions, abnormal muscle tone, abnormal palpebral closure, protrusion of the nictitating membrane, strabismus/protrusion of the palpebral reflex, bilateral constriction/dilatation of pupil size, abnormal papillary light reflex, increased lacrimation, increased salivation and atypical general reactions. However, these observations were infrequent and occasionally observed in concurrent control animals or before treatment was initiated.

There were no treatment-related effects on urinary parameters. The mean volume of urine voided by females fed diets containing 200 or 800 ppm at week 12 was statistically significantly decreased; however, as the volumes were similar to those measured before treatment, no dose–response relationship was observed and no statistically significant differences were observed in males at week 12 or in either sex at week 25 or 51, these changes were considered to be incidental and not treatment related.

A statistically significant dose-related increased AST activity (2.03-fold) was noted in week 26 for males at 8000 ppm, which was considered not to be treatment related, because it was largely reflective of an increase for one male; values for the other animals in this group were within the historical control range. Furthermore, the plasma AST activity for the same male in week 52 was similar to that determined before treatment. Changes at week 52 for another male given 8000 ppm were considered to be treatment related (see below) (Table 19).

Increased ALT activity was noted in both males and females at 8000 ppm in weeks 13, 26 and 52, with males achieving statistical significance for the dose–response relationship in weeks 13 and 26. ALT activities were increased in one male (8M: weeks 26 and 52) fed 200 ppm, one female (29F: weeks 26 and 52) given 800 ppm and two males (18M and 19M: weeks 13, 26 and 52) and one female (38F: weeks 13, 26 and 52) at 8000 ppm (Table 19).

Statistically significantly higher ALP activities were observed at week 52 (2.73-fold) in males at 4000 ppm and at week 13 (males: 2.83-fold; females: 2.27-fold), week 26 (males: 2.64-fold; females: 2.64-fold) and week 52 (males: 3.59-fold; females: 3.36-fold) in both sexes at 8000 ppm, compared with concurrent controls. The values for one male (10M: weeks 13 and 26) at 800 ppm and two males (13M: week 26; 15M: weeks 13, 26 and 52) at 4000 ppm were above normal expectations (Table 19).

Table 19. Key results of the 1-year dietary toxicity study in dogs

		Males							Females						
		0 ppm	200 ppm	800 ppm	4 000 ppm	8 000 ppm	HCD[a] Mean	HCD[a] Range	0 ppm	200 ppm	800 ppm	4 000 ppm	8 000 ppm	HCD[a] Mean	HCD[a] Range
Body weight (kg)		14.64	15.22	13.29	14.10	13.70	–	–	12.92	12.71	12.63	13.36	11.05	–	–
Body weight gain, weeks 0–52 (kg)		2.97	3.88	1.82	2.58	2.66	–	–	2.68	2.36	2.57	3.19	0.81[b]	–	–
Feed consumption (g/animal per week)		2 107	2 110	2 112	2 110	2 111	–	–	2 067	1 920	2 089	1 993	2 094	–	–
Blood chemistry															
AST (IU/L)	w13	32	38	29	34	42	32	20–50	28	28	26	32	36	–	–
	w26	30	30	29	29	61*			26	30	28	23	28		
	w52	29	30	29	27	37			26	24	29	28	30		
ALT (IU/L)	w13	38	42	44	46	136*[DR]	–	–	29	36	38	39	79	–	–
	w26	42	48	48	43	113*[DR]			31	36	46	31	57		
	w52	40	53	51	47	128			29	35	50	33	59		
ALP (IU/L)	w−1	91	87	127	109	116	–	–	121	99	89	97	101	–	–
	w13	63	68	103	120	178*			91	76	70	91	207*		
	w26	53	52	100	120	140*			75	79	66	78	198**		
	w52	41	43	73	112**	147**			55	64	53	69	185**		
GGT (IU/L)	w13	2	2	3	3	4	–	–	2	3	3	2	3	–	–
	w26	3	5	3	5	3			2	2	4	3	4		
	w52	3	3	4	4	4			3	3	3	4	5*[DR]		
Total protein (g/L)	w13	55	55	56	57	57*[DR]	–	–	60	58	57	56	55**	57	50–66
	w26	58	58	59	59	58			62	62	60	61	57*		
	w52	58	59	56	59	59			61	61	59	59	57*[DR]		

		Males							Females						
		0 ppm	200 ppm	800 ppm	4 000 ppm	8 000 ppm	HCD[a] Mean	HCD[a] Range	0 ppm	200 ppm	800 ppm	4 000 ppm	8 000 ppm	HCD[a] Mean	HCD[a] Range
Albumin (g/L)	w13	35	36	36	37	36	–	–	39	37	38	37	35*DR	–	–
	w26	36	35	34	35	33			39	38	36	37	34*		
	w52	35	35	33	37	33			39	37	37	36	34**		
A/G ratio	w13	1.8	1.9	1.8	1.9	1.6	–	–	1.9	1.9	1.9	2.1	1.8	–	–
	w26	1.6	1.6	1.4	1.4	1.4			1.6	1.6	1.5	1.6	1.5		
	w52	1.5	1.5	1.5	1.7	1.3			1.8	1.6	1.6	1.6	1.5		
Cholesterol (mmol/L)	w13	7.1	5.9	6.6	6.9	6.1	–	–	8.3	7.0	6.1	5.9	6.4*DR	–	–
	w26	6.7	5.7	6.7	6.7	5.8			8.2	6.3*	5.9**	6.6	6.0*		
	w52	6.0	5.1	5.7	6.3	5.5			7.5	6.1	5.5*	5.9	5.4*		
Triglycerides (mmol/L)	w13	0.37	0.28	0.45	0.53	0.48**DR	0.35	0.17–0.71	0.59	0.63	0.50	0.49	0.51	–	–
	w26	0.33	0.30	0.44	0.49*	0.45			0.51	0.48	0.48	0.52	0.50		
	w52	0.40	0.36	0.42	0.53	0.52*DR			0.55	0.60	0.60	0.58	0.52		
Calcium (mmol/L)	w13	2.83	2.79	2.76	2.81	2.80	–	–	2.89	2.88	2.86	2.84	2.70*	2.7	2.35–3.00
	w26	2.66	2.67	2.60	2.66	2.61			2.74	2.76	2.72	2.74	2.59*		
	w52	2.63	2.63	2.57	2.69	2.57			2.71	2.70	2.66	2.66	2.53*		
Absolute organ weights (g)															
Brain		87.4	86.8	84.2	84.3	78.8*	84.7	74.9–100	76.6	83.1	77.2	75.8	71.5	–	–
Kidney		73.9	65.0	61.7	60.6*	60.0*	62.8	51.0–79.6	57.3	58.5	58.4	53.1	54.9	–	–
Liver		437	425	409	484	488**DR	–	–	429	437	437	448	455	–	–
Thymus		11.9	9.92	7.74	8.38	6.72	9.10	3.42–19.9	9.60	9.43	10.9	11.2	7.38	8.2	3.9–13.7

	Males							Females						
	0 ppm	200 ppm	800 ppm	4 000 ppm	8 000 ppm	HCD[a] Mean	HCD[a] Range	0 ppm	200 ppm	800 ppm	4 000 ppm	8 000 ppm	HCD[a] Mean	HCD[a] Range
Thyroid	1.01	1.14	0.95	1.18	1.20	–	–	1.10	1.28	1.10	1.33	1.38	0.94	0.63–1.39
Spleen	55.7	45.7	39.9	44.6	55.5	55.6	27.9–81.4	30.7	46.1	39.0	39.6	41.7	54.2	45.8–61.8
Heart	124	121	107	105*	113	110.2	76.9–142	103	101	97.4	98.8	99.3	–	–
Testes/epididymides	32.3	25.2	24.3*	27.5	25.3	27.7	20.2–33.5	–	–	–	–	–	–	–
Relative organ weights (%)														
Brain	0.6	0.6	0.6	0.6	0.6	0.64	0.46–0.82	0.6	0.7	0.6	0.6	0.7	–	–
Kidney	0.50	0.42	0.46	0.43	0.43	0.47	0.39–0.60	0.44	0.46	0.46	0.40	0.50	–	–
Liver	3.0	2.8	3.1	3.4	3.6**[DR]	–	–	3.3	3.4	3.5	3.4	4.2*	–	–
Thymus	0.080	0.065	0.057	0.059	0.049	0.069	0.03–0.11	0.073	0.076	0.088	0.083	0.064	0.07	0.04–0.10
Thyroid	0.007	0.008	0.007	0.008	0.009	–	–	0.009	0.010	0.009	0.010	0.013	0.0069	0.0049–0.0093
Spleen	0.389	0.298	0.299	0.310	0.407	0.35	0.22–0.48	0.236	0.359	0.313	0.298	0.371	0.34	0.29–0.42
Adrenals	0.010	0.010	0.010	0.010	0.011	–	–	0.013	0.015	0.017	0.009*	0.017*	0.014	0.010–0.019
Heart	0.8	0.8	0.8	0.7	0.8	0.82	0.71–0.89	0.8	0.8	0.8	0.7	0.9	–	–
Testes/epididymides	0.220	0.166*	0.186	0.196	0.184	0.18	0.15–0.20	–	–	–	–	–	–	–
Microscopic findings: liver														
No. examined	*4*	*4*	*4*	*4*	*4*			*4*	*4*	*4*	*4*	*4*		

	Males							Females						
	0 ppm	200 ppm	800 ppm	4 000 ppm	8 000 ppm	HCD[a]		0 ppm	200 ppm	800 ppm	4 000 ppm	8 000 ppm	HCD[a]	
						Mean	Range						Mean	Range
Hepatocyte hypertrophy	0	0	0	2	3*	–	–	0	0	0	1	4*	–	–
Hepatocyte pigment	1	0	1	3	4	–	–	1	1	0	1	4	–	–
Pigmented macrophages	1	0	0	0	2	–	–	1	0	0	1	2	–	–
Portal fibrosis/bile duct proliferation	0	0	0	0	1	–	–	0	0	0	0	0	–	–
Centrilobular degeneration	0	0	0	0	1	–	–	0	0	0	0	1	–	–
Agonal congestion/haemorrhage	0	0	0	0	1	–	–	0	0	0	0	3	–	–

A/G: albumin/globulin; ALP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; DR: dose–response test (a significant trend was reported only where none of the pairwise comparisons was significant); GGT: gamma-glutamyl transferase; HCD: historical control data; IU: international units; ppm: parts per million; w: Week; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ (analysis of variance, dose–response and Dunnett's)

[a] Historical control data were generated from January 1998 to March 2007. For organ weights, the data were obtained from 2–6 studies, with between 3 and 14 animals. For the blood biochemistry parameters, the data were obtained from 2–39 studies, with between 2 and 294 animals.

[b] This reduced growth rate was due to just one female exhibiting an overall body weight loss of 6.0%.

Source: Beck (2012b)

Increased GGT activity was seen in one male (8M: week 26) fed diets containing 200 ppm mandestrobin, one female (30F: week 26) given 800 ppm, one male (15M: weeks 13, 26 and 52) offered 4000 ppm and one male (18M: weeks 13 and 52) and two females (38F and 39F: week 52) fed 8000 ppm. The increased GGT activity in females at 8000 ppm attained statistical significance for the dose–response relationship (Table 19).

Total cholesterol was statistically significantly decreased at week 26 in females fed diets containing 200 (0.77-fold), 800 (0.72-fold) or 8000 ppm (0.73-fold) and at week 52 in females offered 800 (0.73-fold) or 8000 ppm (0.72-fold), compared with concurrent controls. As the mean values were similar to those determined before treatment was initiated, the differences at week 26 are considered not to be toxicologically important (Table 19).

Disturbances to ALP (800 ppm only), GGT and/or total cholesterol in animals offered 200 or 800 ppm were considered not to be toxicologically relevant, as they were small in magnitude, inconsistent over time and between sexes and/or lacked a histopathological correlate. However, the increased ALP activities in males offered 4000 ppm at week 52 and in males and females offered 8000 ppm at weeks 13, 26 and 52 were considered to be treatment related. Increased GGT activities in males given 4000 ppm in weeks 13, 26 and 52, in males given 8000 ppm in weeks 13 and 52 and in females given 8000 ppm in week 52 were considered to be treatment related. In addition, increased ALT activity in both sexes at 8000 ppm was considered to be treatment related.

Statistically significantly higher triglyceride levels were observed at week 26 (1.49-fold) in males fed 4000 ppm. This was considered not to be toxicologically relevant, as the mean value was within the historical control range (0.17–0.71 mmol/L). Concentrations were increased in two males (week 13: 13M and 15M; week 26: 15M; week 52: 13M) offered 4000 ppm and three males (week 13: 18M and 20M; week 52: 19M) offered 8000 ppm. The increased triglyceride level for one male offered 4000 ppm and two males offered 8000 ppm at week 13 were considered not to be toxicologically relevant, as the values for these animals at week 52 were similar to those determined before treatment was initiated. However, the increases for one male given 4000 ppm at weeks 13 and 52 and one male fed 8000 ppm at week 52 were considered to be treatment related (Table 19).

Decreased albumin levels were noted in females offered 8000 ppm at weeks 26 and 52. Decreased total protein and calcium levels in females offered 8000 ppm at weeks 13 and 26 and at weeks 13, 26 and 52, respectively, were considered not to be treatment related, but reflective of decreased albumin concentrations (Meuten, 1982). Additionally, all individual values were within the historical control ranges, or differences were similar to those recorded pretreatment. Occasional differences reached statistical significance in other parameters; however, these changes were not considered to be treatment related, as they were slight, within the historical control range or comparable to pretest values, or there were no significant differences between sexes or collection points (Table 19).

Absolute kidney weights were statistically significantly decreased in males offered 4000 ppm (0.82-fold) or 8000 ppm (0.81-fold), compared with concurrent controls. Spleen weights (absolute and relative) were above normal expectations in one male (17M) and one female (38F) at 8000 ppm. The values for one control male (3M) were also high. Absolute brain weights were decreased in males at 8000 ppm (0.90-fold), compared with concurrent controls. Absolute thyroid/parathyroid weights were above normal expectations in one female (36F) fed 4000 ppm and one female (38F) at 8000 ppm. Absolute and relative thymus weights were low in one male (10M) at 800 ppm and two males (17M and 20M) and one female (39F) offered 8000 ppm, compared with concurrent controls (Table 19).

Lower absolute and/or relative testes/epididymides weights were recorded for one male (11M) at 800 ppm and one male (17M) at 8000 ppm, compared with concurrent controls. A statistically significant decrease (0.75-fold) in absolute testes/epididymides weight was seen for males offered 800 ppm, compared with concurrent controls. In addition, the relative testes/epididymides weight for males at 200 ppm was statistically significantly lower (0.75-fold) than for concurrent controls. When compared with concurrent controls, mean relative adrenal weights (0.69-fold and 1.31-fold) were statistically significantly different for females at 4000 and 8000 ppm, respectively. Absolute heart weight was statistically significantly decreased in males at 4000 ppm (0.84-fold), compared with concurrent controls (Table 19).

Organ weight changes in the spleen, thyroid/parathyroid, thymus, testes/epididymides, adrenals and heart were considered not to be toxicologically relevant, as they were inconsistent between the sexes, there were no macroscopic or microscopic correlates, values were within historical control ranges or there was a lack of a clear dose–response relationship. Decreases in absolute kidney weight and brain weight were considered of doubtful toxicological relevance, as the relative weights were not statistically significantly different, all individual values were within historical control limits, no changes were observed in females and no related clinical chemistry parameters or macroscopic or microscopic correlates were present.

Absolute liver weights were increased in males at 4000 ppm (1.11-fold) and 8000 ppm (1.12-fold) and in females at 8000 ppm (1.06-fold), compared with controls. Relative liver weights were increased in males at 4000 ppm (1.13-fold) and 8000 ppm (1.20-fold) and in females at 8000 ppm (1.27-fold), compared with controls. Changes in relative liver weight at 8000 ppm were statistically significant. Dark liver was observed for one male and three females fed 8000 ppm and correlated with findings recorded microscopically. There were no other macroscopic findings suggestive of treatment-related effects (Table 19).

In the liver, hepatocyte hypertrophy was recorded for males and females fed diets containing 4000 or 8000 ppm mandestrobin. Hepatocyte hypertrophy was characterized by hepatocytes in the centrilobular/midzonal area, with increased pale eosinophilic staining cytoplasm. Hepatocyte hypertrophy recorded for females offered 4000 ppm was considered not to be adverse on the basis that no other histopathological findings were recorded and there were no associated changes in blood biochemistry. Increased levels of hepatocyte pigment were recorded for males fed 4000 and 8000 ppm and females fed 8000 ppm. Hepatocyte pigment was characterized by the presence of small golden brown cytoplasmic granules, primarily in hepatocytes in the centrilobular and periportal zones. The incidence of hepatocyte hypertrophy and hepatocyte pigment achieved statistical significance in males and females offered 8000 ppm. There was also a marginal increase in pigmented macrophages in males and females at 8000 ppm. Pigmented macrophages were characterized by macrophages in the portal area and in the hepatic sinusoids, with dark brown cytoplasmic pigment. The hepatocyte pigment and macrophage pigment were identified as lipofuscin using Schmorl's and Long Ziehl-Neelsen stains. Additionally, centrilobular degeneration was recorded for one male and one female offered 8000 ppm. Centrilobular degeneration was characterized by the presence of degenerating hepatocytes in the centrilobular zone, with occasional single-cell necrosis. Portal fibrosis/bile duct proliferation was recorded for one male offered 8000 ppm. It was characterized by the presence of proliferating bile duct cells and fibroblasts, with an overall increase in fibrous tissue in the portal tracts. Agonal congestion/haemorrhage was recorded for one male and three females at 8000 ppm, which correlated with the dark liver observed during the macroscopic examination. However, this finding was considered to be secondary to the hepatocyte hypertrophy, rather than a direct effect of mandestrobin treatment. The increase in lipofuscin in this study was considered to be related to hepatocyte hypertrophy and/or cellular degeneration (Table 19).

The decreased incidence of stomach mineralization in treated males and the increased incidence of sublingual gland inflammatory cell foci in treated females, compared with controls, achieved statistical significance. Both stomach mineralization and inflammatory cell foci in the sublingual salivary gland are commonly seen as background findings. There was no dose-related response for either of the findings, and these statistically significant findings were considered not to be of biological relevance.

The NOAEL was 800 ppm (equal to 19.2 mg/kg bw per day), based on increased relative liver weights, hepatocyte hypertrophy and hepatocyte pigment and disturbances to clinical biochemistry parameters (increased ALP, GGT and triglycerides) in males at 4000 ppm (equal to 92.0 mg/kg bw per day) (Beck, 2012b).

(b) Dermal application

Rats

In a study of dermal toxicity, mandestrobin (purity 93.4%) was applied repeatedly for 28 consecutive days to the skin of male and female Wistar rats at a dose of 100, 300 or 1000 mg/kg bw per day for 6 hours per day. Clinical signs and mortality were observed twice daily, before and after the administration. Body weights and feed consumption were measured weekly. Ophthalmoscopy was conducted before the start of administration and in week 4. Haematology and clinical chemistry were conducted at necropsy, after fasting, and urine analysis was performed in week 4. Bone marrow smears were prepared for all animals but not examined, because no related findings were evident during haematology or histopathology. At the end of the study, all animals were terminated and subjected to macroscopic examination. Organs were weighed, and histopathology was performed on an extensive list of tissues.

No mortality or clinical signs were noted during the study.

No effects were noted on body weight, feed consumption, ophthalmoscopy, haematology and clinical chemistry, necropsy, organ weights or histopathology.

As no treatment-related effects were noted, the NOAEL was 1000 mg/kg bw per day, the highest dose tested (Ogata, 2011).

2.3 Long-term studies of toxicity and carcinogenicity

Mice

Mandestrobin (purity 93.4%) was administered to four groups of 51 male and 51 female CD-1 mice (6–7 weeks old) at a dietary concentration of 0, 700, 2000 or 7000 ppm (equal to 0, 82.5, 238.8 and 823.9 mg/kg bw per day for males and 0, 99.2, 280.3 and 994.0 mg/kg bw per day for females, respectively) for 78 weeks. In addition to these animals (designated as the "main group"), 12 mice of each sex per group were treated at a dietary concentration of 0, 700, 2000 or 7000 ppm (equal to 0, 88.4, 255.0 and 883.3 mg/kg bw per day for males and 0, 104.0, 325.0 and 1045.1 mg/kg bw per day for females, respectively) for 52 weeks only, for interim sacrifice (designated as the "satellite group").

Dose, homogeneity and stability in the diet were confirmed by chemical analysis. Animals were checked for viability/mortality twice each day, and clinical signs were observed at least once daily. Body weight and feed consumption were recorded weekly up to week 16 and every 4 weeks thereafter. Blood samples for haematology were collected from all satellite group animals at week 52 and from all main group animals at week 78. A detailed gross necropsy was performed at termination and for all mice found dead or killed in extremis. The following organ weights were measured for all animals in the interim sacrifice group and for at least 10 animals of each sex per group in the main groups: adrenals, brain, heart, kidneys, liver, ovaries, spleen, testes and epididymides, and uterus with cervix. An extensive list of tissues was examined histologically, including gross lesions and tissue masses.

No treatment-related effect on survival was noted (Table 20). The incidences and causes of morbidity and mortality in controls and treated animals were generally similar and consistent with the usual pattern of causes of demise in mice of this strain. Clinical signs in moribund animals generally included hunched posture, sluggishness, pale appearance, laboured/rapid respiration, swollen abdomen and/or thin appearance. Effects on body weight gain were noted only in the 7000 ppm females of the satellite group; however, this effect was not replicated in females in the main dose group and therefore was not considered to be treatment related. No effects on feed consumption were noted. No statistically significant treatment-related effects on haematology parameters were noted at either week 52 (interim) or week 78 (main study). A small number of control and treated animals killed during the treatment period or surviving to termination had elevated white blood cell counts, generally due to increased neutrophils and/or lymphocytes, which generally correlated microscopically with the presence of haemolymphoreticular tumour.

Table 20. Key results of the long-term toxicity and carcinogenicity study in mice

	Males				Females			
	0 ppm	700 ppm	2 000 ppm	7 000 ppm	0 ppm	700 ppm	2 000 ppm	7 000 ppm
Mortality[a] (satellite groups)	2/12	0/12	1/12	2/12	2/12	0/12	1/12	0/12
Survival (%) (satellite groups)	83	100	92	83	83	100	92	100
Mortality[a] (main groups)	13/51	12/51	9/51	13/51	18/51	16/51	16/51	14/51
Survival (%) (main groups)	75	76	82	75	65	69	69	73
Organ weights								
Liver, week 52 (g)	2.34	2.47	2.43	2.59	1.75	1.80	1.93	1.97
% of control	–	*5.6*	*3.8*	*10.7*	–	*2.9*	*10.3*	*12.6*
Liver, week 52 (% of body weight)	4.42	4.67	4.76	5.10**	4.07	4.19	4.41	5.10**
% of control	–	*5.7*	*7.7*	*15.4*	–	*2.9*	*8.4*	*25.3*
Liver, week 78 (g)	2.40	2.55	2.54	2.76 DR*	1.93	2.20	2.32	2.09
% of control	–	*6.3*	*5.8*	*15.0*	–	*14.0*	*20.2*	*8.3*
Liver, week 78 (% of body weight)	4.59	4.85	4.81	5.24**	4.42	4.65	4.96	4.90
% of control	–	*5.7*	*4.8*	*14.2*	–	*5.2*	*12.2*	*10.9*

DR: dose–response test (a significant trend was reported only where none of the pairwise comparisons was significant); ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (analysis of variance, dose–response and Dunnett's)

[a] No. of animals that died prior to termination / no. of animals in group.

Source: Beck (2012c)

Liver weight increases were noted in the high-dose males and females. Relative liver weight increases attained statistical significance at both interim and terminal sacrifices in males and at interim sacrifice only in females (Table 20).[4] The increase in liver weights was considered to be non-adverse in the absence of histological indicators of an adverse effect on the liver.

No macroscopic or non-neoplastic histopathological effects were noted. No treatment-related effect on tumour incidence was noted.

The NOAEL for toxicity was 7000 ppm (equal to 823.9 mg/kg bw per day), the highest dose tested, based on the absence of adverse effects. No carcinogenic potential of mandestrobin was observed (Beck, 2012c).

Rats

Mandestrobin (purity 93.4%) was administered to groups of 50 male and 50 female Wistar rats (6 weeks of age) at a dietary concentration of 0, 400, 2000, 7000 or 15 000 ppm (equal to 0, 21.0, 105.1, 375.6 and 804.3 mg/kg bw per day for males and 0, 26.7, 135.2, 475.0 and 1016.2 mg/kg bw per day for females, respectively) for 104 weeks (main groups: carcinogenicity cohort). In addition to these animals, 20 rats of each sex per group were treated at a dietary concentration of 0, 400, 2000, 7000 or 15 000 ppm (equal to 0, 25.5, 130.3, 448.8 and 991.8 mg/kg bw per day for males and 0, 31.3, 151.4,

[4] The mode of action for the liver weight increase was considered to be due to liver enzyme induction, via activation of the CAR by mandestrobin, as evidenced by work presented in section 2.6(c) below.

535.3 and 1138.9 mg/kg bw per day for females, respectively) for 52 weeks for interim sacrifice (satellite groups: chronic toxicity cohort).

Doses, homogeneity and stability in the diet were confirmed by analysis. Animals were checked for mortality and clinical signs daily. Body weight and feed consumption were recorded once weekly during acclimatization and up to week 16 and every 4 weeks thereafter. Ophthalmoscopic examinations were performed on all animals before treatment and on control and highest-dose satellite animals in week 50. A FOB was conducted on all rats of the satellite groups in week 51. Blood samples (after fasting) were drawn from all animals of the satellite groups in weeks 13, 26 and 52 and from 10 animals of each sex per main group in weeks 78 and 104. For the chronic toxicity cohort, the full set of haematological and clinical chemistry parameters was determined, whereas for the carcinogenicity cohort, only total and differential white cell count were determined in blood samples. Urine samples were collected from all satellite group animals in weeks 12, 25 and 51. All animals, either found dead or terminated, were subjected to detailed necropsy and collection of tissues. The following organs were weighed: adrenals, brain, heart, kidneys, liver, ovaries, spleen, testes and epididymides, thymus, and uterus and cervix. An extensive list of tissues was examined histologically, including gross lesions and tissue masses.

No treatment-related effects on clinical signs, FOB or ophthalmoscopy were noted. In the carcinogenicity cohort, decreased and dose-responsive mortality was observed in all female treatment groups. In males of the carcinogenicity cohort, the mortality was lower at 400 and 7000 ppm; however, as this was not dose responsive, the decreased mortality was considered not to be treatment related. In the chronic toxicity cohort, body weight suppression was observed in males at 15 000 ppm during the first 24 weeks, accompanied by reduced body weight gain (15%) from start to week 13 and at week 40 (8.1%). Body weight in females at 15 000 ppm was lower than in controls at all measurement intervals (9%). In these females, body weight gain was lower from the start to week 52 (16.4%). In both sexes given diets containing 15 000 ppm, feed consumption was lower over the 52-week treatment period, statistically significantly in females for pairwise comparison and in males for dose–response relationship. The statistically significant increases in body weight, body weight gain and feed consumption in males at 400 ppm were considered incidental, as this finding was not repeated in the carcinogenicity cohort. In the carcinogenicity cohort, decreases in body weight and/or body weight gain in both sexes were observed at 15 000 ppm. In the carcinogenicity cohort, feed consumption decreased in males at 15 000 ppm, attaining statistical significance for pairwise comparison from weeks 1 to 52 and for dose–response relationship from weeks 1 to 104. In females, feed consumption was statistically significantly decreased for dose–response relationship at 15 000 ppm over the 104-week treatment period. However, there was a body weight decrease in females of up to 19% at 2000 ppm (at week 68 and from week 76 onwards), up to 25% at 7000 ppm (at week 24 and from week 40 onwards) and up to 36% at 15 000 ppm (at week 6 and from week 11 onwards). Statistically significant decreases in overall body weight gains (start to week 104) were observed for females fed 2000, 7000 or 15 000 ppm (18.1%, 18.2% and 32.8%, respectively), compared with controls. In males, body weight gain was statistically significantly decreased at 15 000 ppm, by 11% from the start to week 52 and by 18% from the start to week 104, compared with controls (Tables 21 and 22).

Haematology investigation in the chronic toxicity cohort (Table 21) showed a treatment-related change in haemoglobin in males and females at 15 000 ppm and in females at 7000 ppm. However, these changes relative to controls were generally small (~5%), and values were within historical control ranges. In addition, no findings were observed in related end-points, such as red blood cells, reticulocytes or morphological changes in blood-forming organs. Therefore, these decreases were considered not to be of toxicological relevance. All other statistically significant differences between controls and treated groups were consistent with normal variability, small, inconsistent between the sexes, lacking temporal consistency and/or unrelated to dose and therefore considered not to be treatment related. In the carcinogenicity cohort (Table 22), all statistically significant differences in haematological end-points between the controls and treated groups were small, inconsistent between sexes, lacking temporal consistency and/or unrelated to dose and therefore considered to be of no toxicological importance. A small number of control and treated animals killed during the treatment period or surviving to termination had elevated white blood cell counts, generally due to increased

Table 21. Key results of the combined chronic toxicity and carcinogenicity study in rats: chronic toxicity cohort

	Males							Females						
						HCD							HCD	
Parameter	0 ppm	400 ppm	2 000 ppm	7 000 ppm	15 000 ppm	Mean	Range	0 ppm	400 ppm	2 000 ppm	7 000 ppm	15 000 ppm	Mean	Range
Mortality[a]	1/20	1/20	1/20	0/20	0/20	–	–	0/20	0/20	1/20	1/20	0/20	–	–
Survival (%)	95	95	95	100	100	–	–	100	100	95	95	100	–	–
Body weight (g)														
Week 0	184.7	189.1	182.8	180.1	180.0	–	–	140.2	140.9	143.5	141.0	138.7	–	–
Week 52	486.9	532.0*	486.3	470.7	451.5	–	–	275.8	265.6	272.7	268.8	251.9*	–	–
Body weight gain (g)														
Weeks 0–28	251.4	285.2*	249.8	239.6	225.0	–	–	110.6	105.2	105.5	106.6	101.4	–	–
Weeks 0–52	302.5	343.8*	302.7	290.6	271.5	–	–	135.6	124.8	129.4	128.3	113.3**	–	–
Feed consumption (g/animal per week)														
Weeks 1–28	155.7	166.2*	157.0	149.0	148.3	–	–	119.9	115.4	114.9	115.5	111.3*[DR]	–	–
Weeks 1–52	154.5	163.9*	155.5	147.5	147.8	–	–	119.4	114.9	114.4	115.1	110.4*	–	–
Haematology (week 52)														
Hb (g/dL)	16.1	16.2	16.1	16.0	15.6**	16.5	15.1–18.0	15.6	15.4	15.2	14.9***	14.7***	15.6	14.6–16.3
MCH (pg)	17.7	17.5	17.7	17.4	16.9**	–	–	19.0	18.7	18.5*	18.8	18.4**	–	–
MCHC (g/dL)	35.6	35.2	35.2	35.0	34.5**	–	–	35.9	35.4	35.0***	35.2**	34.5***	–	–
PT (s)	22.3	21.3*	21.5	21.1*	20.0***	–	–	21.5	21.9	21.8	21.4	21.5	–	–
LUC (10^9/L)	0.0	0.0	0.0	0.0	0.1*	–	–	0.0	0.0	0.0	0.0	0.0	–	–
Blood chemistry (week 52)														
ALP (IU/L)	59	52	54	49*	50	–	–	27	28	24	19**	16***	–	–
Calcium (mmol/L)	2.64	2.62	2.63	2.66	2.63	–	–	2.69	2.62**	2.69	2.74*	2.78***	–	–

Parameter	Males							Females						
	0 ppm	400 ppm	2 000 ppm	7 000 ppm	15 000 ppm	HCD Mean	HCD Range	0 ppm	400 ppm	2 000 ppm	7 000 ppm	15 000 ppm	HCD Mean	HCD Range
Chloride (mmol/L)	105	104*	103***	102***	102***	–	–	103	103	103	103	102*[DR]	–	–
Inorganic phosphate (mmol/L)	1.2	1.3	1.3	1.3	1.4**	–	–	0.9	0.9	0.8	0.9	1.0	–	–
Total protein (g/L)	68	69	69	70	69	–	–	72	71	73	75*	74*	–	–
Albumin (g/L)	44	45	45	47*	46	–	–	53	51	52	54	54**[DR]	–	–
Globulin (g/L)	24	24	23	23	23	–	–	19	20	20	21*	20	–	–
A/G	1.8	1.9	1.9	2.0*	2.0	–	–	2.8	2.6	2.7	2.6	2.7	–	–
Total bilirubin (µmol/L)	1.7	1.9	1.8	1.7	1.5	–	–	2.8	2.4	2.4	2.7	2.1**	–	–
GGT (IU/L)	2	2	2	2	8***	–	–	2	2	2	2	6***	–	–
Total cholesterol (mmol/L)	2.3	2.3	2.4	2.7	2.9*	–	–	2.0	1.8	2.3	3.1***	3.0***	–	–
Glucose (mmol/L)	6.3	6.1	5.4**	5.7	5.9	–	–	6.1	5.5	5.5*	5.2***	4.8***	–	–
Organ weights														
Absolute liver weight (g)	9.63	11.22**	10.494	10.77*	12.26***	–	–	6.22	5.96	6.41	7.26**	7.71***	–	–
% change			*9*	*12*	*27*					*3*	*17*	*24*		
Relative liver weight (%)	2.01	2.16	2.22*	2.35***	2.81***	–	–	2.33	2.34	2.44	2.83***	3.15***	–	–
% change			*10*	*17*	*40*					*5*	*21*	*35*		
Absolute kidney weight (g)	2.15	2.26	2.099	2.066	2.169	–	–	1.47	1.41	1.42	1.40	1.40	–	–
Relative kidney weight (%)	0.45	0.43	0.44	0.45	0.49**	–	–	0.55	0.55	0.54	0.54	0.57	–	–
% change					*10*									
Absolute brain weight (g)	2.16	2.19	2.14	2.14	2.06**	–	–	1.96	1.97	1.96	1.93	1.95	–	–
% change					*5*									
Relative brain weight (%)	0.45	0.42	0.54	0.47	0.47**[DR]	–	–	0.74	0.77	0.75	0.75	0.80*	–	–
% change					*4*							*8*		

Parameter	Males 0 ppm	Males 400 ppm	Males 2 000 ppm	Males 7 000 ppm	Males 15 000 ppm	Males HCD Mean	Males HCD Range	Females 0 ppm	Females 400 ppm	Females 2 000 ppm	Females 7 000 ppm	Females 15 000 ppm	Females HCD Mean	Females HCD Range
Gross pathology and histopathology														
Liver														
Large	0	0	0	1	6*	–	–	0	0	1	0	2	–	–
Hepatocellular eosinophilia/hypertrophy	0	0	0	15***	20***	–	–	0	0	0	17***	15***	–	–
Hepatocyte vacuolation	1	3	1	3	6	–	–	0	2	3	4*	2	–	–
Agonal congestion/ haemorrhage	2	1	1	5	10	–	–	1	0	1	1	1	–	–
Thyroid														
Follicular cell hypertrophy	1	0	1	9**	18***	–	–	0	0	0	9***	15***	–	–

A/G: albumin/globulin; ALP: alkaline phosphatase; DR: dose–response relationship (a significant trend was reported only where none of the pairwise comparisons was significant); GGT: gamma-glutamyl transferase; Hb: haemoglobin; HCD: historical control data; IU: international units; LUC: large unstained cells; MCH: mean cell haemoglobin; MCHC: mean cell haemoglobin concentration; ppm: parts per million; PT: prothrombin time; *: $P < 0.005$; **: $P < 0.01$; ***: $P < 0.001$ (analysis of variance, dose–response and Dunnett's)

[a] No. of animals that died prior to termination / no. of animals in group.

Source: Beck (2012d)

Table 22. Key results of the combined chronic toxicity and carcinogenicity study in rats: carcinogenicity cohort

	Males							Females						
						HCD (%)							HCD (%)	
Parameter	**0 ppm**	**400 ppm**	**2 000 ppm**	**7 000 ppm**	**15 000 ppm**	**Mean**	**Range**	**0 ppm**	**400 ppm**	**2 000 ppm**	**7 000 ppm**	**15 000 ppm**	**Mean**	**Range**
Mortality[a]	18/50	8/50	18/50	9/50	14/50	–	–	17/50	15/50	14/50	10/50	9/50	–	–
Survival (%)	64	84	64	82	72	–	–	66	70	72	80	82	–	–
Body weight (g)														
Week 0	188.3	187.6	188.7	186.4	188.9	–	–	144.0	141.7	143.4	143.8	144.4	–	–
Week 104	611.5	613.4	570.6	584.5	538.5***	–	–	363.6	353.8	323.2**	323.2**	292.5***	–	–
Body weight gain (g)														
Weeks 0–52	327.5	329.8	331.7	314.5	291.8***	–	–	139.9	143.5	130.7	120.9***	110.8***	–	–
Weeks 0–104	424.0	426.5	384.4	396.7	349.2***	–	–	220.0	214.5	180.1**	179.9**	147.9***	–	–
Feed consumption (g/animal per week)														
Weeks 1–52[b]	152.2	151.2	153.6	150.2	144.6*	–	–	113.5	114.2	113.1	110.6	107.2*[DR]	–	–
Weeks 1–104[b]	148.4	147.4	149.4	147.2	141.9*[DR]	–	–	113.6	114.1	112.8	111.6	108.1*[DR]	–	–
Haematology (week 104)														
WBC (10^9/L)	5.9	4.7**	4.8*	5.3	5.1	–	–	3.4	3.5	4.1	3.5	4.3	–	–
Lymphocytes (10^9/L)	3.9	3.1*	3.3	3.3	3.5	–	–	2.1	2.0	2.1	2.0	2.0	–	–
Eosinophils (10^9/L)	0.1	0.1	0.1	0.1	0.1	–	–	0.1	0.1	0.1	0.1	0.4*	–	–
Organ weights														
Absolute liver weight (g)	11.51	11.87	11.17	13.10	11.85	–	–	6.989	7.262	7.340	7.226	7.748	–	–
Relative liver weight (%)	2.051	2.029	2.042	2.215	2.334*	–	–	2.153	2.038	2.472*	2.446*	2.753***	–	–

Parameter	Males 0 ppm	Males 400 ppm	Males 2 000 ppm	Males 7 000 ppm	Males 15 000 ppm	Males HCD (%) Mean	Males HCD (%) Range	Females 0 ppm	Females 400 ppm	Females 2 000 ppm	Females 7 000 ppm	Females 15 000 ppm	Females HCD (%) Mean	Females HCD (%) Range
Absolute kidney weight (g)	2.420	2.456	2.445	2.694	2.335	–	–	1.677	1.695	1.675	1.560	1.601	–	–
Relative kidney weight (%)	0.433	0.421	0.448	0.456	0.461*	–	–	0.520	0.481	0.569	0.526	0.574*	–	–
Absolute brain weight (g)	2.24	2.20	2.25	2.22	2.23	–	–	2.02	2.00	2.00	2.02	2.02	–	–
Relative brain weight (%)	0.40	0.38	0.41	0.38	0.44	–	–	0.63	0.57	0.69	0.69	0.73**[DR]	–	–
Macroscopic findings: liver														
Large	2	4	4	7	9	–	–	0	2	2	2	3	–	–
Microscopic non-neoplastic findings														
Liver														
Hepatocellular eosinophilia/ hypertrophy														
Decedents	1	1	1	2	2	–	–	1	4	2	7*	5*	–	–
Terminal kill	6	10	12	28***	35***	–	–	15	18	30***	36***	37***	–	–
All animals	7	11	12	30***	37***	–	–	16	22	21**	43***	42***	–	–
Hepatocyte vacuolation														
Decedents	6	2	11	3	12*	–	–	5	3	1	0	4	–	–
Terminal kill	22	37	26	35*	33**	–	–	15	10	18	29*	34***	–	–
All animals	28	39*	37	38	45*	–	–	20	13	19	29	38*	–	–
- Minimal	24	35	33	31	35	–	–	19	12	19	29	36	–	–

Parameter	Males							Females						
						HCD (%)							HCD (%)	
	0 ppm	400 ppm	2 000 ppm	7 000 ppm	15 000 ppm	Mean	Range	0 ppm	400 ppm	2 000 ppm	7 000 ppm	15 000 ppm	Mean	Range
- Slight	3	2	3	7	9	–	–	1	0	0	0	2	–	–
- Moderate	1	2	1	0	1	–	–	0	1	0	0	0	–	–
Bile duct hyperplasia														
Decedents	1	1	0	0	1	–	–	3	6	4	3	1	–	–
Terminal kill	2	5	7	4	4	–	–	10	17	18	26**	18	–	–
All animals	3	6	7	4	5	–	–	13	23	22	29**	19	–	–
Thyroid														
Follicular cell hypertrophy														
Decedents	0	0	0	0	0	–	–	0	0	0	0	0	–	–
Terminal kill	0	2	0	0	10**	–	–	0	0	0	0	3	–	–
All animals	0	2	0	0	10**	–	–	0	0	0	0	3	–	–
Follicular cell hyperplasia														
Decedents	0	0	0	1	4*	–	–	0	1	1	0	0	–	–
Terminal kill	4	11	5	4	9	–	–	1	0	3	3	4	–	–
All animals	4	11	5	5	13*	–	–	1	1	4	3	4	–	–
Kidney														
Papillary mineralization														
Decedents	2	3	2	3	3	–	–	10	4	3	6	4	–	–
Terminal kill	7	8	7	10	13	–	–	17	13	25	30*	26	–	–
All animals	9	11	9	13	16	–	–	27	17	28	36	30	–	–

Parameter	Males							Females						
						HCD (%)							HCD (%)	
	0 ppm	400 ppm	2 000 ppm	7 000 ppm	15 000 ppm	Mean	Range	0 ppm	400 ppm	2 000 ppm	7 000 ppm	15 000 ppm	Mean	Range
Pelvic mineralization														
Decedents	7	4	8	0	8	–	–	14	12	8	8	7	–	–
Terminal kill	22	30	19	30	26	–	–	30	29	34	36	36	–	–
All animals	29	34	27	30	34	–	–	44	41	42	44	43	–	–
Corticomedullary mineralization														
Decedents	0	0	0	0	0	–	–	0	0	3	1	4*	–	–
Terminal kill	0	1	0	0	0	–	–	6	7	14*	16*	14	–	–
All animals	0	1	0	0	0	–	–	6	7	17*	17*	18**	–	–
- Minimal	0	1	0	0	0	–	–	6	6	12	15	13	–	–
- Slight	0	0	0	0	0	–	–	0	1	2	1	1	–	–
Epididymis														
Oligospermia														
Decedents	1	3	1	6	3	–	–	–	–	–	–	–	–	–
Terminal kill	0	1	1	2	7*	–	–	–	–	–	–	–	–	–
All animals	1	4	2	8	10*	–	–	–	–	–	–	–	–	–
Testis								–	–	–	–	–		
Interstitial cell hyperplasia	2	0	1	1	0	–	–	–	–	–	–	–	–	–
Ovary														
Sex cord stromal hyperplasia														
Decedents	–	–	–	–	–	–	–	0	0	0	1	0	–	–

Parameter	Males							Females						
						HCD (%)							HCD (%)	
	0 ppm	400 ppm	2 000 ppm	7 000 ppm	15 000 ppm	Mean	Range	0 ppm	400 ppm	2 000 ppm	7 000 ppm	15 000 ppm	Mean	Range
Terminal kill	–	–	–	–	–	–	–	3	8	5	5	5	–	–
(%)	–	–	–	–	–	–	–	*6*	*16*	*10*	*12*	*10*	*27.1*	*2–48%*[c]
All animals	–	–	–	–	–	–	–	3	8	5	6	5	–	–
Neoplastic microscopic findings														
Testis														
Interstitial cell adenoma	0	0	0	2	3	–	–	–	–	–	–	–	–	–
(%)	0	0	0	4	6	2.3	0–6[d]							
Ovary														
Sex cord stromal tumours	–	–	–	–	–	–	–	2	0	1	4	6**[DR]	–	–
(%)								4	0	2	8	12	See text	
Combined sex cord stromal tumour and hyperplasia[e]	–	–	–	–	–	–	–	5	8	6	8	9	–	–
(%)	–	–	–	–	–	–	–	10	16	12	16	18	–	–

DR: dose–response relationship (a significant trend was reported only where none of the pairwise comparisons was significant); HCD: historical control data; ppm: parts per million; WBC: white blood cells; *: $P < 0.005$; **: $P < 0.01$; ***: $P < 0.001$ (analysis of variance, dose–response and Dunnett's)

[a] No. of animals that died prior to termination / no. of animals in group.
[b] Week 48 excluded from female calculation of feed consumption due to animals fed ad libitum.
[c] Data generated from 2000 to 2008, from eight studies.
[d] Data generated on Han Wistar rats from 2000 to 2008, from eight studies.
[e] The number of animals exhibiting both hyperplasia and tumour is counted as 1.

Source: Beck (2012d)

neutrophils and/or lymphocytes, which generally correlated microscopically with the presence of haemolymphoreticular tumours.

In the chronic toxicity cohort, the activity of GGT was statistically significantly increased in both sexes fed diets containing 15 000 ppm mandestrobin at week 13 (males: 1.5-fold; females: 2-fold), at week 26 (males: 2.5-fold; females: 1.5-fold) and at week 52 (males: 4-fold; females: 3-fold), compared with controls. Total cholesterol was higher at week 13 in animals fed 7000 ppm (males: 29%; females: 38%) and 15 000 ppm (males: 47%; females: 56%), at week 26 in females fed 7000 ppm and 15 000 ppm (50% and 65%, respectively) and at week 52 in females fed 7000 ppm (55%) and in both sexes fed 15 000 ppm (males: 26%; females: 50%), compared with controls. Therefore, the consistent increases seen in females given 7000 ppm and in both sexes fed 15 000 ppm were considered to be toxicologically relevant. As no disturbances were observed on subsequent occasions, the change in males fed 7000 ppm at week 13 was considered not to be toxicologically relevant (Table 21).

All other statistically significant differences between control and treated groups were consistent with normal variability (i.e. within historical control ranges, which, however, are available only for weeks 13 and 26), small, inconsistent between the sexes, lacking temporal consistency and/or unrelated to dose and therefore considered to be incidental.

Urine analysis showed an increased mean volume of voided urine in males fed diets containing 400 ppm at weeks 12 and 51 and in males given 15 000 ppm at week 51. Quantitative measurements indicated that excretion was approximately 64–71% higher. However, as there were no similar effects in females or overt differences in week 25, these changes were considered to be incidental and not treatment related. Increased urine output correlated with a decrease in specific gravity.

In the chronic toxicity cohort, a statistically significant increase in relative kidney weights (10%) was noted in males at 15 000 ppm. A statistically significant decrease in absolute brain weight (5%) was noted in males at 15 000 ppm. In contrast, relative brain weight was increased at 15 000 ppm in males and females, by 4% and 8%, respectively. Changes in kidney and brain weights were considered not to be toxicologically relevant as they were small, there were no macroscopic or microscopic correlates and they were related to body weight suppression. Increased liver weights were noted in treated males at and above 2000 ppm and in females at and above 7000 ppm. In males, absolute liver weights at 2000, 7000 and 15 000 ppm were increased by 9%, 12% and 27%, respectively. In females, absolute liver weights at 7000 and 15 000 ppm were increased by 17% and 24%, respectively. Relative liver weights in males at 2000, 7000 and 15 000 ppm were increased by 10%, 17% and 40%, respectively; in females at 7000 and 15 000 ppm, relative liver weights were increased by 21% and 35%, respectively (Table 21). Mean relative ovary weights were statistically significantly increased (26%) in females at 15 000 ppm. This finding is considered to be incidental, as there was no similar change seen for the females in the carcinogenicity cohort at the terminal kill, and it was not associated with any pathological correlates. Other absolute and relative organ weight changes were considered not to be biologically relevant, as they were small in magnitude, not dose dependent, inconsistent between sexes, spontaneous background changes due to normal interanimal variability and/or lacking a histopathological correlate.

From the macroscopic investigation, a statistically significantly increased incidence of large liver was recorded in males at 15 000 ppm. Large liver was also noted in two females at 15 000 ppm, in one female at 2000 ppm and in one male at 7000 ppm, which generally correlated with findings seen microscopically. Other statistically significant differences between groups for the macroscopic findings, such as mottled liver and red mandibular lymph node, which correlated with agonal congestion/haemorrhage microscopically, were sporadic and not dose related. Increased incidences and severity of hepatocellular eosinophilia/hypertrophy were noted in animals at 7000 and 15 000 ppm and were characterized by enlarged hepatocytes with increased amounts of eosinophilic cytoplasm. This was considered to be an adaptive change associated with mandestrobin metabolism. A minor increase in hepatocyte vacuolation in males at 15 000 ppm, characterized by variable numbers of small to large cytoplasmic vacuoles within scattered hepatocytes, was also recorded, but, according to the study author, the toxicological relevance of this change remained unclear (Table 21). The effects on liver

weight were associated with the blood biochemistry and pathological changes and therefore were considered to be toxicologically relevant.

In the thyroid, increased incidences and severity of follicular cell hypertrophy in animals at 7000 and 15 000 ppm were noted (Table 21). Follicular cell hypertrophy was characterized by follicles with columnar epithelium with increased amounts of apical cytoplasm, with or without a decrease in follicular colloid. This is generally considered to be an adaptive change due to increased thyroid hormone metabolism in the liver and is commonly associated with liver cell hypertrophy.

In the carcinogenicity cohort, statistically significantly increased relative kidney weights were noted at 15 000 ppm for both sexes (males: 7%; females: 10%). Changes in kidney weights were considered not to be toxicologically relevant, as they were small and likely related to body weight suppression. The relative brain weight was increased at 15 000 ppm for both sexes (males: 10%; females: 16%); in females, changes achieved statistical significance. Changes in brain weights were considered not to be toxicologically relevant, as there were no macroscopic or microscopic correlates and they were likely related to body weight suppression. In males, a statistically significant increase in relative liver weight (14%) was observed at 15 000 ppm. In females, statistically significant relative liver weight increases of 15%, 14% and 28% were seen at 2000, 7000 and 15 000 ppm, respectively (Table 22).

From the macroscopic investigation, large liver was variably recorded in males at 7000 and 15 000 ppm, although this was not statistically significant (Table 22).

Microscopic evaluation in all animals showed, in the kidney, an increased papillary mineralization (not statistically significant) in females and males at and above 7000 ppm; however, there was no clear dose–response relationship. Papillary mineralization was characterized by small amounts of dark basophilic mineral within the renal papillary tubules. A significant increase in minimal corticomedullary mineralization in females at and above 2000 ppm was noted. In the absence of other correlated biological changes, this effect was considered to be of minimal toxicological relevance. Corticomedullary mineralization was characterized by small amounts of dark, basophilic material within the tubules and tubular epithelium at the corticomedullary junction (Table 22).

In the liver, increased hepatocellular eosinophilia/hypertrophy in animals at 2000 ppm and above, characterized by enlarged hepatocytes with increased amounts of pale eosinophilic cytoplasm, was noted. Increases in hepatocellular eosinophilia/hypertrophy were statistically significant in males at 7000 and 15 000 ppm and in females at and above 2000 ppm. There was no associated increase in hepatic tumours noted in the study. Increased hepatocyte vacuolation was observed in males (unscheduled and terminal kill) at all doses, achieving statistical significance at 400 and 15 000 ppm, but with no clear dose–response relationship. The increased incidence at all doses was of minimal severity. At 7000 and 15 000 ppm, an increase in slight hepatocyte vacuolation was noted. In females, an increase was observed at 7000 and 15 000 ppm, with the value at the highest dose being statistically significant. Hepatocyte vacuolation was characterized by variable numbers of small to large cytoplasmic vacuoles within scattered hepatocytes. According to the study author, the toxicological relevance of this change in this study was unclear. Bile duct hyperplasia was slightly increased in males at and above 2000 ppm and variably in treated females. This finding was considered not to be toxicologically relevant, as it was not observed in a dose-dependent manner and there was no associated bile duct epithelial degeneration or necrosis, fibrosis or progression to bile duct neoplasia (Table 22).

In the thyroid gland, increased follicular cell hypertrophy and hyperplasia were noted in males and, to a lesser extent, in females at 15 000 ppm; this increased incidence was statistically significant in males at 15 000 ppm. Follicular cell hypertrophy was characterized by follicles with columnar epithelium with increased amounts of apical cytoplasm, with or without a decrease in follicular colloid. This was not accompanied by an increase in thyroid follicular cell tumours and is known to be associated with hepatocellular hypertrophy (Table 22).

In the ovary, a minor increase in sex cord stromal hyperplasia was noted in treated females, but not in a dose-dependent manner and also without statistical significance. Sex cord stromal hyperplasia was characterized by a hyperplastic lesion composed of mixtures of granulosa, theca, luteal or Sertoli

cells within the ovarian parenchyma, with minimal involvement of the surface epithelium. The incidence in treated animals was within current historical background data (Table 22).

Increased oligospermia was recorded in the epididymis of males at 15 000 ppm; however, the finding was primarily noted unilaterally. This was considered to be an age-related change due to the predominantly unilateral nature and, in the absence of any other male reproductive tract findings, not associated with generalized hormonal disruption or mandestrobin toxicity (Table 22).

A reduction in the incidence of benign mammary fibroadenomas and a statistically significant reduction in the incidence of pituitary tumours (adenoma and carcinoma) were noted in females at 15 000 ppm. This correlates with the reduction in fatal tumours observed in female decedents at 15 000 ppm.

An increased incidence of sex cord stromal tumours was recorded in the ovary of females at 7000 and 15 000 ppm. These increased incidences were not statistically significant when analysed using pairwise analysis, but were positive for tests (Peto) of an increasing dose–response relationship. The incidence of sex cord stromal tumour in the ovary exceeded the laboratory historical control range for this strain of rats. However, it is also noted that the incidence in the concurrent control animals exceeded or was close to the upper range of the historical controls (Tables 23 and 24). Simultaneous occurrences (multiplicity) of sex cord stromal tumour were found in two rats at the highest dose of 15 000 ppm.

It is noted that the tumours occurred at doses at which body weight gain was reduced by more than 20%. In addition, higher survival rates of 80% and 82% in females at 7000 and 15 000 ppm, respectively, were observed. This may have contributed to the higher numbers of tumours. In this respect, it is noted that in the historical control data provided, the tumours occurred at a survival greater than 70%.

A dose-related increase in interstitial cell adenomas was noted in the testis of males. The increase was statistically significant in a trend test, but no dose was statistically significantly increased in pairwise comparisons.

The NOAEL for toxicity was 400 ppm (equal to 26.7 mg/kg bw per day), based on effects on body weight and liver (histopathological changes and increased liver weights) in females at 2000 ppm (equal to 135.2 mg/kg bw per day). The NOAEL for carcinogenicity was 7000 ppm (equal to 375.6 mg/kg bw per day), based on an equivocal increase in the incidence of tumours (ovarian sex cord stromal adenomas and testicular interstitial cell adenomas) at the highest dose (Beck, 2012d).

Table 23. First set of historical control data for sex cord stromal hyperplasia and tumours

Parameter	Study 1	Study 2	Study 3	Study 4	Study 5	Study 6	Study 7	Study 8	Total average
Number of animals examined	70	50	50	50	50	50	100	80	480
Survival (%)	78	74	72	74	72	37	45	58	64
Sex cord stromal hyperplasia	31/70	14/49	1/50	24/50	1/50	13/50	26/100	16/59	126/478
(%)	44	28	2	48	2	26	26	27	26.3
Sex cord stromal tumour	1/70	0/49	0/50	2/50	0/50	0/50	0/100	0/59	3/478
(%)	1.4	0	0	4.0	0	0	0	0	0.63

Source: Yamada & Miyata (2012)

Table 24. Second set of historical control data for sex cord stromal hyperplasia and tumours

Parameter	Study 1	Study 2	Study 3	Study 4	Study 5	Study 6	Study 7	Total average
Completed year	2000	2001	2003	2004	2005	2007	2008	2000–08
Pathologist[a]	A	B	B	C	A	D	D	A, B, C, D
Number of animals examined	70	50	50	50	100	60	100	480
Survival (%)	78	74	72	72	79	58	45	68
Sex cord stromal hyperplasia	31/70	14/49	1/50	1/50	31/64	15/59	26/100	120/442
(%)	44	29	2	2	48	27	26	27.1
Sex cord stromal tumour	1/70	0/49	0/50	0/50	2/64	0/59	0/100	3/442
(%)	1.4	0	0	0	3.1	0	0	0.68

[a] The pathologist for the carcinogenicity study on mandestrobin is not the same as the pathologists for studies 1–7 in this table.
Source: Beck (2012d)

2.4 *Genotoxicity*

Mandestrobin was negative for genotoxicity in three in vitro assays (gene mutation, chromosomal aberration and forward mutation assay) in the presence and absence of metabolic activation and in an in vivo assay (mouse bone marrow micronucleus assay). The results of the genotoxicity tests are summarized in Table 25 and described briefly below.

Table 25. Results of genotoxicity studies performed with mandestrobin

Type of study	Organism/cells	Dose/concentration range	Purity (%)	Result	Reference
In vitro					
Gene mutation (Ames test)	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537 and *Escherichia coli* WP2*uvr*A	−S9: 9.77–5 000 µg/plate +S9: 39.1–5 000 µg/plate	93.4	Negative ±S9	Kitamoto (2010a)
Chromosomal aberration assay	Chinese hamster lung cells (CHL/IU)	−S9: 3.91–80.0 µg/mL +S9: 100–150 µg/mL	93.4	Negative ±S9	Kitamoto (2010b)
HPRT forward mutation assay	Chinese hamster V79 cells	−S9: 1.0–50.0 µg/mL +S9: 8.0–144.0 µg/mL	93.4	Negative ±S9	Wollny (2010)
In vivo					
Micronucleus assay	Mouse (CD-1) bone marrow erythrocytes (gavage)	500–2 000 mg/kg bw	93.4	Negative	Kitamoto (2010c)

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

(a) In vitro

Mandestrobin (purity 93.4%) was tested in two separate experiments for its mutagenic potential on *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* strain WP2*uvr*A using the preincubation method at concentrations up to 5000 µg/plate in the absence and presence of S9 mix. Cytotoxicity in strains TA100, TA1535 and TA1537 was seen at and above 313 µg/plate without S9 mix and at and above 625 µg/plate with S9 mix. No cytotoxicity was observed for strains WP2*uvr*A and TA98 with or without S9 mix. Precipitation of the test compound was observed at and above 1250 µg/plate with and without S9 mix. There was no significant dose-related increase in the number of revertant colonies, with or without S9 mix. Positive controls showed a marked increase in the number of revertant colonies. Mandestrobin was not mutagenic under the test conditions (Kitamoto, 2010a).

Mandestrobin (purity 93.4%) was tested for its clastogenic potential in Chinese hamster lung cells (CHL/IU). Based on the solubility limit in dimethyl sulfoxide (DMSO), the highest concentration was set at 1000 µg/mL in the medium. Precipitates were seen in the medium at the beginning of treatment at and above 125 µg/mL and at the end of treatment at and above 500 µg/mL. In a preliminary cytotoxicity assay, marked growth inhibition was seen under all treatment conditions (6-hour exposure ±S9 and 24-hour exposure −S9). In the main test, when cells were exposed for 6 hours, no increase in the incidence of aberrant cells (structural, numerical or polyploid) in any treatment groups without S9 was observed. A suggestive dose-related increase in cells with structural aberrations was seen in the presence of S9 in cells treated for 6 hours. The increase at the highest concentration (3.5%) fell outside of the historical range for the laboratory (3%), but was considered to be of limited biological relevance, as it occurred at a concentration at which a greater than 55% inhibition of cell growth was observed, and cytotoxicity can lead to artefactual clastogenic results. When CHL/IU cells were treated for 24 hours without S9 mix, the negative result of the previous experiment was confirmed. In the presence of S9 mix, a repeat 6-hour treatment resulted in negative findings through a dose series of 100–150 µg/mL. Positive and negative controls gave expected results. Mandestrobin showed little potential to induce chromosomal aberrations under the test conditions (Kitamoto, 2010b).

Mandestrobin (purity 93.4%) was tested in a gene mutation (*HPRT* locus) assay in Chinese hamster V79 cells. In a preliminary cytotoxicity assay, cell survival after 4 hours of treatment was reduced to 16.3% at 15.6 µg/mL without S9 mix and to less than 10% at 250 µg/mL with S9 mix. After 24 hours of treatment, cell survival was reduced to less than 10% at 62.5 µg/mL without S9. Three independent experiments were performed. In experiment I, cells were exposed for 4 hours with and without metabolic activation (S9 mix). Nine doses from 0.25 to 12.0 µg/mL were chosen for treatment without S9 mix, and the six treatments from 1.0 to 10.0 µg/mL were selected for evaluation. Six doses from 8.0 to 172.0 µg/mL were chosen for treatment with S9 mix, and the five treatments from 8.0 to 128.0 µg/mL were selected for evaluation. In experiment IA, cells were exposed for 4 hours with metabolic activation. Seven doses from 16.0 to 172.0 µg/mL were chosen for treatment with S9 mix, and the five treatments from 16.0 to 144.0 µg/mL were selected for evaluation. In experiment II, cells were treated for 24 hours without metabolic activation and for 4 hours with metabolic activation. Nine doses from 1.88 to 70.0 µg/mL were chosen for treatment without S9 mix, and the five doses from 7.5 to 50.0 µg/mL were selected for evaluation. Seven doses from 16.0 to 172.0 µg/mL were chosen for treatment with S9 mix, and the five doses from 16.0 to 144.0 µg/mL were selected for evaluation. Cytotoxic effects occurred at 8.0 µg/mL and above without metabolic activation (4 hours of treatment), at 144 µg/mL and above with metabolic activation (4 hours of treatment) and at 50.0 µg/mL and above without metabolic activation (24 hours of treatment).

No relevant and reproducible increase in mutant colony number per 10^6 cells was noted in the main experiments up to the maximum concentration. Isolated increases in the mutation frequency, exceeding the threshold of 3 times the mutation frequency of the corresponding solvent control, occurred occasionally, but were not reproduced in the parallel cultures under identical conditions and were based on the relatively low solvent controls. Compared with the corresponding negative controls, the 3-fold threshold was not exceeded. In addition, the mean values of mutation frequency between the first and the second cultures at 8.0 µg/mL (experiment I with metabolic activation), 32.0 µg/mL (experiment IA with metabolic activation) and 144.0 µg/mL (experiment II with metabolic activation)

were below the threshold and were within the historical control range. Furthermore, linear regression analysis (least squares) found no significant dose-dependent trend in the mutation frequency, indicated by a *P*-value below 0.05 in all of the experimental groups.

Appropriate positive controls showed a distinct increase in induced mutant colonies. Mandestrobin did not induce gene mutations at the *HPRT* locus in V79 cells (Wollny, 2010).

(b) In vivo

Mandestrobin (purity 93.4%) was tested for its clastogenic potential in an in vivo chromosomal aberration assay in mouse (CD-1) bone marrow. A range-finding toxicity assay was conducted in five mice of each sex per group administered a single mandestrobin dose (10 mL/kg bw) of 0 (0.5% aqueous methylcellulose), 500, 1000 or 2000 mg/kg bw by gavage. Mice were observed for clinical signs and mortality immediately after dosing, at 2.5 hours after dosing and daily for 2 days. Body weights were recorded once daily for 2 days. No abnormal signs were observed in any animals, but a slightly decreased body weight gain was noted in males at 2000 mg/kg bw. However, it was concluded that there were no substantial differences in sex response, and 2000 mg/kg bw was chosen as the highest test dose.

In the micronucleus test, mandestrobin was administered by gavage in 0.5% w/v aqueous methylcellulose to five male mice per group at a dose of 0, 500, 1000 or 2000 mg/kg bw; bone marrow smears were prepared 24 hours later. An additional group of five animals received mandestrobin at 2000 mg/kg bw, and smears were prepared 48 hours after treatment. The positive control material was cyclophosphamide. The incidence of micronucleated cells in 2000 polychromatic erythrocytes (PCEs) was scored for each animal, and the incidence of PCEs in 1000 erythrocytes (PCEs and normochromatic erythrocytes [NCEs]) was also recorded. Observations were performed using a microscope.

No dose-related increase in the incidence of micronuclei was noted. The positive control showed an appropriate increase in micronucleus formation. Although no change in the PCE/NCE ratio was found, a slight decrease in body weight gain at 2000 mg/kg bw indicated systemic absorption. Additionally, the exposure of bone marrow to mandestrobin was seen in the rat ADME studies.

In conclusion, mandestrobin did not cause an increase in the number of micronucleated PCEs in male mice when tested up to a dose of 2000 mg/kg bw (Kitamoto, 2010c).

2.5 Reproductive toxicity

(a) Multigeneration studies

In a dose range–finding study, groups of 10 Wistar rats of each sex were fed mandestrobin (purity 93.4%) at a dietary concentration of 0, 5000, 10 000 or 20 000 ppm (equal to 0, 244.1, 498.5 and 1032.5 mg/kg bw per day for males and 0, 316, 667.8 and 1229.0 mg/kg bw per day for females, respectively). Rats were mated after 4 weeks of exposure, and females were allowed to complete gestation and lactation while being maintained on treated diet. Adult males were terminated after gestation in the females was complete. At 4 days postpartum, litters were standardized to eight pups, and surplus pups were necropsied, then discarded. Adult females were terminated at 21 days postpartum, at weaning of the pups. Pups were terminated at 21 days of age.

No mortality or clinical signs were observed. In males, statistically significant decreases in body weight and body weight gain were noted in the 20 000 ppm group during the dosing period. In females, statistically significant decreases in body weight or body weight gain were noted in the 20 000 ppm group from day 28 of dosing through the gestation period to the lactation period. In females at 10 000 ppm, a statistically significant decrease in body weight or body weight gain was noted on day 28 of dosing and day 20 of gestation (Table 26).

Table 26. Key findings in the preliminary range-finding reproductive toxicity study in rats

	Males				Females			
	0 ppm	**5 000 ppm**	**10 000 ppm**	**20 000 ppm**	**0 ppm**	**5 000 ppm**	**10 000 ppm**	**20 000 ppm**
Parental animals (F_0)								
Body weight (g)								
Before mating (day 0)	217.0	215.9	217.1	216.2	162.6	159.1	160.2	161.2
Before mating (day 28)	324.4	328.9	320.0	302.2*	211.8	205.2	199.3**	201.6*
Gestation day 20	379.3	383.4	374.4	346.8*	329.4	318.4	312.5*	284.3**
Lactation day 21	(day 62)	(day 62)	(day 62)	(day 62)	267.5	277.4	267.8	244.3**
Feed consumption (g/animal per day)								
Before mating (day 7)	17.6	18.0	17.0	13.1**	13.6	11.7**	12.0*	10.1**
Before mating (day 28)	18.4	18.7	18.4	18.1	13.9	13.7	12.2**	13.0
Gestation day 20	17.4	17.9	18.0	17.3	19.7	18.2	19.3	16.7**
Lactation day 21	(day 62)	(day 62)	(day 62)	(day 62)	61.8	57.7	50.3*	36.8**
Necropsy findings								
Number of animals examined	10	10	10	10	10	10	10	10
Liver dark brownish change	0	0	0	10	0	0	1	8
Liver enlargement	0	0	5	10	0	0	1	7
Thyroid enlargement	0	1	1	4	0	0	1	1
Organ weights								
Absolute liver weight (g)	10.8	13.4**	14.7**	16.3**	12.0	14.1	16.4**	19.8**
% change	–	*24*	*36*	*51*	–	*17*	*37*	*65*
Relative liver weight (%)	2.85	3.48**	3.93**	4.71**	4.50	5.11	6.13**	8.15**
% change	–	*22*	*38*	*65*	–	–	*36*	*81*
Absolute thyroid weight (mg)	22.4	29.9*	30.1*	33.4**	18.2	18.3	20.8	19.6
% change	–	*33*	*34*	*49*	–	–	*14*	*8*
Relative thyroid weight (%)	5.92	7.76	8.14*	9.76**	6.82	6.62	7.78	7.94
% change	–	–	*37*	*65*	–	–	*14*	*16*
Absolute ovary weight (mg)	–	–	–	–	93.9	84.7	82.6	64.8**
% change	–	–	–	–	–	–	–	*30*

	Males				Females			
	0 ppm	5 000 ppm	10 000 ppm	20 000 ppm	0 ppm	5 000 ppm	10 000 ppm	20 000 ppm
Relative ovary weight	–	–	–	–	35.11	30.46	30.84	26.55**
% change	–	–	–	–	–	–	–	*24*
Absolute uterus weight (g)	–	–	–	–	0.503	0.490	0.301**	0.229**
% change	–	–	–	–	–	–	*40*	*55*
Relative uterus weight (%)	–	–	–	–	0.18	0.17	0.11**	0.094**
% change	–	–	–	–	–	–	*39*	*48*
Histopathology								
Number of animals examined	10	10	10	10	10	10	10	10
Liver								
Brown pigment in bile duct, focal (grades 1–3)	0	0	1	6	0	0	5	9
Brown pigment deposition in perilobular hepatocytes (grade 1)	0	0	0	3	0	0	2	6
Periductular inflammatory cell infiltration, focal (grade 1)	0	0	1	4	0	0	3	8
Eosinophilic foci of altered hepatocytes (grade 1)	0	0	0	1	0	0	0	1
Centrilobular hypertrophy, hepatocyte (grades 1–3)	0	4 (gr. 1)	10 (gr. 1–3)	10 (gr. 1–3)	0	6 (gr. 1–2)	10 (gr. 1–2)	10 (gr. 2–3)
Bile duct proliferation (grades 1–2)	0	0	0	1	0	0	3	5
Ovary								
Decreased vacuolation in interstitial gland (grade1)	–	–	–	–	0	0	1	7
Uterus								
Atrophy (grade 1)	–	–	–	–	0	0	2	10
Thyroid								

	Males				Females			
	0 ppm	**5 000 ppm**	**10 000 ppm**	**20 000 ppm**	**0 ppm**	**5 000 ppm**	**10 000 ppm**	**20 000 ppm**
Diffuse hypertrophy of follicular cells (grade 1)	0	0	2	5	0	0	0	0
Reproductive performance								
Copulation index (%)	100	100	100	100	100	100	100	100
Fertility index (%)	100	100	100	100	100	100	100	100
Litter examination								
Implantation sites/dam	–	–	–	–	13.5	12.1	11.5	11.0
Offspring at birth/dam	–	–	–	–	13.0	10.4	10.0	10.5
Live offspring at birth/dam	–	–	–	–	13.0	10.4	10.0	10.1*
Viability index (M/F) (day 0)	–	–	–	–	100	100	100	96.5*
Weaning index	–	–	–	–	100	100	100	95
Offspring (F_1)								
Pup body weight (g)								
Day 0	5.7	6.0	5.9	5.6	5.4	5.8	5.6	5.2
Day 21	52.5	49.4	45.0*	26.6**	50.5	47.7	43.4*	25.5**
Pup organ weights on weaning								
Terminal body weight (g)	53.1	49.9	44.9**	26.3**	50.5	48.8	44.3*	25.8**
Absolute brain weight (g)	1.459	1.437	1.438	1.258**	1.424	1.417	1.390	1.228**
% change	–	–	–	*14*	–	–	–	*14*
Relative brain weight (%)	2.76	2.89	3.22*	5.04**	2.83	2.91	3.15	5.03**
% change	–	–	*16*	*83*	–	–	–	*78*
Absolute thymus weight (mg)	202.2	194.1	172.2	75.6**	208.2	206.4	174.0	80.1**
% change	–	–	–	*63*	–	–	–	*38*
Relative thymus weight (% × 10^{-3})	380.6	390.2	380.7	280.4**	411.2	423.8	390.1	297.7**
% change	–	–	–	*26*	–	–	–	*28*
Absolute spleen weight (g)	0.248	0.210	0.194*	0.091**	0.228	0.210	0.189	0.088**
% change	–	–	*22*	*63*	–	–	–	*61*

	Males				Females			
	0 ppm	5 000 ppm	10 000 ppm	20 000 ppm	0 ppm	5 000 ppm	10 000 ppm	20 000 ppm
Relative spleen weight (%)	0.46	0.41	0.43	0.33**	0.45	0.42	0.42	0.33*
% change	–	–	–	*29*	–	–	–	*27*
Absolute uterus weight (mg)	–	–	–	–	36.91	37.98	31.38	25.18**
% change	–	–	–	–	–	–	–	*32*

F: females; F_0: parental generation; F_1: first filial generation; gr.: grade; M: males; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$
Source: Hoshino (2010)

In males, statistically significant increases in absolute and relative liver weights were observed at 5000 ppm (absolute 24%; relative 22%), 10 000 ppm (absolute 36%; relative 38%) and 20 000 ppm (absolute 51%; relative 65%). In females, statistically significant increases in absolute and relative liver weights were observed at 10 000 ppm (absolute 37%; relative 36%) and 20 000 ppm (absolute 65%; relative 81%). Statistically significant increases in absolute and relative thyroid weights were observed in males at 10 000 ppm (absolute 34%; relative 37%) and 20 000 ppm (absolute 49%; relative 65%). In females, statistically significant decreases in absolute and relative uterus weights were noted at 10 000 ppm (absolute 40%; relative 39%) and 20 000 ppm (absolute 55%; relative 48%). In addition, decreased absolute and relative ovary weights were noted at 20 000 ppm (absolute 30%; relative 24%). However, no treatment-related changes in reproductive function, such as mating ability, fertility, pregnancy, parturition or nursing behaviour, were noted in females (Table 26).

Dark brownish change in the liver was noted in one female in the 10 000 ppm group and in 10 males and eight females in the 20 000 ppm group. Enlargement of the liver was noted in five males and one female in the 10 000 ppm group and in 10 males and seven females in the 20 000 ppm group. Enlarged thyroid was noted in one, one and four males and in zero, one and one female in the 5000, 10 000 and 20 000 ppm groups, respectively (Table 26).

Histopathology investigation in adults revealed brown pigment in the bile ducts in one male and five females at 10 000 ppm and in six males and nine females at 20 000 ppm. Brown pigment deposition in the perilobular hepatocytes was noted in two females at 10 000 ppm and in three males and six females at 20 000 ppm. Focal periductular inflammatory cell infiltration was noted in one male and three females at 10 000 ppm and in four males and eight females at 20 000 ppm. Proliferation of the bile ducts was noted in three females at 10 000 ppm and in one male and five females at 20 000 ppm. Eosinophilic focus of the altered hepatocytes was noted in one male and one female at 20 000 ppm. Centrilobular hypertrophy of the hepatocytes was noted in four, 10 and 10 males and six, 10 and 10 females at 5000, 10 000 and 20 000 ppm, respectively. Diffuse hypertrophy of the follicular thyroid cells was noted in two males at 10 000 ppm and in five males at 20 000 ppm. Hypertrophy in the thyroid follicular cells in this study is considered to be an adaptive change caused by the induction of hepatic enzymes. Among eight animals with enlarged thyroids at necropsy, diffuse hypertrophy of the follicular cells was noted in two males at 20 000 ppm. In the other six animals, vacuolation of the follicular cells was noted. It has been reported that vacuolation of thyroid follicular cells is a spontaneous lesion in BrlHan:WIST@Jcl (GALAS) rats (Shimoi et al., 2001). In the present study, this change was also noted in one female in the control group. Therefore, vacuolation of the follicular cells was considered to be incidental. In the ovary, decreased vacuolation in the interstitial gland was noted in one female at 10 000 ppm and in seven females at 20 000 ppm. Uterus atrophy was noted in two females at 10 000 ppm and in 10 females at 20 000 ppm (Table 26).

Mating performance and fertility appeared unaffected at all doses. A statistically significant decrease in the viability index on day 0 was noted in the 20 000 ppm group. Furthermore, a decrease in weaning index (not statistically significant) was noted in the 20 000 ppm group, and this change was

induced by the death of four F_1 animals after the culling in one dam. A statistically significant decrease in the number of live offspring at birth (and as a consequence also in the number of live offspring on day 4 before culling) was noted in the 20 000 ppm group. However, the number of live offspring at birth in the control group was above the mean of the background range of the test facility, and that in the 20 000 ppm group was slightly below the mean of the background range. Accordingly, the decrease in the number of live offspring at birth in the high-dose group was considered to be an incidental change attributable to the increased number of live offspring at birth in the control group and was judged to be toxicologically irrelevant (Table 26).

Pups at 10 000 ppm and 20 000 ppm showed statistically significant decreases in body weight or body weight gain after postnatal day (PND) 4 (before culling) in males and females, which increased in magnitude up to weaning. At necropsy, no abnormal changes attributable to the test substance were noted in either sex. At 20 000 ppm, statistically significant decreases in absolute brain, thymus and spleen weights in both sexes and in absolute uterus weights in females were observed. In addition, absolute spleen weights were also statistically significantly decreased in the mid-dose male pups (10 000 ppm). Furthermore, statistically significant decreases in relative weights of thymus and spleen were noted in both sexes at 20 000 ppm. Statistically significant increases in relative brain weights (in mid- and high-dose males as well as in high-dose females) and in relative uterus weights (in high-dose females) were considered to result from excessive lower body weight at necropsy (Table 26).

A top dietary concentration of 10 000 ppm was considered appropriate for the main study (Hoshino, 2010).

The main two-generation rat reproductive toxicity study was conducted with mandestrobin (purity 93.4%) at dietary concentrations of 0, 1000, 3000 and 10 000 ppm (equal to 0, 47.77, 145.7 and 511.7 mg/kg bw per day for males and 0, 65.68, 200.3 and 672.0 mg/kg bw per day for females, respectively). Groups of 26 male and 26 female Wistar rats were allocated to treatment groups (F_0 generation) and were exposed to treated diets 10 weeks prior to mating and then during gestation and lactation, until scheduled termination. At the end of the lactation period (21 days postpartum), 24–26 male and female pups were selected to become the F_1 parental animals. These animals were again permitted a 10-week premating phase starting at approximately 28 days of age and then mated; females were allowed to rear their litters to form the F_2 generation. The F_2 animals were monitored until weaning, and the study was then terminated. Exposure to treated diet continued throughout the study, including the premating, mating, gestation and lactation periods of the F_0 and F_1 generations. The F_2 pups were indirectly exposed to test substance until weaning (day 21 postpartum).

Mortality and clinical signs were checked at least once daily for all animals. Body weights and feed consumption were determined weekly throughout the study until termination for males except during the mating period. For females, body weight and feed consumption were recorded weekly during the premating periods, on days 0, 7, 14 and 20 postcoitum and on days 0, 4, 7, 14 and 21 postpartum. The age and body weight at which vaginal opening or preputial separation occurred were recorded for F_1 generation weanlings selected for breeding the F_2 generation. Sperm analysis was performed for all males per group (F_0 and F_1 animals), comprising assessment of motility, morphology and sperm count. A record of mating of females was made by daily examination of the vaginal smears for spermatozoa and/or appearance of a vaginal plug throughout the pairing period. The day on which evidence of mating was observed was considered to be day 0 postcoitum. Towards the end of the gestation period (gestation days [GDs] 21–25), females were examined twice daily for signs of parturition. Females without litters by GD 25 were killed and necropsied on GD 26. Day 0 of lactation was the day on which a female had delivered all its pups. All F_0 and F_1 males were terminated 5 and 6 weeks postcoitum, respectively. All F_0 and F_1 females were terminated on day 21 of lactation. On PND 4, litter size was randomly adjusted to eight pups (equal sex ratio, in principle). Litters with fewer than eight pups were maintained as they were. The pups culled at the litter size adjustment were subjected to necropsy. The following organ weights were recorded from all F_0 and F_1 parent animals on day 21 postpartum or shortly thereafter: brain, pituitary, thyroid, liver, kidney, adrenal, spleen, testis, epididymis, prostate, seminal vesicle, ovary and uterus. Full histopathological examination was carried out on the following organs/tissues of

all males and females in the control and 10 000 ppm groups: pituitary, adrenal, testis, epididymis, seminal vesicle, coagulating gland, prostate, ovary, fallopian tube, uterus and vagina. In addition, the livers of males and females and the thyroids of males in the F_0 and F_1 generations and the kidneys of F_0 females were also examined. Because treatment-related changes were seen in livers of top-dose F_0 and F_1 males and females, in thyroids of top-dose F_0 and F_1 males and in adrenals of top-dose F_1 females, the thyroids and livers of F_0 and F_1 animals and adrenals of F_1 females in the 1000 and 3000 ppm groups were also examined histologically.

In parental animals from the F_0, F_1 and F_2 generations, no clinical signs were noted during the study.

In F_1 animals, one female (#60425-6) died on PND 13 in the 10 000 ppm group. In F_2 animals, one male and one female (#20411-43, #70404-58) died on PND 5 in the 10 000 ppm group. Before litter size adjustment (PND 4), death was observed sporadically in a few pups in all dose groups, including the control groups for both F_1 and F_2 pups.

In F_0 males, body weight gain was suppressed at 10 000 ppm, with significant differences in body weight and body weight gain throughout the observation period. Reduced feed consumption was also noted in males at 10 000 ppm from premating days 28 to 63 of dosing (Table 27).

Table 27. Key findings of the two-generation reproductive toxicity study in rats (first generation)

	Males				**Females**			
Finding	**0 ppm**	**1 000 ppm**	**3 000 ppm**	**10 000 ppm**	**0 ppm**	**1 000 ppm**	**3 000 ppm**	**10 000 ppm**
F_0 parental animals								
Dose (mg/kg bw per day)								
Premating (weeks 0–2)	0	56.15	166.3	559.1	0	62.48	195.3	628.5
Postmating/gestation	0	43.27	132.0	452.1	0	60.19	186.2	602.8
Lactation	–	–	–	–	0	162.8	511.0	1 634.6
Feed consumption (g/animal per day)								
Premating (week 10, ending day 70)	17.8	17.5	17.3	17.0	12.9	12.3	12.4	12.5
Premating (mean over weeks 4–9)	18.0	17.8	17.4	16.6**	12.7	12.4	12.9	12.4
Lactation (day 7)	–	–	–	–	41.8	39.1	41.5	36.0*
Lactation (day 14)	–	–	–	–	47.6	45.2	48.3	44.3
Lactation (day 21)	–	–	–	–	60.4	58.2	60.0	58.8
Body weight (g)								
Before mating (day 0)	185.8	185.7	185.9	185.8	134.6	135.8	135.9	135.8
Before mating (day 70)	393.8	391.2	386.2	369.9**	221.1	225.4	225.1	220.4
Gestation day 20	438.7	437.7	436.1	415.0**	326.1	327.0	329.2	324.3
Lactation day 21	(day 119)	(day 119)	(day 119)	(day 119)	268.1	272.7	273.8	273.9
Body weight gain (g)								
Premating (days 0–70)	208.0	205.5	200.3	184.1**	86.5	89.7	89.2	84.6
Gestation (days 0–7)					24.2	21.0	21.8	20.8
Gestation (days 0–14)					48.2	44.0	43.9	42.7**

	Males				Females			
Finding	**0 ppm**	**1 000 ppm**	**3 000 ppm**	**10 000 ppm**	**0 ppm**	**1 000 ppm**	**3 000 ppm**	**10 000 ppm**
Gestation (days 0–20)	252.9 (days 0–119)	252.0 (days 0–119)	250.2 (days 0–119)	229.2** (days 0–119)	103.3	101.0	103.3	102.0
Reproductive performance								
Number of pairs	–	–	–	–	26	26	26	26
Mating index (%)	–	–	–	–	100	100	100	96.2 (25/26)
Mean precoital time (days)	–	–	–	–	2.9	3.2	2.5	3.6
Fertility index (%)	–	–	–	–	96.2 (25/26)	100	100	96.0 (24/25)
Gestation index (%)	–	–	–	–	100	100	100	100
Gestation duration (days)	–	–	–	–	22.1	22.0	22.0	22.0
Implantations (mean)	–	–	–	–	13.0	12.2	13.2	13.0
Live young at birth	–	–	–	–	12.2	11.7	12.2	12.3
Birth index (%)	–	–	–	–	93.78	95.86	92.64	95.40
Organ weights								
Absolute liver weight (g)	12.65	13.04	14.12**	15.53**	10.42	11.45*	12.24**	17.24**
% change	–	–	*12*	*23*	–	*10*	*17*	*65*
Relative liver weight (%)	2.87	2.97	3.23**	3.73**	3.89	4.21*	4.47**	6.30**
% change	–	–	*12*	*30*	–	*8*	*15*	*75*
Absolute kidney weight (g)	2.536	2.545	2.555	2.499	1.987	1.992	1.961	2.095**
% change	–	–	–	–	–	–	–	*5*
Relative kidney weight (%)	0.577	0.580	0.585	0.599	0.742	0.731	0.717	0.766**
% change	–	–	–	–	–	–	–	*3*
Absolute thyroid weight (mg)	23.75	24.04	25.29**	28.97**	20.98	21.90	21.67	21.55
% change	–	–	*6*	*22*	–	–	–	–
Relative thyroid weight (%)	5.45	5.48	5.78*	7.02**	7.85	8.00	7.93	7.88
% change	–	–	*6*	*29*	–	–	–	–
Absolute ovary weight (mg)	–	–	–	–	91.34	90.35	97.48	82.33
Relative ovary weight (%)	–	–	–	–	34.14	33.21	35.66	30.06*
% change	–	–	–	–	–	–	–	*12*
Absolute uterus weight (g)	–	–	–	–	0.45	0.54	0.51	0.38*
% change	–	–	–	–	–	–	–	*16*
Relative uterus weight (%)	–	–	–	–	0.167	0.198	0.187	0.137**
% change	–	–	–	–	–	–	–	*18*
Gross findings								
No. of animals examined	*26*	*26*	*26*	*26*	*25*	*26*	*26*	*24*
Kidney: greenish change, cortex	0	0	0	0	0	0	0	5*

Finding	Males				Females			
	0 ppm	1 000 ppm	3 000 ppm	10 000 ppm	0 ppm	1 000 ppm	3 000 ppm	10 000 ppm
Liver: dark brownish change	0	0	0	0	0	0	0	24**
Liver: enlargement	0	0	0	0	0	0	0	20**
Histopathology								
Liver (no. examined)	*26*	*26*	*26*	*26*	*25*	*26*	*26*	*24*
Brown pigment, bile duct/periportal								
Grade 1	0	0	0	9**	0	0	0	10**
Grade 2	0	0	0	0	0	0	0	7
Grade 3	0	0	0	0	0	0	0	3
Brown pigment deposition, hepatocyte, perilobular								
Grade 1	0	0	0	0	0	0	0	4*
Cell infiltration, inflammatory, focal, periductular								
Grade 1	0	0	0	4*	0	0	0	10**
Proliferation, bile duct								
Grade 1	0	0	0	0	0	0	0	5**
Grade 2	0	0	0	0	0	0	0	4
Grade 3	0	0	0	0	0	0	0	3
Hypertrophy, hepatocyte, diffuse								
Grade 1	0	0	15**	20**	0	6*	17**	1**
Grade 2	0	0	0	4	0	0	0	21
Grade 3	0	0	0	0	0	0	0	2
Thyroid (no. examined)	*26*	*26*	*26*	*26*	*0*	*0*	*0*	*0*
Hypertrophy, follicular cell diffuse								
Grade 1	0	0	0	4*	–	–	–	–
F1 pups								
Body weight (g)								
PND day 0	5.8	5.8	5.8	5.8	5.5	5.5	5.4	5.5
PND day 7	16.2	15.9	16.3	14.7*	15.7	15.5	15.7	14.2*
PND day 14	32.5	32.0	32.3	28.0**	31.6	31.2	31.4	27.1**
PND day 21	52.0	51.1	50.3	43.8**	50.3	49.5	48.5	41.9**
Before mating (day 91)	390.7	391.6	381.7	353.0**	224.0	229.5	225.7	211.8
Gestation day 20	465.5	464.1	452.5	428.0**	340.8	349.5	346.3	328.9
Lactation day 21	(day 147)	(day 147)	(day 147)	(day 147)	277.4	289.9*	290.1*	279.0
Body weight gain (g)								
PND day 7	6.2	6.0	6.2	5.2**	6.0	5.8	6.0	5.0**
PND day 14	22.5	22.0	22.2	18.4**	21.9	21.5	21.6	17.9**
PND day 21	42.0	41.2	40.2	34.3**	40.5	39.9	38.8	32.7**

Finding	Males				Females			
	0 ppm	1 000 ppm	3 000 ppm	10 000 ppm	0 ppm	1 000 ppm	3 000 ppm	10 000 ppm
F_1 pup development								
Day of cleavage of balanopreputial gland (males)	41.1	41.8	42.0	42.7**	–	–	–	–
Day of vaginal opening	–	–	–	–	31.0	30.9	31.0	32.5*
Body weight (g) at cleavage of balanopreputial gland/vaginal opening	173.96	181.38	179.62	167.17	94.96	96.46	95.5	89.33**

bw: body weight; F_0: parental generation; F_1: first filial generation; grades: 1, minimal; 2, mild; 3, moderate; PND: postnatal day; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$
Source: Matsuura (2012)

In F_1 animals at 10 000 ppm, although birth weights were almost the same as those in the control group, postnatal body weight gain was suppressed in males and females before weaning, and statistically significant differences in body weight and body weight gain were noted in both sexes from PNDs 7 to 21 (Table 27). After weaning, suppressed body weight gain continued in males until the terminal necropsy, with statistically significant differences in body weight and body weight gain at almost all measurement points. In females, statistically significantly lower body weights also continued after weaning. However, a statistically significant difference in body weight gain was observed only on PND 28 (data not shown). Statistically significantly lower feed consumption was observed sporadically in males and females at 10 000 ppm (data not shown).

In F_2 animals at 10 000 ppm, statistically significant differences in body weight gain were noted in both sexes from PNDs 7 to 21 and in body weight from PNDs 14 to 21 (Table 28).

Table 28. Key findings of the two-generation reproductive toxicity study in rats (second generation)

Finding	Males				Females			
	0 ppm	1 000 ppm	3 000 ppm	10 000 ppm	0 ppm	1 000 ppm	3 000 ppm	10 000 ppm
F_1 animals								
Dose (mg/kg bw per day)								
Premating (weeks 0–2)	0	84.73	254.5	881.2	0	90.11	247.9	929.3
Postmating/gestation	0	47.77	145.7	511.7	0	65.68	200.3	672.0
Lactation	–	–	–	–	0	163.4	504.6	1 687.5
Reproductive performance								
Number of pairs	–	–	–	–	25	26	26	24
Mating index (%)	–	–	–	–	100	100	100	100
Mean precoital time (days)	–	–	–	–	2.6	2.6	2.3	2.0
Fertility index (%)	–	–	–	–	100	96.2	92.3	100
Gestation index (%)	–	–	–	–	100	100	100	100
Gestation duration (days)	–	–	–	–	22.2	22.0	22.0	21.8**
Implantations (mean)	–	–	–	–	13.8	13.5	13.1	13.2
Live young at birth	–	–	–	–	13.0	12.9	12.5	12.3

Finding	Males				Females			
	0 ppm	**1 000 ppm**	**3 000 ppm**	**10 000 ppm**	**0 ppm**	**1 000 ppm**	**3 000 ppm**	**10 000 ppm**
Birth index (%)	–	–	–	–	93.96	95.29	95.44	93.04
Organ weights at weaning								
Body weight (g)	*52.0*	*51.4*	*50.5*	*44.0***	*50.6*	*49.8*	*48.8*	*41.6***
Absolute spleen weight (g)	0.266	0.248	0.232*	0.199**	0.252	0.250	0.232	0.200**
% change	–	–	*13*	*25*	–	–	–	*21*
Relative spleen weight (%)	0.51	0.48	0.45*	0.44**	0.49	0.50	0.47	0.48
% change			*12*	*14*	–	–	–	–
Absolute thymus weight (mg)	204.1	199.0	203.4	167.8**	208.8	206.2	208.5	172.1**
% change	–	–	–	*18*	–	–	–	*18*
Relative thymus weight ($\times 10^{-3}$ %)	392.9	387.5	403.2	380.1	413.0	414.28	426.5	412.4
Absolute uterus weight (mg)	–	–	–	–	40.38	40.38	42.70	34.60*
% change	–	–	–	–	–	–	–	*14*
Relative uterus weight ($\times 10^{-3}$ %)	–	–	–	–	80.0	81.9	87.13	84.14
Organ weights for F_1 parental animals								
Absolute liver weight (g)	13.86	14.00	14.62	15.97**	11.14	12.30**	13.33**	16.96**
% change	–	–	–	*15*	–	*10*	*20*	*52*
Relative liver weight (%)	2.97	3.00	3.216**	3.74**	4.01	4.25	4.59**	6.10**
% change	–	–	*8*	*26*	–	–	*14*	*52*
Absolute thyroid weight (mg)	25.32	25.12	26.02	32.41*	23.61	22.06	20.62	21.03
% change	–	–	–	*28*	–	–	–	–
Relative thyroid weight ($\times 10^{-3}$ %)	5.45	5.39	5.75	7.72**	8.49	7.65	7.12	7.55
% change	–	–	–	*42*	–	–	–	–
Absolute ovary weight (mg)	–	–	–	–	97.10	95.83	98.25	84.40**
% change	–	–	–	–	–	–	–	*13*
Relative ovary weight (%)	–	–	–	–	35.09	33.03	33.84	30.28**
% change	–	–	–	–	–	–	–	*14*
Absolute spleen weight (g)	0.688	0.709	0.701	0.733	0.612	0.693	0.634	0.661
% change	–	–	–	–	–	–	–	–
Relative spleen weight (%)	0.14	0.15	0.15	0.17**	0.22	0.24	0.21	0.23
% change	–	–	–	*21*	–	–	–	–

Finding	Males				Females			
	0 ppm	1 000 ppm	3 000 ppm	10 000 ppm	0 ppm	1 000 ppm	3 000 ppm	10 000 ppm
Gross findings								
Liver: dark brownish change	0/25	0/26	0/26	0/24	0/25	0/25	0/24	20/24**
Liver: enlargement	0/25	0/26	0/26	0/24	0/25	0/25	0/24	6/24**
Histopathology								
Liver (no. examined)	*25*	*26*	*26*	*24*	*25*	*25*	*24*	*24*
Brown pigment, bile duct/periportal								
Grade 1	0	0	10**	22**	0	0	11**	5**
Grade 2	0	0	0	2	0	0	0	16
Grade 3	0	0	0	0	0	0	0	3
Brown pigment deposition, hepatocyte, perilobular								
Grade 1	0	0	0	0	0	0	0	5*
Cell infiltration, inflammatory, focal, periductular								
Grade 1	0	0	0	24*	0	0	3	24**
Proliferation, bile duct								
Grade 1	0	0	0	0	0	0	0	7**
Grade 2	0	0	0	0	0	0	0	8
Grade 3	0	0	0	0	0	0	0	1
Hypertrophy, hepatocyte, diffuse								
Grade 1	0	0	6*	19**	0	4*	18**	11**
Grade 2	0	0	0	4	0	0	0	13
Adrenals (no. examined)	*25*	*0*	*0*	*24*	*25*	*25*	*24*	*24*
Hypertrophy, cortical cell, fascicular zone								
Grade 1	0	–	–	0	0	0	0	5*
F_2 animals								
Body weight (g)								
PND day 0	5.8	5.7	5.9	5.8	5.5	5.5	5.6	5.4
PND day 7	16.0	15.8	15.9	15.0	15.5	15.3	15.5	14.3
PND day 14	32.9	31.7	32.4	29.6**	32.1	31.0	31.8	28.5**
PND day 21	53.5	51.6	52.2	46.3**	51.4	50.3	50.4	44.5**
Body weight gain (g)								
PND day 7	6.1	5.8	6.0	5.4**	5.9	5.6	5.7	5.1**
PND day 14	23.0	21.7	22.4	20.0**	22.5	21.3	22.0	19.4**
PND day 21	43.6	41.7	42.2	36.7**	41.9	40.7	40.6	35.4**
F_2 pup organ weights at weaning								
Absolute thymus weight (mg)	212.0	204.9	201.5	182.5**	210.7	209.0	205.3	179.0**

Finding	Males 0 ppm	Males 1 000 ppm	Males 3 000 ppm	Males 10 000 ppm	Females 0 ppm	Females 1 000 ppm	Females 3 000 ppm	Females 10 000 ppm
% change	–	–	–	*14*	–	–	–	*15*
Relative thymus weight ($\times 10^{-3}$ %)	395.0	392.7	384.13	388.0	406.4	408.9	401.9	400.5
Absolute spleen weight (g)	0.273	0.253	0.245	0.209**	0.274	0.262	0.234	0.203**
% change	–	–	–	*24*	–	–	–	*26*
Relative spleen weight (%)	0.50	0.48	0.46	0.44**	0.52	0.50	0.46*	0.45**
% change	–	–	–	*12*	–	–	*12*	*14*

bw: body weight; F_2: second filial generation; grades: 1, minimal; 2, mild; 3, moderate; PND: postnatal day; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$
Source: Matsuura (2012)

No treatment-related changes were observed in the estrous cycles in females of any treated group of both the F_0 and F_1 generations. All mating pairs in each group in both generations showed evidence of copulation by 14 days after the start of mating, except for one pair in the 10 000 ppm group in the F_0 generation. Therefore, the test compound was judged to have no effect on mating ability.

In F_0 and F_1 animals, no treatment-related changes were observed in the mating ability or fertility of males or females of any treated group. In the F_0 generation, all the mating pairs of each group, except for one pair in the 10 000 ppm group, copulated during the first or second estrous stage of females within 13 days after the start of mating. There were no statistically significant differences in the copulation index, in the number of estrous stages without copulation or in the days until copulation between the control and treated groups. In the F_1 generation, there were only one and two non-pregnant females at 1000 and 3000 ppm, respectively, and no statistically significant difference was found in the fertility index between the control and treated groups.

In both F_0 and F_1 generations, no treatment-related changes suggesting effects on spermatogenesis were detected in any treated group. All parameters, including sperm motility, spermatid counts, sperm counts and the incidences of abnormal sperm and tailless sperm, were comparable between the control and treated groups, with no statistically significant differences. Furthermore, no abnormalities were observed in males that had no copulation or did not impregnate their mating partners, except for one male in the control group of the F_0 generation, which showed low sperm motility of 40%.

No treatment-related differences were found in the number of implantations or offspring born alive, birth index or gestation index between the control and treated groups in both the F_0 and F_1 generations. In the F_1 generation, the gestation length in the 10 000 ppm group (21.8 days) was statistically significantly shorter than in the control group (22.2 days) (Table 28). However, there was no treatment-related change in gestation duration for F_0 dams (Table 27). In F_1 dams, the difference from the control group in gestation duration was small, and the gestation duration of all dams in the 10 000 ppm group was 21 or 22 days, which is within the normal range (21–23 days) of this strain. Moreover, the mean gestation duration in the 10 000 ppm group was almost equal to the lower limit of the historical control range of the test facility (mean duration: 21.9–22.2 days; range of individual duration: 21–23 days; 2002–2009), whereas that in the control group was equal to the upper limit of the historical control range. Therefore, the statistically significant difference observed in the gestation duration in the 10 000 ppm group was considered to be a variation within the normal range and was judged to be of no toxicological relevance.

In F_1 and F_2 pups, no treatment-related changes were detected in the number of offspring born and born alive, sex ratio, live birth index, viability index on days 4, 7 and 14, and weaning index (Tables 27 and 28).

External examination of pups revealed no anomalies in F_1 or F_2 pups of any group, including the pups that died.

At 10 000 ppm, preputial separation (cleavage of balanopreputial gland) in F_1 males and vaginal opening in F_1 females showed a statistically significant delay of 1.6 and 1.5 days on average, compared with the control group (Table 27). The body weights in the 10 000 ppm group on the corresponding days were lower than those in the control group in both sexes (males, 96.1% of the control value; females, 94.1% of the control value), and a statistically significant difference was observed in females. In males, besides the slight (not statistically significant) body weight decrease at cleavage, body weight was consistently statistically significantly lower than that of controls from culling to day 91 after birth (~15%). Similarly, body weight gains were also statistically significantly depressed, but less severely. Females showed the same pattern. It is noted that no observations or examinations suggested effects on any reproductive function, including estrous cycle, sperm parameters and histopathological findings. Overall, this slight delay in sexual maturation in both sexes was considered to be related to growth retardation.

Pathological examination revealed treatment-related changes in the liver of both sexes and the thyroid of males in both generations and the adrenals of F_1 females. At necropsy, dark brownish change and enlargement of the liver were noted in F_0 and F_1 females in the 10 000 ppm group (Tables 27 and 28).

Liver weights were increased in males in the 3000 and 10 000 ppm groups and in females in the 1000 ppm group and above in both generations. In addition, increases in thyroid weights were observed in F_0 males in the 3000 and 10 000 ppm groups and in F_1 males in the 10 000 ppm group, as were decreases in ovary weights in F_0 and F_1 females and uterus weights in F_0 females in the 10 000 ppm group (Tables 27 and 28). In F_0 animals, treatment-related increases in absolute and relative liver weights were observed in males at 3000 ppm (absolute 12%; relative 12%) and 10 000 ppm (absolute 23%; relative 30%). In females, absolute and relative liver weights were increased at 1000 ppm (absolute 10%; relative 8%), 3000 ppm (absolute 17%; relative 15%) and 10 000 ppm (absolute 65%; relative 75%), compared with controls. In females, absolute and relative kidney weights were statistically significantly decreased at 10 000 ppm (absolute 5%; relative 3%). In males, absolute and relative thyroid weights were increased with statistical significance at 3000 ppm (absolute 6%; relative 6%) and 10 000 ppm (absolute 22%; relative 29%), compared with controls. In females at 10 000 ppm, relative ovary weights were decreased by 12% compared with controls, and absolute and relative uterus weights were decreased by 16% and 18% compared with controls, respectively (Table 27). In addition, significantly higher relative weights of the brain and seminal vesicle were observed in the 10 000 ppm group. However, the absolute weights of both organs were comparable to those in the control group, with no statistically significant differences; therefore, these changes were considered to be due to the lower body weights at necropsy in this group and were judged to be of no toxicological relevance.

In F_1 pups at weaning, statistically significant decreases in the absolute and relative weights of the spleen were noted in males at 3000 ppm (absolute 13%; relative 12%) and 10 000 ppm (absolute 25%; relative 14%). In addition, at 10 000 ppm, statistically significantly lower absolute weights (18%) were observed in the thymus in both sexes and in the spleen (21%) and uterus (14%) in females (Table 28), as were statistically significantly higher relative weights of the brain in both sexes in the 10 000 ppm group. However, the changes were judged to be of no toxicological relevance, as absolute organ weight change was not accompanied by relative organ weight change and vice versa; therefore, they were considered to be due to the lower body weights at necropsy in this group.

In F_1 adults, males had statistically significantly increased absolute and relative liver weights (absolute 15%; relative 26%) at 10 000 ppm and relative liver weight (8%) at 3000 ppm. Females showed increased absolute liver weight at 1000, 3000 and 10 000 ppm, by 10%, 20% and 52%, respectively, whereas relative liver weights were increased at 3000 ppm and 10 000 ppm by 14% and 52%, respectively. In males, absolute and relative thyroid weights were statistically significantly increased at 10 000 ppm by 28% and 42%, respectively. In females, absolute and relative ovary weights were statistically significantly decreased by 13% and 14%, respectively, at 10 000 ppm. In addition, in males, statistically significantly higher relative weights of several other organs (brain, spleen, kidney,

adrenal, testis, seminal vesicle and epididymis) were observed in the 10 000 ppm group. However, the absolute weights of these organs were comparable to those in the control group, with no statistically significant differences; therefore, these changes were considered to be due to the lower body weights at necropsy in this group and were judged to be of no toxicological relevance. Furthermore, in females, there were statistically significantly lower absolute brain weight in the 10 000 ppm group and relative brain weights in the 1000 and 3000 ppm groups, relative pituitary weights in the 3000 and 10 000 ppm groups, and relative left adrenal weight in the 3000 ppm group. However, they were changes observed only in absolute or relative weight, not both, with a lack of clear dose dependency; therefore, they were judged not to be treatment related (Table 28).

In F_2 pups at weaning, statistically significant decreases in the absolute and relative weights of the spleen were noted in males and females at 10 000 ppm (males: absolute 24%, relative 12%; females: absolute 26%, relative 14%). Moreover, the spleen weights of females in the 3000 ppm group also tended to be lower than those in the control group, and a statistically significant difference was noted in the relative weights (12%). In addition, statistically significantly decreased thymus weights were observed in males and females, by 14% and 15%, respectively, in the 10 000 ppm group (Table 28), and statistically significantly higher relative brain weights were seen in both sexes in the 10 000 ppm group. However, these changes were observed only in the absolute or relative weight, not both, and the corresponding relative or absolute weights of these organs were similar to those of the control group; therefore, they were considered to be due to the lower body weights at necropsy in this group and were judged to be of no toxicological relevance.

In F_1 females, ovarian follicle count revealed a statistically significant increase in the number of medium-sized follicles in the 10 000 ppm group. In addition, although there was no statistically significant difference, the number of large follicles also tended to increase in the 10 000 ppm group. However, the number of small follicles, which include primordial follicles, and the total number of ovarian follicles in the 10 000 ppm group were comparable with those in the control group, with no statistically significant differences. Moreover, the percentage of ovarian follicles at each developmental stage in the 10 000 ppm group was almost equal to that in the control group, indicating no effects on oogenesis. In addition, there were no treatment-related changes suggesting impairment of ovarian function in any reproductive observations, including estrous cycles and the number of implantations, and no histopathological changes were detected in the ovaries in this group. Consequently, the increase in number of medium-sized ovarian follicles was judged to be incidental and of no toxicological relevance.

In histopathological examination, findings included brown pigment in the bile duct/periportal area in F_0 males and females in the 10 000 ppm group and F_1 males and females in the 3000 and 10 000 ppm groups, focal periductular inflammatory cell infiltration in F_0 males and females and F_1 males in the 10 000 ppm group and F_1 females in the 3000 and 10 000 ppm groups, and brown pigment deposition in the perilobular hepatocytes and proliferation of the bile duct in F_0 and F_1 females in the 10 000 ppm group. The brown pigments were considered to be porphyrin pigments, because the pigment stained dark blue with Schmorl and negative for Hall and Berlin blue and had birefringence, according to an examination using a polarizing lens. Diffuse hypertrophy of the hepatocytes was also observed in males in the 3000 and 10 000 ppm groups and in females in the 1000 ppm group and above in both generations. However, this change, accompanied by increased liver weights, was considered to be a physiological adaptation to the repeated administration of the test compound, and only the adaptive changes were observed in F_0 and F_1 females in the 1000 ppm group, without any other change. In addition, hypertrophy of the follicular cells of the thyroid was considered to be secondary to the hepatocellular hypertrophy observed in F_0 males in the 10 000 ppm group, and hypertrophy of the cortical cells in the fascicular zone of the adrenal was suggestive of a stress-related change in F_1 females in the 10 000 ppm group. Besides, greenish change in the cortex and increased kidney weights without histopathological changes were observed in F_0 females in the 10 000 ppm group. In contrast, no histopathological changes were detected in the reproductive organs (including the ovary and uterus) of males or females in either generation.

Mandestrobin showed no evidence of an effect on fertility or reproductive function. Among offspring, there were no treatment-related changes in the viability index, number of offspring or birth

weights, although postnatal body weight gain was suppressed in both sexes of F_1 and F_2 offspring in the 10 000 ppm group. A slight delay in sexual maturation attributed to growth retardation was noted in both sexes (vaginal opening in F_1 females and preputial separation in F_1 males) in the 10 000 ppm group. Slightly lower spleen weights at weaning were also observed in F_1 male offspring and F_2 female offspring in the 3000 and 10 000 ppm groups and in F_2 male offspring in the 10 000 ppm group; however, they completely recovered to the control level in F_1 adult animals. No treatment-related changes were found in any external features, clinical signs or necropsy findings at weaning of F_1 or F_2 offspring (Table 28).

The NOAEL for parental toxicity was 1000 ppm (equal to 47.77 mg/kg bw per day), based on increased liver weights, diffuse liver hypertrophy, brown pigment in the bile duct/periportal area in both sexes in the F_1 generation and periductular inflammatory cell infiltration in F_1 females at 3000 ppm (equal to 145.7 mg/kg bw per day).

The NOAEL for offspring toxicity was 1000 ppm (equal to 47.77 mg/kg bw per day), based on decreased spleen weights in F_1 male and F_2 female pups.

The NOAEL for reproductive toxicity was 10 000 ppm (equal to 511.7 mg/kg bw per day), the highest dose tested (Matsuura, 2012).

(b) Developmental toxicity

Rats

In a dose range–finding developmental toxicity study, seven Wistar rats of each sex per dose group were administered mandestrobin (purity 93.4%) orally by gavage (in 0.5% w/v methylcellulose) at a dose of 0, 250, 500 or 1000 mg/kg bw per day during GDs 6–19. Females were cohabited with males on a one-to-one basis for mating. Presumed-pregnant females (GD 0) were allocated to each dose group. All animals were examined twice daily to detect dead or moribund animals. Additionally, animals were observed immediately after dosing and at 0.5, 1, 2 and 4 hours after dosing for signs of reaction to treatment. Body weight was recorded on GDs 4, 6, 7, 8, 9, 12, 15, 17, 19 and 20. Feed consumption was recorded for GDs 4–5, 6, 7, 8, 9–11, 12–14, 15–16, 17–18 and 19. A necropsy was performed on GD 20. The ovaries and the uteri were examined for pregnancy status, gravid uterus weight, number of corpora lutea and the number and intrauterine position of implantations (subdivided into live fetuses, early intrauterine deaths, late intrauterine deaths and dead fetuses). Individual fetal and placental weights were recorded, and fetuses were examined externally and sexed. No claim of GLP compliance was made, but the study was conducted in accordance with current requirements.

All treated animals showed piloerection on GDs 6–7. On GD 7, piloerection was seen in all treated animals at half an hour post-dosing for up to 1 hour post-dosing only. No significant adverse signs of maternal or embryo/fetal toxicity were observed at any of the doses.

No treatment-related effects on body weight or feed consumption were observed. In the caesarean section examination, there were no adverse effects on uterus weight, numbers of pregnancies, corpora lutea and implantations, the mean incidence of preimplantation and postimplantation losses, or litter size.

There was no effect of treatment on sex ratio, mean litter weight, mean placental weight or mean fetal weight. In the 250 mg/kg bw per day dose group, there were two malformed fetuses in two litters; one fetus had a severely reduced orbit of the eye, and the other fetus had no patent anal opening. There was one malformed fetus in the 500 mg/kg bw per day dose group, with a severely displaced umbilical opening associated with absent muscle tissue. At 1000 mg/kg bw per day, the incidence of the variation haematoma of the lower jaw was higher than in the control group, but this was an isolated finding and was not considered likely to be an adverse effect of treatment. Overall, there was no effect of treatment on the mean incidence of external fetal variations and malformations.

Based on the results of this study, a top dose of 1000 mg/kg bw per day was recommended for the main developmental toxicity study (Rhodes, 2009a).

In the main developmental toxicity study, mandestrobin (purity 93.4%) was administered in 0.5% aqueous methylcellulose to groups of 24 presumed pregnant Wistar rats by gavage at a dose of 0, 100, 300 or 1000 mg/kg bw per day from GDs 6 to 19. All females were observed twice daily for mortality and signs of toxicity. Feed consumption was recorded for GDs 3–5, 6, 7, 8, 9–11, 12–14, 15–16, 17–18 and 19. Body weights were recorded on GDs 3, 6, 7, 8, 9, 12, 15, 17, 19 and 20. On GD 20, all dams were terminated. Postmortem examination, including gross macroscopic examination of all internal organs, with emphasis on the uterus, uterine contents, position of fetuses in the uterus and number of corpora lutea, was performed, and the data were recorded. All organs or tissues showing grossly visible abnormalities were preserved. The uteri (and contents) and placentae of all females with live fetuses were weighed at necropsy. Fetuses were removed from the uterus, sexed, weighed individually and examined for gross external abnormalities. Approximately one half of the fetuses in each litter, selected by systematic sampling, were dissected, and the viscera were examined. They were then eviscerated, the carcasses were processed to stain the ossified skeleton by the Alizarin technique, the cartilage was processed with Alcian Blue staining and the skeletons were examined. The remaining fetuses were placed in Bouin's solution. At examination, the head was removed, and the coronal sections of the head were examined. The remaining portion of the fetus was examined by dissection and preserved, with the head sections.

No mortality was observed in the study. Post-dosing observations showed mouth rubbing (and very occasionally salivation) at 300 and 1000 mg/kg bw per day immediately after dosing only, but not at 0.5 and 1 hour post-dosing. This post-dosing observation was seen with increasing frequency at 300 mg/kg bw per day from GD 9 onwards and at 1000 mg/kg bw per day from GD 7 onwards. This observation was considered a common finding in rodents in oral (gavage) prenatal developmental toxicity studies and likely is related to taste aversion rather than evidence of toxicity.

Feed consumption was statistically significantly lower for the 1000 mg/kg bw per day group on GDs 6, 7 and 8 (days 1, 2 and 3 of dosing). Over the entire dosing period, mean feed consumption was similar in all groups. Mean body weight gain and mean gravid uterus weight adjusted for body weight were unaffected by treatment. There was no adverse effect of treatment on mean uterine/implantation data (Table 29).

Table 29. Key findings of the developmental toxicity study in rats

Finding	**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**
Maternal body weight (g), day 20	303.2	300.2	309.9	300.4
Maternal body weight gain, days 6–20 (%)	41.4	39.2	43.3	38.4
Feed consumption (g/rat per day)				
Day 6	19.1	17.8	18.6	16.7*
Day 7	20.5	19.2	19.4	17.7**
Day 8	20.2	19.7	20.3	17.7*
Days 6–18	21.4	20.8	21.0	20.5
Number of females mated	24	24	24	24
Number of non-pregnant females	0	1	0	0
Number of pregnant females (%)	24 (100)	23 (95.8)	24 (100)	24 (100)
Number of females with live fetuses	24	23	24	24
Mean number of early intrauterine deaths/dam (number of dams affected)	0.3 (7)	0.8 (10)	0.4 (8)	0.7 (14*)
% postimplantation loss/dam (dams affected)	2.8 (7)	8.6 (10)	4.4 (10)	6.9 (14*)

Finding	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
Mean litter size	10.5	10.0	11.2	10.0
Mean fetal weight (g)	3.75	3.77	3.77	3.83
Mean placental weight (g)	0.48	0.5	0.48	0.5
Sex ratio (% males)	50.0	47.3	53.8	55.6
Total number of fetuses with external or visceral variations, *n* (%)	77 (30.9)	78 (35.7)	103 (37.4)	101 (42.4)
Litter incidence	24/24	23/23	24/24	24/24
Fetuses with external or visceral malformations, *n* (%)	0	0	0	2 (0.7)
Litter incidence	0	0	0	2/24
Kidney, severely increased pelvic cavitation	0	0	0	2/24
Total number of fetuses with skeletal variations, *n* (%)	122 (94.8)	108 (93.7)	128 (96.4)	120 (99.2)
Total number of fetuses with skeletal malformation, *n* (%)	0	0	1 (0.8)	3 (2.9)
Litter incidence	–	–	1/24	3/24 DR* F+
Rib cartilage shortened	0/24	0/23	0/24	1/24
Sternebrae, cleft xiphoid cartilage	0/24	0/23	0/24	1/24
Vertebral cervical arch and centrum, additional ossification site fused	0/24	0/23	1/24	0/24
Vertebral cervical arch, additional cartilaginous ventral plate fused	0/24	0/23	0/24	1/24
Total number of fetuses with malformations, *n* (%)	0	0	1 (0.4)	5 (2.1)
Litter incidence	–	–	1/24	5/24* F+

bw: body weight; DR: significant dose–response test; F+: Cochran-Armitage and Fisher's exact (upper tail); *: $P < 0.05$; **: $P < 0.01$
Source: Rhodes (2012a)

There were no treatment-related effects on the number of pregnancies, the mean numbers of corpora lutea or implantations or the mean incidence of preimplantation and postimplantation losses. The number of dams with early intrauterine deaths or postimplantation loss at 1000 mg/kg bw per day showed an apparent statistically significant increase; however, both mean number of early intrauterine deaths and mean percentage of postimplantation loss showed no dose–response relationship or statistical significance.

There was no effect of treatment on sex ratio, mean litter weight, mean placental weight or mean fetal weight. Malformations were noted in one fetus in the group receiving 300 mg/kg bw per day and five fetuses from five litters in the group receiving 1000 mg/kg bw per day. In the fetus from the 300 mg/kg bw per day group, vertebral cervical arch and centrum, additional ossification site fused was noted. Among the five fetuses with malformation in the 1000 mg/kg bw per day group, one had cleft xiphoid cartilage in the sternebrae, one shortened rib cartilage and one additional cartilaginous vertebra plate fused in the vertebral cervical arch, and two had severely increased pelvic cavitation in the kidney. Historical control data for this latter visceral malformation indicated that this abnormality is a rare finding (Table 30).

Table 30. Historical control data (Charles River) for kidney, severely increased pelvic cavitation

	% fetal incidence		No. of studies	No. of litters	No. of fetuses	Years covered
	Mean	**Range**				
1	0.33	0–0.8	3	65	598	Unknown
2	0.13	0–0.8	6	113	1 064	2002–2011
3	0.04 (cumulative)	–	–	–	2 243	2002–2011

Source: Rhodes (2012a)

The NOAEL for maternal toxicity was 300 mg/kg bw per day, based on reduced feed consumption at 1000 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 300 mg/kg bw per day, based on an increase in a limited number of visceral and skeletal malformations at 1000 mg/kg bw per day (Rhodes, 2012a).

Rabbits

In a dose range–finding (non-GLP-compliant) developmental toxicity study, mandestrobin (purity 93.4%) was administered in 0.5% aqueous methylcellulose by gavage to presumed-pregnant female New Zealand white rabbits (seven per group) at a dose of 0, 250, 500 or 1000 mg/kg bw per day from GD 7 to GD 28. Observations were recorded at least 6 times per day during the dosing period. Feed consumption was recorded daily from GD 3 to GD 29, and body weights were measured on GDs 3, 7, 8, 9, 12, 15, 17, 19, 22, 25, 28 and 29. Females were terminated on GD 29, and a gross examination was performed. Gravid uterine weight was measured. The ovaries and uteri were removed and examined, and the following data were recorded: pregnancy status, gravid uterus weight, number of corpora lutea and the number and intrauterine position of implantations. Individual fetal and placental weights were recorded, and fetuses were examined externally and sexed by internal gonadal inspection.

One animal of the 1000 mg/kg bw per day dose group presented the clinical observation "head tilt"; however, veterinary examination revealed no treatment-related effects. Mean body weight, mean body weight gain and mean gravid uterus weight were unaffected by treatment.

A statistically significant dose–response relationship for body weight loss among animals treated at 500 and 1000 mg/kg bw per day was observed on the first day of dosing. However, this was considered not to be treatment related owing to the body weight loss seen prior to treatment in these groups. Mean feed consumption at the high dose was very slightly lower than that of controls throughout the study, but was statistically significantly lower only on the first day of dosing. There was no effect of treatment on sex ratio, mean litter weight or mean placental weight. In the high-dose group, mean fetal weight was slightly, but not statistically significantly, lower than that of controls, and this was considered to be a result of the higher litter size at this dose.

There was no effect of treatment on the mean incidence of external fetal variations and malformations. In the high-dose group, there was one malformed fetus with spina bifida, severely malformed head structures and severely flexed forelimb wrist joints. This was an isolated finding and was not considered to be related to treatment by the study author.

Although statistical significance was not reached, there were more fetuses with variations in the high-dose group (slightly enlarged bilateral eye bulge, upper incisor not erupted).

On the basis of this study, a top dose of 1000 mg/kg bw per day was recommended for the main developmental toxicity study (Rhodes, 2009b).

In the main developmental toxicity study, mandestrobin (purity 93.4%) was administered (in 0.5% aqueous methylcellulose) by gavage to 24 presumed-pregnant female New Zealand white rabbits

per group at a dose of 0, 100, 300 or 1000 mg/kg bw per day from GD 7 to GD 28. Observations were recorded at least twice daily during the dosing period and at least once daily during the pre-dosing and post-dosing period. Feed consumption was measured daily from GDs 3 to 29, and body weights were recorded on GDs 3, 7, 8, 9, 12, 15, 17, 19, 22, 25, 28 and 29. Females were terminated on GD 29, and a gross examination was performed. Gravid uterine and placental weights were measured, and fetuses were removed by caesarean section. The number of corpora lutea, number of implantations, number of live fetuses, and number and intrauterine position of embryonic/fetal deaths (including time of resorptions) were recorded. Each live fetus was weighed and examined externally. The heads and hearts of approximately half of the fetuses in each litter were fixed in Bouin's solution. Serial sections of the head were examined and preserved in 10% neutral buffered formalin. Several coronal slices of each heart were made to reveal the internal structure. These heart sections were examined and then preserved in neutral buffered formalin. All fetuses were dissected and sexed, and the viscera were examined. The remaining skeletons were examined for skeletal abnormalities.

No maternal animals died during the study period. One animal in the 300 mg/kg bw per day group aborted its pregnancy on GD 20 (Table 31). This abortion was not considered to be an effect of treatment because it was in one animal only and was not seen at the high dose. Clinical observations were generally unremarkable and showed no dose-related trend. Group mean body weight gains during the study were highly variable, with no marked adverse effect seen. Mean feed consumption at all doses was slightly lower than in controls, and this was statistically significant at 300 and 1000 mg/kg bw per day. These slight differences from controls are considered unlikely to be indicative of significant systemic toxicity to the pregnant rabbit because in the pre-dosing period, feed consumption at 1000 mg/kg bw per day was also slightly lower than in controls, and the difference was comparable with that during the treatment phase.

Table 31. Key findings of the developmental toxicity study in rabbits

Finding	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	HCD[a]	
					Mean	Range
Number of females inseminated	24	24	24	24	–	–
Number of non-pregnant females	1	1	2	6	–	–
Number of pregnant females (%)	23 (95.8)	23 (95.8)	22 (91.7)	18 (75.0)	–	–
Accidental death	0	0	0	0	–	–
Abortion/premature delivery	0	0	1	0	–	–
Total litter resorptions	0	0	0	1	–	–
Number of litters for evaluation	23	23	21	17	–	–
Uterine data[b]						
Mean number of corpora lutea	10.3	10.1	10.0	9.7 (9.4)	10.4	10.0–11.9
Mean number of implantations	8.9	9.4	8.8	8.8 (8.4)	8.6	7.6–9.4
Mean % preimplantation loss	15.0	7.7	12.9	9.1 (13.0)	17.3	9.5–27.6
Mean number of intrauterine deaths early	0.6	0.9	0.8	0.9 (0.9)	0.58	0.4–0.8
Mean number of intrauterine deaths late	0.2	0.3	0.2	0.3 (0.3)	0.2	0.1–0.3
Mean number of dead fetuses	0.0	0.0	0.0	0.1 (0.1)	0.0	0.0

Finding	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	HCD[a]	
					Mean	Range
Mean % postimplantation loss	8.7	13.6	11.0	13.3 (18.1)	9.1	7.1–14.2
Number of dams affected	10	17*	11	11	–	–
Mean number of live fetuses	8.0	8.2	7.8	7.5 (7.1)	7.8	6.9–8.7
Maternal body weight (kg)						
Day 7	3.32	3.28	3.31	3.44	–	–
Day 29 (surviving does)	3.83	3.72	3.70	3.87	–	–
Number of implantations/doe	8.9	9.4	8.8	8.8	–	–
Resorption and fetal death (%)	8.7	13.6	11.0	13.3	–	–
Mean litter size (live fetuses)	8.0	8.2	7.8	7.5	7.8	6.9–8.7
Mean fetal weight (g)						
Males	44.4	43.9	43.0	45.9	–	–
Females	45.1	43.4	41.7	45.4	–	–
Sex ratio (% males)	49.1	50.3	57.6	50.0	49.7	45.1–50.7
Fetuses with external or visceral malformations, *n* (%)	7 (3.3)	4 (1.5)	2 (1.2)	2 (1.7)	(2.6)	–
Litter incidence	6/23	3/23	2/21	2/17	–	–
Fetuses with external or visceral variations, *n* (%)	98 (55.3)	85 (46.0)	80 (49.4)	54 (43.0)	(48.7)	–
Litter incidence	22/23	23/23	21/21	15/17	–	–
Fetuses with skeletal malformation, *n* (%)	3 (1.5)	4 (1.7)	7 (4.9)	2 (1.6)	(2.7)	–
Litter incidence	2/23	3/23	6/21	2/17	–	–
Fetuses with skeletal variations, *n* (%)	148 (79.8)	142 (75.0)	114 (70.8)	110 (85.8)	(73.9)	–
Litter incidence	22/23	23/23	21/21	17/17	–	–
Total number of fetuses with malformations, *n* (%)	8 (4.3)	6 (3.2)	9 (5.5)	3 (2.3)	–	–
Litter incidence	6/23	4/23	7/21	3/17	–	–

bw: body weight; *n*: number; *: $P < 0.05$ (Cochran-Armitage and Fisher's exact, upper tail)

[a] Historical control data were derived from six embryo/fetal studies performed with New Zealand white rabbits at Covance from April 2004.

[b] The animal with total embryo/fetal loss was excluded from the analysis; values in parentheses contain this doe in the analysis.

Source: Rhodes (2012b)

The number of pregnancies was 23, 23, 22 and 18, and there were 23, 23, 21 and 17 surviving females with litters on GD 29 at 0, 100, 300 and 1000 mg/kg bw per day, respectively (Table 31). The distribution of non-pregnant animals in the high-dose group (*n* = 6) was considered a chance event by the study author and unrelated to treatment, as any increase in very early postimplantation losses would have been seen as an increase in this parameter in the mean data. Implantation in rabbits occurs at days 7–7.5 after insemination, at the beginning of the dosing period.

One animal receiving 1000 mg/kg bw per day (number 93) had total embryo/fetal loss; this isolated incidence was not considered to be an effect of treatment by the study author. The mean numbers of corpora lutea and implantations and the mean incidence of preimplantation and postimplantation losses showed no effect of treatment. The number of dams with postimplantation loss at 100 mg/kg bw per day showed an apparent statistical significance; however, all data were within expected ranges, and this was not considered to be biologically relevant.

Mean litter size was unaffected by treatment (Table 31).

There was no effect of treatment on sex ratio, mean litter weight, mean placental weight or mean fetal weight.

Overall, there was no effect of treatment on the incidence of fetal variations and malformations, which were all within expected ranges for this strain of rabbit (Table 31). Malformations were noted in eight fetuses from six litters in the control group, six fetuses from four litters in the group receiving 100 mg/kg bw per day, nine fetuses from seven litters in the group receiving 300 mg/kg bw per day and three fetuses from three litters in the group receiving 1000 mg/kg bw per day.

In conclusion, administration of mandestrobin by oral gavage to pregnant rabbits elicited no systemic toxicity to the maternal rabbits. There was no evidence of embryo or fetal toxicity at any dose tested. Based on these observations, the NOAELs for both maternal and embryo/fetal toxicity were 1000 mg/kg bw per day, the highest dose tested (Rhodes, 2012b).

2.6 Special studies

(a) Neurotoxicity

Acute neurotoxicity

In a preliminary acute neurotoxicity study, mandestrobin (purity 93.4%) was administered to groups of three Wistar rats of each sex by a single oral gavage dose of 0, 300, 1000 or 2000 mg/kg bw in 0.5% w/v aqueous methylcellulose. Observations were made at 1, 2, 3, 4, 5, 6, 7 and 8 hours after treatment. Body weights were determined on the day of dosing prior to treatment and were similar across all groups. On the day following dosing, animals were euthanized.

No mortality was noted. Clinical signs included statistically significantly higher mean defecation count (1.3 counts) and number of urine pools (1.0 pool) for males at 2000 mg/kg bw at approximately 1 hour following dosing on study day 0. However, these findings were transient, the magnitude of change relative to the control group was small and there were no correlating findings for the 2000 mg/kg bw males. In males from the control group, a urine pool count of 0.7 was reported at 8 hours. Therefore, these changes were not considered test compound related. No neurotoxic effects were noted.

On the basis of these results, doses of 500, 1000 and 2000 mg/kg bw were selected for evaluation of the acute neurotoxic potential of mandestrobin. It was also determined that the time at which FOB and locomotor activity would be observed in the definitive study was 8 hours post-dosing on study day 0 (time of peak effect) (Herberth, 2011a).

In the main acute neurotoxicity study, groups of 12 Wistar rats of each sex were given a single oral gavage dose of mandestrobin (purity 93.4%) in 0.5% w/v aqueous methylcellulose at 0, 500, 1000 or 2000 mg/kg bw. Clinical observations were performed once daily on all animals, except on days of FOB assessments. Body weights were recorded at least weekly. FOB and locomotor activity testing were carried out during the week of acclimation, at the time of peak effect (8 hours post-dosing) on study day 0, and again on days 7 and 14. On day 15, animals were terminated and submitted for neuropathology.

No mortality, clinical signs or macroscopic or microscopic findings were noted at necropsy. Body weights were unaffected by treatment. Home cage, handling, open field, sensory, neuromuscular and physiological parameters were unaffected by test compound administration.

In locomotor activity, statistically significantly lower mean overall total and ambulatory counts in the 2000 mg/kg bw males and statistically significantly lower mean total and ambulatory counts in the 1000 and 2000 mg/kg bw males during the second subinterval (11–20 minutes) were noted at the time of peak effect on study day 0, but were unaffected on study day 7 or 14. There were no other findings to corroborate this effect, which was therefore attributed to transient systemic toxicity at the high dose, rather than typical neurotoxicity. No other statistically significant changes were noted in 1000 mg/kg bw males during the other subintervals; therefore, the decreases were attributed to better habituation observed in this group and not to mandestrobin administration. Statistically significantly lower mean overall ambulatory counts were noted for 2000 mg/kg bw females at the time of peak effect on study day 0, primarily due to reductions in ambulatory counts during the first two subintervals (0–10 and 11–20 minutes). Although there were no differences in total motor activity counts noted for these animals on study day 0, similar reductions in ambulatory counts were noted for the 2000 mg/kg bw males at this evaluation; therefore, these reductions were attributed to mandestrobin administration. No other mandestrobin-related effects on locomotor activity were noted in 2000 mg/kg bw females on study day 7 or 14 (Table 32).

During FOB assessments, on study day 7, home cage parameter observations recorded statistically significantly fewer females in the 1000 mg/kg bw group sitting or standing normally in the home cage; however, this behaviour was not observed at 2000 mg/kg bw. Therefore, this decrease was not attributed to mandestrobin administration. On study day 7, open field parameter observations recorded a statistically significant decrease in the number of rearing counts for 2000 mg/kg bw females. This decrease was attributed to a single female in this group that did not rear during the evaluation, whereas rearing counts for the remaining females in the 2000 mg/kg bw group were similar to those observed in the control group. The number of rearing counts of the corresponding female was similar to that of animals in the control group on study days 0 and 14. Therefore, the decrease observed on study day 7 was not attributed to mandestrobin administration.

Table 32. FOB and locomotor activity findings of the acute neurotoxicity study in rats

			Males				Females			
End-point	**Day**	**Min**	**0 mg/kg bw**	**500 mg/kg bw**	**1 000 mg/kg bw**	**2 000 mg/kg bw**	**0 mg/kg bw**	**500 mg/kg bw**	**1 000 mg/kg bw**	**2 000 mg/kg bw**
Home cage parameter: Mean no. of animals sitting or standing normally	0	–	5	6	3	3	6	6	4	2
	7	–	2	4	5	2	8	4	2[#]	4
	14	–	3	5	3	5	5	7	9	8
Open field parameter: Mean no. of animals rearing	0	–	6.5	7.3	6.8	5.5	12.3	7.9	9.2	8.8
	7	–	9.7	8.7	10.5	8.0	15.8	12.8	12.0	11.4*
	14	–	10.6	12.3	13.2	11.4	17.0	16.6	15.1	16.9
Locomotor activity counts: Total counts	0	0–10	1 146	1 094	1 052	979	1 275	1 283	1 186	1 115
		11–20	570	568	379[+]	300[+]	482	409	467	435
		21–30	258	194	136	131	145	214	168	126
		31–40	118	177	121	118	120	261	99	89
		41–50	82	143	106	127	168	209	219	152

End-point	Day	Min	Males 0 mg/kg bw	Males 500 mg/kg bw	Males 1 000 mg/kg bw	Males 2 000 mg/kg bw	Females 0 mg/kg bw	Females 500 mg/kg bw	Females 1 000 mg/kg bw	Females 2 000 mg/kg bw
		51–60	94	93	124	63	149	159	177	125
	Cum.	–	2 268	2 267	1 918	1 718+	2 339	2 536	2 316	2 042
Locomotor activity counts: Ambulatory counts	0	0–10	363	331	318	286	445	460	414	348
		11–20	121	100	49+	31+	105	87	97	84
		21–30	33	20	8	10	16	46	16	11
		31–40	2	25	21	6	20	66	9	16
		41–50	1	16	18	8	26	22	31	17
		51–60	2	6	9	1	11	27	19	5
	Cum.	–	522	498	424	343+	623	708	587	480+

bw: body weight; Cum: cumulative; #: $P < 0.05$ (Fisher's exact test); *: $P < 0.05$ (Dunnett's test); +: $P < 0.05$ (sequential linear trend test)
Source: Herbert (2011b)

The NOAEL for neurotoxicity was 1000 mg/kg bw, based on decreased overall locomotor activity (total and/or ambulatory counts) at 2000 mg/kg bw. The NOAEL for systemic toxicity was 2000 mg/kg bw, the highest dose tested (Herberth, 2011b).

Subchronic neurotoxicity

In a dose range–finding study, mandestrobin (purity 93.4%) was given to four groups of five Crl:WI(HAN) rats of each sex per group at a dietary concentration of 0, 1500, 5000 or 15 000 ppm (equal to 0, 132, 430 and 1200 mg/kg bw per day for males and 0, 135, 430 and 1305 mg/kg bw per day for females) for 28 days. All animals were observed twice daily for mortality and moribundity. Clinical examinations were performed daily, and detailed physical examinations were performed approximately weekly. Individual body weights were recorded twice weekly, and feed consumption was recorded weekly. At the scheduled termination, complete necropsies were conducted, and the brain was collected from all animals.

All animals survived. There were no test substance–related clinical observations or macroscopic findings in any of the treated groups. A marginally lower body weight (2–4%) was noted in both sexes given 15 000 ppm during the first week of treatment. A statistically significantly lower body weight gain (15%) was recorded in both sexes given 15 000 ppm during the first week of treatment (days 0–3). However, no effect on body weight was observed through the end of dosing, as body weights for the 15 000 ppm groups were similar to those of the vehicle control group. Therefore, these body weight changes were not considered to be adverse. A marginally, but not statistically significantly, reduced feed consumption was reported in males given 15 000 ppm during the first week of treatment. It is noted that feed consumption was similar to that of controls from day 7 to 28. There were no treatment-related effects evidenced by the macroscopic investigation, and no effect on brain weight.

The NOAEL for systemic toxicity was 15 000 ppm (equal to 1200 mg/kg bw per day), the highest dose tested (Hosako, 2011a). Based on the results of the study, the dose of 15 000 ppm was considered appropriate for subsequent neurotoxicity testing.

In a subchronic neurotoxicity study, mandestrobin (purity 93.4%) was administered orally to 12 Wistar rats of each sex per group at a dietary concentration of 0, 1500, 5000 or 15 000 ppm (equal to 0, 99, 338 and 1024 mg/kg bw per day for males and 0, 122, 415 and 1223 mg/kg bw per day for

females, respectively) for 13 weeks. All animals were observed twice daily for mortality and moribundity. Detailed physical examinations, feed consumption and body weights were recorded once weekly, beginning 1 week prior to test diet administration. Ophthalmoscopy was performed in all animals during acclimatization and in week 12. A FOB, including locomotor activity testing, was performed during acclimatization and in weeks 1, 3, 7 and 12. At termination of the study, all animals were euthanized and submitted for neuropathology.

No mortality or clinical signs were noted. One male in the 5000 ppm group and one female in the control group were found in the incorrect cages and were euthanized and sent to necropsy on study day 58. No treatment-related effects were noted during ophthalmoscopy or neuropathology.

Test compound–related, statistically significantly reduced mean body weight gains were noted for males offered diet containing 15 000 ppm of the test compound during study days 0–7, 14–21 and 28–35. As a result, the mean body weight gain of males in the high-dose group was somewhat lower (not statistically significantly) than in the control group over the entire treatment period (study days 0–91), and mean body weights in the high-dose group were 4.8–9.3% lower than in the control group beginning on study day 7 and continuing until the end of the treatment period; differences in mean body weight were statistically significant on study days 35–56. Mean body weight gains were unaffected by test compound exposure thereafter. Mean body weight gain for males offered diet containing 15 000 ppm of the test compound was statistically significantly higher than in the control group during study days 77–84; however, this was transient, and an increase in mean body weight gain was not considered toxicologically relevant. Mean body weights and body weight gains for males offered diet containing 1500 and 5000 ppm of the test compound and for females at all test compound concentrations were unaffected by test compound exposure. Statistically significant differences from the control group were transient, did not occur in a dose-related manner, did not affect mean body weights and/or did not affect overall mean body weight gains (Table 33).

Statistically significantly reduced mean feed consumption (evaluated as g/animal per day and g/kg bw per day) was noted for males offered diet containing 15 000 ppm of the test compound during study days 0–7 (g/animal per day and g/kg bw per day), days 21–28 (g/animal per day) and days 28–35 (g/animal per day). In addition, slightly lower (not statistically significantly) mean feed consumption (g/animal per day) was noted during study days 7–21. These results corresponded to reduced mean body weight gains noted for these males during this period and were considered to be test substance related (Table 33).

Table 33. Body weight, body weight gain and feed consumption in a subchronic neurotoxicity study in rats

		Males				Females			
End-point	**Study day(s)**	**0 ppm**	**1 500 ppm**	**5 000 ppm**	**15 000 ppm**	**0 ppm**	**1 500 ppm**	**5 000 ppm**	**15 000 ppm**
Mean body weight (g)	0	190	190	188	188	133	129	130	132
	35	344	340	338	313*	199	192	196	201
	42	359	353	353	327*	204	197	204	205
	49	380	374	374	345*	215	204	212	212
	56	389	383	384	353*	219	208	215	216
	91	435	430	427	400	234	228	234	234
Mean body weight gain (g)	0–7	38	38	37	29**	15	18	18	19
	7–14	42	41	41	39	16	16	19	19
	14–21	32	32	32	26*	18	13**	12**	16

End-point	Study day(s)	Males				Females			
		0 ppm	1 500 ppm	5 000 ppm	15 000 ppm	0 ppm	1 500 ppm	5 000 ppm	15 000 ppm
	21–28	21	20	18	16	7	8	9	9
	28–35	21	19	21	15**	9	9	9	6
	0–91	245	239	237	211	101	99	104	102
Mean feed consumption (g/animal per day)	0–7	22	22	21	19**	15	15	15	16
	7–14	23	23	23	22	15	15	16	17**
	14–21	24	23	23	22	16	16	16	17
	21–28	24	23	23	21*	16	15	16	17
	28–35	25	23	24	22**	17	16	18	16

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Dunnett's test)
Source: Herberth (2012)

The only statistically significant difference during home cage observations was recorded in the 1500 ppm group and consisted of a lower number of females that were alert and oriented towards the observer and a higher number of females that were sitting or standing normally. However, these transient findings were not considered to be abnormal in the home cage, had no dose–response relationship and therefore were not considered to be treatment related.

Handling, sensory and physiological parameters were unaffected by test compound administration. During open field parameter observations, a lower mean time to first step was recorded in all treated females during study week 1, but was not considered to be treatment related by the study author, as this was a small magnitude of change, findings were noted only during the week 1 assessment, there was no dose–response relationship and no effect was observed in males. A higher mean rearing count was also noted in 5000 ppm males during study week 3, but was also not considered to be treatment related, as there was no dose–response relationship. Reduced forelimb grip strength was recorded in 15 000 ppm females during study week 7, but was not considered to be treatment related, as these results were not noted during study weeks 3 and 12 and there were no additional effects on related parameters. In addition, statistically significantly lower mean rotarod performance during week 1 and lower mean forelimb grip strength during week 3 were noted in 1500 ppm females, but were not considered to be treatment related, as no dose–response relationship was evident. Locomotor activity patterns (mean ambulatory and total motor activity counts) were unaffected by test diet consumption. Statistically significant differences were limited to lower mean total counts in 15 000 ppm females during 51–60 minutes in study week 1, higher mean cumulative total counts in 15 000 ppm males in study week 7, and higher mean ambulatory counts in 15 000 ppm females during 21–30 minutes in study week 12. However, findings were transient or did not affect mean overall counts, and no remarkable shifts in the pattern of habituation occurred in any of the test compound–exposed groups when the animals were evaluated in study weeks 1, 3, 7 and 12. Therefore, they were considered not to be treatment related.

The NOAEL for systemic toxicity was 5000 ppm (equal to 338 mg/kg bw per day), based on transient decreases in body weight, body weight gain and feed consumption at 15 000 ppm (equal to 1024 mg/kg bw per day). The NOAEL for neurotoxicity was 15 000 ppm (equal to 1024 mg/kg bw per day), the highest dose tested, based on the absence of neurotoxic effects (Herberth, 2012).

(b) Immunotoxicity

In a dose range–finding immunotoxicity study, mandestrobin (purity 93.4%) was administered to four groups of Han Wistar rats (eight females per group) at a dietary concentration of 0, 1500, 5000 or 15 000 ppm (equal to 0, 135, 436 and 1340 mg/kg bw per day, respectively) for 28 days. A concurrent

positive control group (group 5) was offered the basal diet on a comparable regimen. The assessment of immunotoxicity was based primarily on the results of a splenic antibody-forming cell (AFC) assay to assess the T cell–dependent antibody response (TDAR) to sheep red blood cells (sRBCs). All TDAR group animals (groups 1–5) were immunized with an intravenous injection of sRBCs on study day 24. A positive control group (group 5) received cyclophosphamide monohydrate (CPS) at a dose of 50 mg/kg bw per day on study days 24 through 27. All animals were observed twice daily for mortality and moribundity. Detailed clinical observations were performed during pretest and once weekly during the study. Following 28 days of dose administration, all animals were euthanized, and blood samples for possible immunoglobulin M (IgM) antibody analysis (AFC assay) were collected. At the scheduled necropsy, the spleens from all animals were weighed.

All animals survived to the scheduled necropsy. There were no test compound–related clinical observations or macroscopic findings in any of the test compound–treated groups. Test compound–related lower body weights, body weight gains (3–7%) and feed consumption were noted at 15 000 ppm during the first week of test diet administration and attributed to unpalatability. However, body weights and feed consumption were similar to those of the vehicle control group throughout the remainder of the study. Statistically significantly lower mean body weight gains compared with the vehicle control group were noted in the positive control group from study days 24 to 28. As a result, lower mean body weight (5.3%) compared with the vehicle control group was noted, and lower mean cumulative body weight gain was noted from study days 0 to 28. Lower body weights are consistent with the known effects of CPS.

No test compound–related changes in absolute or relative adrenal gland, brain, spleen or thymus weight were observed when compared with the vehicle control group. There were no test compound–related effects on spleen cell number or the response to sRBCs, as measured by AFC IgM specific activity (AFC/10^6 spleen cells) and total spleen activity (AFC/spleen), at any dose tested.

In the absence of mandestrobin-related effects on the AFC response, the NOAEL for the humoral immune response in the TDAR groups was considered to be 15 000 ppm (equal to 1340 mg/kg bw per day), the highest dose tested. No adverse effects were observed for the general toxicity parameters evaluated (Hosako, 2011a).

In the main immunotoxicity study, mandestrobin (purity 93.4%) was administered to four groups of 10 female Han Wistar rats at a dietary concentration of 0, 1500, 5000 or 15 000 ppm (equal to 0, 147, 471 and 1419 mg/kg bw per day, respectively) for 28 days. The assessment of immunotoxicity was based primarily on the results of a splenic AFC assay to assess the TDAR to sRBCs. A positive control group (group 5) received CPS at a dose of 50 mg/kg bw per day on study days 24 through 27. All animals (groups 1–5) were immunized with an intravenous injection of sRBCs on study day 24. Animals were observed twice daily for mortality and moribundity. Clinical examinations were performed daily, and detailed physical examinations were performed approximately weekly. Individual body weights were recorded twice weekly, and feed consumption was recorded weekly. Following 28 days of dose administration, all animals were euthanized. Blood samples for possible serum IgM antibody analysis were collected from all animals at the scheduled necropsy. Complete necropsies were conducted on all animals, and the spleen, thymus, lymph nodes, Peyer’s patches and adrenal glands were collected from all animals. Spleen, thymus and adrenal gland weights were recorded. Liver and kidney weights were not recorded.

No mortality or clinical signs were noted during the study.

Body weights were unaffected by test compound administration. However, a statistically significantly higher mean body weight gain was noted at 15 000 ppm (study days 3–7), and higher mean cumulative body weight gains were noted at 5000 ppm (study days 0–7, 0–10 and 0–14) and 15 000 ppm (study days 0–10 and 0–14), compared with the vehicle control group. Differences in body weight gain were probably due to biological variability and were not considered related to test compound administration owing to the lack of a clear dose–response trend, the transient nature and the fact that the cumulative body weight gains at the end of the study (study day 28) were not affected at either

dietary concentration. Statistically significantly lower mean body weight gains and cumulative body weight gains were noted in the positive control group from study days 24 to 28 and 0 to 28, respectively, compared with the vehicle control group. These lower body weight gains were consistent with the known effects of CPS. Feed consumption was unaffected by test compound administration.

There were no mandestrobin-related gross observations. A small thymus was noted in 9/10 animals in the positive control (CPS) group at the scheduled necropsy; however, these findings were consistent with the known effects of CPS and correlated with the lower thymus weights.

Higher relative spleen weights were recorded at 15 000 ppm; however, absolute spleen weights were not affected. There were no mandestrobin-related effects on adrenal gland or thymus weights. There were no statistically significant effects on spleen cell number, and mandestrobin did not significantly suppress the humoral immune response to the T cell–dependent antigen sRBCs when evaluated as either specific activity (AFC/10^6 spleen cells) or total activity (AFC/spleen). The positive control agent produced satisfactory results.

The NOAEL for immunotoxicity was 15 000 ppm (equal to 1419 mg/kg bw per day), the highest dose tested, based on the absence of mandestrobin-related effects on the AFC response. No adverse effects were observed for general toxicity (Hosako, 2011b).

(c) Mechanistic studies

Several in vivo and in vitro mechanistic studies were performed (as summarized in Table 34) to investigate the mode of action of the liver and thyroid effects of mandestrobin and to address possible hormonal effects of mandestrobin.

Table 34. Summary of mechanistic studies with mandestrobin

Study	Species/test cells	Results	Reference
In vivo studies			
Short-term study	Rat (males and females)	*Liver*: ↑ in liver weight with diffuse, hepatocellular hypertrophy, proliferation of liver smooth endoplasmic reticulum, ↑ in liver CYP2B and T_4-UGT activities and ↑ in replicative DNA synthesis in a dose-dependent and reversible manner *Thyroid*: hypertrophy, ↓ in serum T_4 levels and ↑ in TSH (findings were reversible)	Asano (2012e)
Short-term study	Mouse (males)	↑ in liver weight and induction of CYP2B activity	Yamada (2012b)
In vitro studies			
Steroidogenesis assay	Human adrenocortical NCI-H295R cell line	No influence on testosterone or estradiol production	Kubo (2012)
Reporter gene assays	hERα-HeLa-9903 and hAR-HeLa 4-11 cell lines	Mandestrobin and its metabolites (5-COOH-S-2200, 4-OH-S-2200, 5-CH_2OH-S-2200 and 5-CA-S-2200-NHM) did not show agonistic or antagonistic effects on hERα or hAR-induced transcriptional activation	Suzuki (2012)

CYP2B: cytochrome P450 2B; DNA: deoxyribonucleic acid; hAR: human androgen receptor; hERα: human estrogen receptor alpha; T_4: thyroxine; TSH: thyroid stimulating hormone; UGT: uridine diphosphate glucuronosyltransferase

In repeated-dose toxicity studies, the primary target organ of mandestrobin was the liver in all species examined. The thyroid was also a target organ in the rat, but not in the mouse or dog. The primary liver finding in the rat was hypertrophy (increased liver weight and/or hepatocellular hypertrophy), and the main thyroid finding was follicular cell hypertrophy. However, no tumorigenicity was observed in rat or mouse carcinogenicity studies.

In conjunction with evidence from the literature, two in vivo studies were performed (one in rats, one in mice) to gain insight into the mechanistic basis of the liver and thyroid effects observed in the main studies in rats.

Two in vitro studies were performed to address possible hormonal effects of mandestrobin and its metabolites: 5-COOH-S-2200, 4-OH-S-2200, 5-CH_2OH-S-2200 and 5-CA-S-2200-NHM.

Short-term study in rats

In a non-GLP-compliant mechanistic study, groups of 10 male and 10 female Wistar rats were fed diets containing mandestrobin (purity 93.4%) at a concentration of 0 (control), 400, 2000, 7000 or 15 000 ppm (equal to 0, 23.3, 115.7, 378.9 and 744.4 mg/kg bw per day for males and 0, 25.7, 131.2, 420.2 and 811.8 mg/kg bw per day for females, respectively) for 7 days. The following parameters were measured at necropsy: serum thyroxine (T_4), triiodothyronine (T_3) and thyroid stimulating hormone (TSH) concentrations, CYP4A activity, CYP2B activity, T_4-UGT, replicative DNA synthesis of hepatocytes (determined as 5-bromo-2′-deoxyuridine [BrdU] incorporation), microscopy of liver and thyroid and ultrastructural analysis of hepatocytes using electron microscopy. To evaluate the time course of alterations at 15 000 ppm, 10 rats of each sex per group were also fed diets containing 0 or 15 000 ppm (equal to 796.3 mg/kg bw per day for males and 952.4 mg/kg bw per day for females) mandestrobin for 14 days. Data from both the 7- and 14-day treatment groups were compared to determine whether enhancement or attenuation of alterations was observed. To evaluate the reversibility of any findings, 10 rats of each sex per group were also fed diets containing 0 or 15 000 ppm (equal to 804.9 mg/kg bw per day for males and 896.1 mg/kg bw per day for females) mandestrobin for 7 days followed by a 7-day recovery period. As a positive control for CAR activation, groups of rats were treated with 1000 ppm phenobarbital (PB).

Treatment with mandestrobin caused no deaths, severe toxicity or changes in clinical signs.

Body weight gain during the first 4 days of treatment was statistically significantly lower in both sexes administered 15 000 ppm in both the 7- and 14-day treatment groups, and this resulted in lower body weights. After the first 4 days, however, body weight gain was generally equivalent to or higher than the control level, but total body weight gain remained lower (8% [not statistically significant] in males and 50% in females) at 15 000 ppm in both the 7- and 14-day treatment groups. In the recovery group, no remarkable findings for evaluation of reversibility were observed, because no findings were observed after the 7-day treatment with mandestrobin. Males at 7000 ppm also had decreased body weight and body weight gain after the 7-day treatment.

Although suppression of body weight gains was observed in females administered PB on day 8 in both the 7- and 14-day treatment groups, the recovery group demonstrated no difference from controls at day 8. Statistically significantly suppressed or a tendency towards suppression of feed consumption was observed in both sexes administered 15 000 ppm, especially in the early phase of the treatment period; however, these alterations were not observed after the 14-day treatment. Less severe changes were observed in both sexes at 7000 ppm. In the recovery group, feed consumption was increased after cessation of treatment with mandestrobin.

No remarkable consistent changes were observed in the PB group in any phase.

***Liver*:** After 7 days of treatment with mandestrobin, statistically significantly increased absolute liver weights for both sexes at 15 000 ppm and relative liver weights for males at 7000 ppm and above and for females at 2000 ppm and above were observed. After the 14-day treatment, statistically significant increases were observed in the absolute and relative liver weights in both sexes at 15 000 ppm and in the PB group. In the recovery group, statistically significant increases were observed in

several parameters: absolute and relative liver weights in males administered 15 000 ppm, and relative liver weights in males administered PB. However, all findings were less severe than those after the 7-day treatment (Table 35).

Table 35. Key findings of the short-term mechanistic study in rats

	Males						Females					
Finding	**0 ppm**	**400 ppm**	**2 000 ppm**	**7 000 ppm**	**15 000 ppm**	**PB**	**0 ppm**	**400 ppm**	**2 000 ppm**	**7 000 ppm**	**15 000 ppm**	**PB**
Organ weights												
Relative liver weight (% of control value)												
7 days	100	99	103	110**	124**	124**	100	99	106*	110**	121**	118**
14 days	100	ND	ND	ND	130**	135**	100	ND	ND	ND	128**	127**
7 days + 7 days of recovery	100	ND	ND	ND	111	109*	100	ND	ND	ND	108	105
Relative thyroid weight (% of control value)												
7 days	100	108	112	104	119	119	100	106	103	132**	131**	122*
14 days	100	ND	ND	ND	117*	127**	100	ND	ND	ND	133**	121**
7 days + 7 days of recovery	100	ND	ND	ND	102	124**	100	ND	ND	ND	112	105
Hepatic enzymatic activity												
CYP2B activity (% of control value)												
7 days	100	148	289*	872*	1 358*	2 510*	100	112	305*	2 581*	7 987*	26 948*
7 days + 7 days of recovery	100	ND	ND	ND	135	235**	100	ND	ND	ND	108	334**
CYP4A activity (% of control value)												
7 days	100	98	99	120	124	167**	100	94	86	89	85	129*
7 days + 7 days of recovery	100	ND	ND	ND	141**	121	100	ND	ND	ND	76*	77*
UGT activity (% of control value)												
7 days	100	123*	130	150*	148*	191*	100	95	100	117	136**	123*
7 days + 7 days of recovery	100	ND	ND	ND	113	136**	100	ND	ND	ND	109	110
Serum hormone levels												
Serum [T_4] (% of control value)												
7 days	100	106	106	90	81*	88	100	100	96	92	84	67**
14 days	100	ND	ND	ND	72**	71**	100	ND	ND	ND	75*	54**
7 days + 7 days of recovery	100	ND	ND	ND	90*	102	100	ND	ND	ND	109	108

Finding	Males						Females					
	0 ppm	**400 ppm**	**2 000 ppm**	**7 000 ppm**	**15 000 ppm**	**PB**	**0 ppm**	**400 ppm**	**2 000 ppm**	**7 000 ppm**	**15 000 ppm**	**PB**
Serum [TSH] (% of control value)												
7 days	100	85	87	78	87	123	100	103	110	149	248**	167**
14 days	100	ND	ND	ND	146	159	100	ND	ND	ND	202	174
7 days + 7 days of recovery	100	ND	ND	ND	92	111	100	ND	ND	ND	122	98
Serum [T_3] (% of control value)												
7 days	100	100	100	80	100	100	100	100	100	100	100	100
14 days	100	ND	ND	ND	80	100	100	ND	ND	ND	71**	71**
7 days + 7 days of recovery	100	ND	ND	ND	100	117*	100	ND	ND	ND	114	114
Histopathology												
Hepatocyte centrilobular hypertrophy												
7 days	0/10	0/10	0/10	0/10	0/10	10/10**	0/10	0/10	0/10	0/10	0/10	10/10**
14 days	0/10	ND	ND	ND	0/10	10/10**	0/10	ND	ND	ND	0/10	10/10**
7 days + 7 days of recovery	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	1/10
Hepatocyte diffuse hypertrophy												
7 days	0/10	0/10	1/10	8/10**	9/10**	0/10	1/10	1/10	5/10	6/10*	8/10**	0/10
14 days	2/10	ND	ND	ND	10/10**	0/10	0/10	ND	ND	ND	5/10*	0/10
7 days + 7 days of recovery	2/10	ND	ND	ND	3/10	1/10	0/10	ND	ND	ND	0/10	0/10
SER proliferation												
7 days	0/2	ND	ND	ND	1/2	ND	0/2	ND	ND	ND	1/2	ND
Hepatocyte intracellular lipid droplet												
7 days	0/2	ND	ND	ND	0/2	ND	0/2	ND	ND	ND	1/2	ND
BrdU labelling indices (% of control value)												
7 days	100	98	182	233**	389**	388**	100	192	197	225*	241**	308**
14 days	100	ND	ND	ND	138*	110	100	ND	ND	ND	111	174*
Thyroid diffuse follicular hypertrophy												
7 days	1/10	1/10	2/10	2/10	3/10	5/10	0/10	1/10	1/10	4/10*	6/10**	3/10
14 days	2/10	ND	ND	ND	5/10	6/10	0/10	ND	ND	ND	5/10*	3/10
7 days + 7 days of recovery	1/10	ND	ND	ND	2/10	4/10	0/10	ND	ND	ND	0/10	0/10

BrdU: 5-bromo-2′-deoxyuridine; CYP: cytochrome P450; ND: not determined; PB: phenobarbital; ppm: parts per million; SER: smooth endoplasmic reticulum; T_3: triiodothyronine; T_4: thyroxine; TSH: thyroid stimulating hormone; *: $P < 0.05$; **: $P < 0.01$

Source: Asano (2012e)

After the 7-day treatment, an increased incidence of enlarged liver was found in males receiving mandestrobin at 15 000 ppm and in females receiving PB at 1000 ppm. After the 14-day treatment, the incidences of enlarged liver were increased in males and females receiving mandestrobin at 15 000 ppm and in males and females receiving PB at 1000 ppm.

After the 7-day treatment, the incidences of diffuse hypertrophy of hepatocytes were increased in females at 2000 ppm and in both sexes at 7000 and 15 000 ppm. Centrilobular hypertrophy of hepatocytes was observed in all animals of the PB group. After the 14-day treatment, the incidences of diffuse hypertrophy of hepatocytes were increased in both sexes at 15 000 ppm. Centrilobular hypertrophy of hepatocytes was observed in all animals of the PB groups (Table 35).

In the recovery group, the incidences of diffuse hypertrophy of hepatocytes were not disturbed among males administered mandestrobin at 15 000 ppm or PB at 1000 ppm. Centrilobular hypertrophy of hepatocytes was observed in one female administered PB at 1000 ppm (Table 35). There was no evidence of necrosis or increased apoptosis in any of the groups at any time point. In addition, the incidences of brown pigment in bile duct were 4/10 for males and 1/10 for females at 15 000 ppm after the 7-day treatment and 2/10 for males at 15 000 ppm after the 14-day treatment. No pigment was observed in the recovery animals. The morphology of hepatocytes was assessed in controls and the highest-dose group using electron microscopy. After the 7-day treatment, proliferation of smooth endoplasmic reticulum (SER) was observed in the hepatocytes from both sexes in the 15 000 ppm mandestrobin-treated groups (Table 35). Peroxisome size and number were not changed in the treatment groups. Enlarged lipid droplets in the hepatocytes were observed in one female administered mandestrobin at 15 000 ppm.

After 7 days of treatment, statistically significant increases in the BrdU labelling indices were noted in animals at 7000 ppm and above and in the PB group. After 14 days of treatment, statistically significant increases were still observed in males administered 15 000 ppm and in females administered PB, but were attenuated compared with the 7-day treatment (Table 35).

After 7 days of treatment, statistically significant increases in CYP2B activity were observed in both sexes at 2000 ppm and above and in the PB group. In the recovery group, a statistically significant increase in CYP2B activity was still observed relative to controls in both sexes of the PB group; however, it was clear that some recovery had taken place, with a decrease in activity compared with the peak at 7 days of treatment (Table 35).

After 7 days of treatment, a statistically significant increase in CYP4A activity was observed in both sexes of the PB group; however, no statistically significant alterations were observed in the mandestrobin groups. In the recovery group, a statistically significant increase in CYP4A activity was observed in males that had been administered 15 000 ppm, and a statistically significant decrease in CYP4A activity was observed in females that had been administered 15 000 ppm or PB (Table 35). However, these alterations are marginal and likely to be due to individual variation. Therefore, they are considered not to be of toxicological relevance.

After 7 days of treatment, statistically significant increases in T_4-UGT activity were observed in males administered mandestrobin at 400 ppm and higher (except for 2000 ppm) and females administered 15 000 ppm. (Although the 2000 ppm group did not show statistical significance, the value was deemed to represent an increase over the controls.) A statistically significant increase in T_4-UGT activity was observed in both sexes of the PB group. In the recovery group, a clear decrease in T_4-UGT activity was detected (Table 35).

All changes, including liver weight, enzyme induction and hepatocellular hypertrophy, showed partial recovery 7 days after cessation of mandestrobin treatment, indicating that the alterations of rat liver by mandestrobin are reversible, similar to the situation with PB.

Thyroid**:** After the 7-day treatment, statistically significant increases were observed in the absolute and relative thyroid weights in females at 7000 ppm and above. After the 14-day treatment, statistically significant increases were observed in the absolute and relative thyroid weights in both sexes at 15 000 ppm and the PB group (Table 35).

After the 7-day treatment, the incidences of diffuse follicular cell hypertrophy were statistically significantly increased in females at 7000 and 15 000 ppm mandestrobin. This finding was also observed in the PB group. After 14 days, the incidences of diffuse follicular cell hypertrophy were increased for males and females at 15 000 ppm. This finding was also observed in the PB group. After the 7-day recovery period, the incidences of diffuse follicular cell hypertrophy remained minimally elevated for males at 1000 ppm PB (Table 35).

After 7 days of treatment with mandestrobin, a decrease in the serum concentration of T_4 was observed in both sexes administered 15 000 ppm (statistically significant only in males), and a statistically significant increase in TSH was observed in females administered 15 000 ppm. Statistically significant decreases in the serum concentration of T_4 and an increase in TSH were observed in females administered PB (Table 35).

After 14 days of treatment, statistically significant decreases in the serum concentration of T_4 and an increase in the serum concentration of TSH were observed in both sexes of the 15 000 ppm mandestrobin and PB groups. Statistically significant decreases in the serum concentrations of T_3 were observed in females of the same groups (Table 35).

In the recovery group, statistically significant decreases in the serum concentration of T_4 were observed in males administered 15 000 ppm mandestrobin, and statistically significant increases in T_3 were observed in males administered PB (Table 35).

Overall, in females treated with mandestrobin for 7 days, a slight, dose-related decrease in T_4 concentration, a slight, dose-related increase in TSH and a dose-related increase in the incidence of diffuse follicular cell hypertrophy were observed in females, but not all were statistically significant. These effects were not seen in males after the 7-day treatment, but were observed in both sexes after the 14-day treatment. Upon cessation of treatment, T_4, TSH and thyroid follicular cell hypertrophy findings had partially reversed by 7 days after the last dose. The serum concentration reductions of T_4 and TSH and changes in thyroid morphology were associated with the induction of hepatic T_4-UGT, suggesting that mandestrobin affected rat thyroid due to perturbation of the hypothalamus–pituitary–thyroid axis by induction of hepatic UGT activity, which depletes T_4. These findings were consistent with those caused by PB.

In conclusion, mandestrobin administration in the rat resulted in increased liver weight, diffuse hepatocellular hypertrophy with proliferation of SER, increased CYP2B activity and a transient increase in the rate of replicative DNA synthesis, with a dose–response relationship and reversibility. Mandestrobin increased T_4-UGT activity and secondarily perturbed the hypothalamus–pituitary–thyroid hormone axis. These effects are similar to those of PB, a known CAR activator. Therefore, it is reasonable to conclude that mandestrobin is a hepatic enzyme inducer via CAR activation in the rat, in a manner similar to PB (Asano, 2012e).

Short-term study in mice

In a second mechanistic non-GLP-compliant study, mandestrobin (purity 93.4%) was administered to 10 male CD-1 mice at a dietary concentration of 0 (control) or 7000 ppm (equal to 814 mg/kg bw per day) for 7 days. Hepatic CYP2B activity (determined by 7-pentoxyresorufin *O*-depentylase [PROD] activity) was then examined. In addition, replicative DNA synthesis of hepatocytes (determined by BrdU labelling index) was examined, because CAR activators often increase the BrdU labelling index of hepatocytes during the early phase of treatment.

No mortality or clinical signs were noted. No changes in body weight or feed consumption were recorded that could confound the evaluation of hepatic CYP2B induction or BrdU labelling.

Absolute liver weight tended to increase, but not statistically significantly (1.06-fold relative to control value), whereas relative liver weight was statistically significantly increased by mandestrobin treatment (1.07-fold relative to control value) (Table 36). No clear alterations in gross pathology of the liver were observed.

Table 36. Key findings of the mechanistic study in mice

Finding	0 ppm	7 000 ppm
Absolute liver weight (% of control value)	100	106
Relative liver weight (% of control value)	100	107*
S9 protein content of liver (% of control value)	100	115*
Hepatic CYP2B activity (% of control value)	100	171**
BrdU labelling index of hepatocytes (% of control value)	100	108
Liver gross pathology (incidence with no remarkable findings)	10/10	10/10
Liver histopathology (incidence)		
Eosinophilic change/hepatocyte hypertrophy	0/10	3/10
Focal hepatocyte brownish pigment	0/10	2/10
Focal mononuclear cell infiltration	2/10	4/10
Focal necrosis	2/10	3/10

BrdU: 5-bromo-2′-deoxyuridine; CYP2B: cytochrome P450; ppm: parts per million; S9: 9000 × *g* supernatant fraction of rat liver homogenate; *: $P < 0.05$; **: $P < 0.01$
Source: Yamada (2012b)

Slight eosinophilic change/hypertrophy of hepatocytes was observed in 3/10 animals treated with mandestrobin at 7000 ppm. Hepatic CYP2B activity was also statistically significantly increased by mandestrobin treatment (1.71-fold relative to control value). No clear alterations were observed in replicative DNA synthesis of hepatocytes.

Overall, treatment of the mouse with mandestrobin resulted in increased liver weight with slight eosinophilic change/hypertrophy of hepatocytes and increased CYP2B activity. Therefore, it is concluded that mandestrobin is a weak hepatic enzyme inducer via at least CAR activation in the mouse. In contrast to PB, however, mandestrobin did not enhance replicative DNA synthesis of hepatocytes during the early phase of treatment (Yamada, 2012b).

In conclusion, the effects observed in the two in vivo studies were compared with CAR-mediated induction of liver enzymes and subsequent perturbations of thyroid hormones. The proposed phenobarbital-like mode of action for mandestrobin tested in the in vivo rat mechanistic study is considered to satisfy the Bradford Hill criteria of dose and temporal concordance, biological plausibility, coherence, strength, consistency and specificity for thyroid follicular cell hypertrophy. Although the phenobarbital-like mode of action could theoretically operate in humans, the markedly different susceptibility for thyroid abnormality renders it non-relevant for humans. Furthermore, no increased tumour rates were observed up to the highest doses tested in the long-term toxicity and carcinogenicity studies.

In vitro hormonal activity studies

Mandestrobin (purity 93.4%) was evaluated for its effects on androgen and estrogen production in a non-GLP-compliant steroidogenesis assay. H295R cells cultured in 24-well plates were incubated with mandestrobin in triplicate for 48 hours. For the evaluation of testosterone and estradiol production, four (runs 1, 2, 3 and 4) and three (runs 1, 2 and 3) independent experiments were performed, respectively. DMSO was used as the vehicle at a final concentration of 0.1%. Positive controls (forskolin, a known inducer, and prochloraz, a known inhibitor) were evaluated concurrently with each run to confirm the changes in testosterone and estradiol levels in the assay. Ten micromoles of forskolin per litre increased the levels of testosterone and estradiol by more than 1.5-fold and 7.5-fold,

respectively, compared with the solvent control. One micromole of prochloraz per litre decreased the levels of both testosterone and estradiol by less than 0.5-fold compared with the solvent control. These results indicated that present assay systems were validated by the positive control experiments. Testosterone and estradiol levels were measured using enzyme-linked immunosorbent assay systems. In run 1, mandestrobin showed cytotoxicity to H295R cells at 100 µmol/L. Therefore, the maximum mandestrobin concentration was set to 30 µmol/L in subsequent runs. For testosterone, through runs 1–4, a significant change in testosterone production relative to the controls was seen only at 30 µmol/L in run 2, but not in runs 3 and 4. For estradiol, no significant change was observed in runs 1 and 2. In run 3, the production of estradiol was statistically significantly increased at 10 µmol/L, but not at either 3 or 30 µmol/L. Consequently, a consistent, dose-related increase in estradiol was not observed.

It is concluded that mandestrobin does not influence testosterone or estradiol production in H295R cells at concentrations up to 30 µmol/L (Kubo, 2012).

In a second in vitro study, mammalian cell–based luciferase reporter gene assays were performed for detecting estrogenic, anti-estrogenic, androgenic and anti-androgenic effects of mandestrobin (purity 93.4%) and its metabolites: 5-COOH-S-2200 (purity 99.7%), 4-OH-S-2200 (purity 99.9%), 5-CH_2OH-S-2200 (purity 99.9%) and 5-CA-S-2200-NHM (purity 94.9%) on human estrogen receptor alpha (hERα)– and human androgen receptor (hAR)–mediated mechanisms. In order to determine the appropriate concentration ranges of substances to be tested, the test substances were evaluated by cytotoxicity assays (100 pmol/L to 100 µmol/L), the concentrations at or above the cytotoxic concentration being excluded from the evaluation. Based on the results of the cytotoxicity assays, the maximum concentrations of mandestrobin, 5-COOH-S-2200, 4-OH-S-2200, 5-CH2OH-S-2200 and 5-CA-S-2200-NHM were 1, 100, 10, 10 and 100 µmol/L in the hERα assays, respectively, and 10, 100, 10, 100 and 100 µmol/L in the hAR assays, respectively.

In the reporter gene assay for hERα, positive controls for hERα (agonist: 17β-estradiol; antagonist: 4-hydroxytamoxifen) showed marked agonistic and antagonistic activities, respectively. However, mandestrobin and its metabolites showed neither agonistic nor antagonistic activity in the assays. In the reporter gene assay for hAR, positive controls for hAR (agonist: dihydrotestosterone; antagonist: hydroxyflutamide) showed marked agonistic and antagonistic activities, respectively. However, neither agonistic nor antagonistic activity was observed for mandestrobin and its metabolites.

Overall, mandestrobin and its metabolites did not show agonistic or antagonistic effects on hERα or hAR, indicating that mandestrobin and its metabolites did not have any impact on hERα- and hAR-mediated transcriptional activities in vitro (Suzuki, 2012).

It is concluded that mandestrobin had no influence on testosterone or estradiol production and that mandestrobin and its metabolites 5-COOH-S-2200, 4-OH-S-2200, 5-CH_2OH-S-2200 and 5-CA-S-2200-NHM did not have any effects on estrogen- or androgen-mediated reporter gene activity.

(d) Toxicity studies on metabolites and impurities

An assessment of the representativeness (i.e. impurity profile) of the batches used in the toxicity studies was performed. According to the impurity profile, the batch used in the toxicity studies represents a worst-case scenario, as it contains impurities that are not present in the commercial specification. Only four impurities are present in both the batch used in the toxicity studies and the commercial specification. All of them are at comparable levels; therefore, their toxicological properties can be considered to be covered by the toxicity studies.

2-COOH-S-2200

2-COOH-S-2200 (2-({2-[(1*RS*)-1-methoxy-2-(methylamino)-2-oxoethyl]benzyl}oxy)-4-methylbenzoic acid) is a rat, hen and goat metabolite and is also present in groundwater. Results of the toxicity studies with this metabolite are summarized in Table 37.

Table 37. Results of toxicity studies with the metabolite 2-COOH-S-2200

Study	Species	Purity (%)	Results	Reference
Acute oral toxicity study	Rat (Slc:WistarHannover/Rcc)	99.0	LD_{50} > 2 000 mg/kg bw	Asano (2012a)
Ames test	*Salmonella typhimurium*, *Escherichia coli*	99.0	Not mutagenic	Kitamoto (2012a)
In vitro chromosomal aberration assay	Chinese hamster lung (CHL/IU) cells	99.0	Weak potential to induce chromosomal aberrations only at highly toxic dose of 2 200 µg/mL	Kitamoto (2012b)
In vitro mammalian gene mutation assay	Chinese hamster V79 cells	99.0	Not mutagenic	Wollny (2011a)
In vivo micronucleus assay	Mouse	99.0	Not genotoxic	Tanaka (2012)

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

In an acute oral toxicity study, a single oral dose of 2-COOH-S-2200 (purity 99.0%), as a suspension in 0.5% aqueous methylcellulose solution, of 2000 mg/kg bw was administered by gavage to six female rats. Clinical and mortality observations were made at 10 and 30 minutes and 1, 2 and 4 hours after treatment and once daily for 14 days thereafter. Body weights were determined on the day of dosing (day 0), day 7 and day 14. On day 14, animals were euthanized, and all organs and tissues were examined macroscopically. No mortality or clinical signs were noted during the study. Body weights were not affected, and no gross abnormalities at necropsy were found. Therefore, the oral LD_{50} of 2-COOH-S-2200 in female rats was greater than 2000 mg/kg bw (Asano, 2012a).

In a reverse gene mutation assay in bacteria (Ames test), 2-COOH-S-2200 (purity 99.0%) was tested on four strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) and one strain of *Escherichia coli* (WP2*uvr*A) in the presence and absence of S9 mix. As neither cytotoxicity nor precipitation was noted in a dose range–finding assay, doses ranged from 156 to 5000 µg/plate with and without S9 mix in the main assays. There was no statistically significant dose-related increase in the number of revertant colonies with or without S9 mix. Positive controls showed the appropriate increase in the number of revertant colonies. 2-COOH-S-2200 was not mutagenic under these test conditions (Kitamoto, 2012a).

The clastogenic potential of 2-COOH-S-2200 (purity 99.0%) was examined in an in vitro chromosomal aberration test using Chinese hamster lung (CHL/IU) cells with and without metabolic activation (S9 mix). In a preliminary cytotoxicity assay, marked growth inhibitions were noted in both the presence and absence of S9 mix at 3500 µg/mL after 6 and 24 hours of exposure. Precipitation was noted after 6 hours of treatment at the concentration of 3500 µg/mL with and without S9 mix. In the chromosomal aberration assay, doses ranged from 550 to 3000 µg/mL without S9 mix and from 700 to 2800 µg/mL with S9 mix. When the CHL/IU cells were exposed for 6 hours to 2-COOH-S-2200 with and without S9 mix, no increase in chromosomally aberrant cells (structural or numerical) was observed. When the CHL/IU cells were exposed for 24 hours without S9 mix, a small increase in structural aberrant cells (but not numerical) was observed only at the high concentration of 2200 µg/mL (equivalent to 6.4 mmol/L). All negative and positive control cultures gave values of chromosomal aberrations within the expected range. 2-COOH-S-2200 has a weak potential to induce chromosomal

aberrations only at the high concentration of 2200 µg/mL (equivalent to 6.4 mmol/L) in CHL/IU cells under the test conditions (Kitamoto, 2012b). It should also be noted that this high concentration exceeds the current Organisation for Economic Co-operation and Development (OECD)–recommended top concentration of 2000 µg/mL (2 mg/mL) (OECD, 2014).

If the relative increase in cell count (RICC) is used to estimate cytotoxicity, as is highly recommended in the latest OECD test guideline, the clastogenic effects occur only at a concentration at which significant cytotoxicity is observed and are considered an artefact of cytotoxicity, as outlined in Kirkland (1998). The result is a classic example of a "false positive", as outlined in Kirkland (1998), for what he terms "high toxicity [in vitro] clastogens". Relative cell count (RCC) was used in the study with 2-COOH-S-2200 as the indicator for cytotoxicity. The RCC index is now well known to underestimate cytotoxicity and often leads to overdosing. The use of the RICC index is therefore more appropriate, as discussed by Galloway et al. (2011). As shown in Table 38, the RICC for 2200 µg/mL (where the response was seen) was just 21.2% of the control value, which is considerably in excess of the top-dose toxicity required by OECD Test Guideline 473. 2-COOH-S-2200 is therefore a "high toxicity [in vitro] clastogen" (i.e. the positive result occurred only at >60% toxicity). The other in vitro assays were negative, which is also typical of this type of false-positive result discussed by Kirkland (1998). There was also no response at lower concentrations. The response seen at 2200 µg/mL (with precipitate: maximum soluble concentrate estimated at approximately 1600 µg/mL) was practically as intense as for the positive control, but reduced to zero at 1100 µg/mL. This would be extremely unusual for a classic non-threshold genotoxic carcinogen. It is considered, therefore, that the result in this assay is an artefact of the high-dose "high toxicity" and is of little biological relevance. In addition, no in vivo clastogenic activity was identified in the mouse micronucleus assay on 2-COOH-S-2200, as described below.

Table 38. Growth rates using RCC and RICC in the in vitro chromosomal aberration test with 2-COOH-S-2200 (24-hour exposure, without S9 mix)

Group	Dose (µg/mL)	Cell number	RCC[a] (%)	RICC[b] (%)
At the start of treatment[c]	–	3.35×10^5	–	–
Control	0	9.54×10^5	100	100
2-COOH-S-2200	550	8.17×10^5	85.7	77.9
	1 100	7.58×10^5	79.5	68.3
	2 200	4.66×10^5	48.8	21.2

RCC: relative cell count; RICC: relative increase in cell count

[a] RCC = (Number of cells in treated cultures / number of cells in control cultures) × 100.

[b] RICC = (Increase in number of cells in treated cultures [final − starting] / increase in number of cells in control cultures [final − starting]) × 100.

[c] The cell number is available in raw data.

Source: Adapted from Kitamoto (2012b)

Metabolite 2-COOH-S-2200 (purity 99.0%) was evaluated in a gene mutation assay at the *HPRT* locus in V79 cells of the Chinese hamster. The study was performed in two independent experiments using identical experimental procedures. In the first experiment, the treatment period was 4 hours with and without metabolic activation. The second experiment was performed with a treatment time of 4 hours with metabolic activation and 24 hours without metabolic activation. As no relevant cytotoxic effect occurred up to the 3500 µg/mL concentration with or without S9 mix, the main assay was conducted at concentrations ranging from 109.4 to 3500 µg/mL with and without S9 mix. Again, the high concentration substantially exceeds the current OECD-recommended top concentration of 2000 µg/mL (2 mg/mL) (OECD, 2014).

Precipitation of the test item at the end of treatment was observed by the unaided eye at 3500 µg/mL in both main experiments with and without metabolic activation. Negative and positive controls

were within the expected historical range. Neither a relevant nor reproducible increase in mutant colony cells (≥3 times control values) was observed in the main experiments up to the maximum concentration with and without metabolic activation. In the 24-hour experiment in the absence of S9, a modest 3-fold increase in mutation frequency was seen in one cell culture, but was not seen in the second culture from the same experiment. No increases were seen in two separate experiments that employed 4-hour exposures with or without S9. Overall, it was concluded that 2-COOH-S-2200 did not induce gene mutations at the *HPRT* locus in V79 cells under the experimental conditions and is considered non-mutagenic in this assay (Wollny, 2011a).

In an in vivo bone marrow micronucleus test, 2-COOH-S-2200 (purity 99.0%) was administered by gavage to groups of six male CD-1 mice at a dose of 0, 500, 1000 or 2000 mg/kg bw in the 24-hour treatment groups and to six male CD-1 mice at 2000 mg/kg bw in the 48-hour treatment group. Bone marrow smears were prepared 24 and 48 hours (five animals per group) after dosing and analysed for the presence of micronuclei by microscopy. There were an additional mitomycin C positive control group (smears prepared at 24 hours) and two solvent negative control groups (smears prepared at 24 and 48 hours). The incidence of micronuclei in 2000 polychromatic erythrocytes (PCEs) and the incidence of PCEs in 500 erythrocytes (including normochromatic erythrocytes [NCEs] and PCEs) were recorded for each animal.

There were no signs of toxicity as a result of the administration of 2-COOH-S-2200 in treated animals. The frequencies of micronucleated PCEs (MNPCEs) after administration of 2-COOH-S-2200 were 0.31%, 0.36% and 0.46% at 500, 1000 and 2000 mg/kg bw, respectively. This apparent increase was not statistically significant. From individual animal data, only two animals had the %MNPCE exceeding the upper limit of the negative historical controls (0.56%): one at 500 mg/kg bw (0.7%) and one at 2000 mg/kg bw (0.75%). In the 1000 mg/kg bw group, all animals had the %MNPCE falling within the negative historical controls. There was no change in the PCE/(PCE+NCE) ratio.

The positive control induced marked statistically significant increases in micronuclei. 2-COOH-S-2200 showed no potential to induce micronuclei in mouse bone marrow cells (Tanaka, 2012).

The following evidence was presented that the target organ is reached at sufficient levels: in the rat ADME study performed with the parent molecule (Kendrick & Farrell, 2012), mandestrobin-derived radioactivity in bone marrow peaked at 234 ng equivalents/g (male) and 217 ng equivalents/g (female) at 0.5 hour post-dosing (T_{max}) in the 5 mg/kg bw dose group and 7455 ng equivalents/g (male) and 3828 ng equivalents/g (female) at 8 hours post-dosing (T_{max}) in the 1000 mg/kg bw dose group. It was still measureable in the bone marrow of male rats at 36 hours after treatment at the high dose, indicating reasonable exposure over the whole 24-hour period. These results indicated that mandestrobin-derived radioactivity reached the bone marrow at significant and sufficient concentration at the low and high doses, respectively; therefore, the negative outcome of the study can be considered to be reliable.

There is no estimated change to the physicochemical properties of the parent compound (octanol–water partition coefficient, molecular weight, water solubility) by the addition of the carboxyl group that would significantly reduce exposure of the bone marrow to 2-COOH-S-2200 when compared with the parent (Table 39). The ADME study with the parent compound suggests that 2-COOH-S-2200 is formed in the gut and liver and absorbed and excreted in urine, ostensibly unchanged, indicating that systemic exposure is not obstructed by the liver. This, coupled with the increased water solubility but unchanged lipophilicity of the metabolite, would potentially increase exposure of the bone marrow: higher concentrations compared with parent compound could be carried in the plasma.

As such, there is no reason to expect that exposure of the bone marrow to 2-COOH-S-2200 would be less than that of the parent compound or at least would not be reduced to an extent that would render the in vivo micronucleus study with 2-COOH-S-2200 invalid.

Table 39. Comparison of the physicochemical properties of 2-COOH-S-2200 with those of mandestrobin

Properties	Mandestrobin	2-COOH-S-2200
Molecular weight (g/mol)	313.39	343.38
Aqueous solubility at 20 ºC (mg/L)	15.8	607[a]
Log octanol–water partition coefficient	3.51/3.44	2.53[a,b]

[a] Estimated at 25 °C using EPISUITE.
[b] New estimate not in dossier, effectively the un-ionized form.

5-COOH-S-2200

5-COOH-S-2200 (3-({2-[(1*RS*)-1-methoxy-2-(methylamino)-2-oxoethyl]benzyl}oxy)-4-methylbenzoic acid) is a rat, hen and goat metabolite. It is also present in soil, groundwater, surface water and crops (including rotational). Results of toxicity studies with this metabolite are summarized in Table 40.

Table 40. Results of toxicity studies with the metabolite 5-COOH-S-2200

Study	Species	Purity (%)	Results	Reference
Acute oral toxicity study	Rat (Slc:WistarHannover/Rcc)	97.6	300 mg/kg bw < LD_{50} < 2 000 mg/kg bw	Asano (2012b)
Ames test	*Salmonella typhimurium*, *Escherichia coli*	97.6	Not mutagenic	Kitamoto (2012c)
In vitro chromosomal aberration assay	Chinese hamster lung (CHL/IU) cells	97.6	Not clastogenic	Kitamoto (2012d)
In vitro mammalian gene mutation assay	Chinese hamster V79 cells	97.6	Not mutagenic	Wollny (2011b)

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

In an acute oral toxicity study in rats, 5-COOH-S-2200 (purity 97.6%) was administered, as a suspension in 0.5% aqueous methylcellulose solution, by gavage to three or six female rats in a single oral dose of 300 or 2000 mg/kg bw. Clinical and mortality observations were made at 10 and 30 minutes and 1, 2 and 4 hours after treatment and once daily for 14 days thereafter. Body weights were determined on the day of dosing (day 0), day 7 and day 14. On day 14, animals were euthanized, and all organs and tissues were examined macroscopically. All three animals treated at 2000 mg/kg bw died, and clinical signs included lateral position and bradypnoea. All six animals treated at 300 mg/kg bw survived the observation period with no clinical signs. All animals gained weight. Retention of foamy fluid in the trachea was observed in two animals at 2000 mg/kg bw, and "uncollapse" and pale in all lobes of the lung were observed in one animal at 300 mg/kg bw during the gross pathological examination. The oral LD_{50} of 5-COOH-S-2200 in rats was therefore greater than 300 mg/kg bw but less than 2000 mg/kg bw (Asano, 2012b).

In a reverse gene mutation assay in bacteria (Ames test), 5-COOH-S-2200 (purity 97.6%) was tested on four strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) and one strain of *Escherichia coli* (WP2*uvr*A) in the presence and absence of S9. As neither cytotoxicity nor precipitation was noted up to 5000 µg/plate in a dose range–finding assay, concentrations ranged from 156 to 5000 µg/plate with and without S9 mix. There was no statistically significant dose-related increase in the number of revertant colonies, with or without S9 mix. Positive controls showed the

appropriate increase in the number of revertant colonies. 5-COOH-S-2200 was not mutagenic under these test conditions (Kitamoto, 2012c).

The clastogenic potential of 5-COOH-S-2200 (purity 97.6%) was examined in an in vitro chromosomal aberration test using Chinese hamster lung (CHL/IU) cells, with and without metabolic activation (S9 mix). In a preliminary cytotoxicity assay, relevant cytotoxicity was noted after 6 hours of treatment at 1750 µg/mL with S9 mix and after 24 hours of treatment at and above 875 µg/mL without S9 mix. Precipitation was noted in the medium at the end of treatment at 3500 µg/mL. Main assays were conducted with concentrations ranging from 219 to 3500 µg/mL without S9 mix and from 438 to 1750 µg/mL with S9 mix. The 3500 µg/mL concentration substantially exceeds the current OECD-recommended top concentration of 2000 µg/mL (2 mg/mL) (OECD, 2014). When the CHL/IU cells were exposed for 6 or 24 hours to 5-COOH-S-2200 with and without S9 mix, no increase in chromosomally aberrant cells (structural or numerical) was observed. All negative and positive control cultures gave values of chromosomal aberrations within the expected range. 5-COOH-S-2200 has no potential to induce chromosomal aberrations in CHL/IU cells under the test conditions (Kitamoto, 2012d).

Metabolite 5-COOH-S-2200 (purity 97.6%) was evaluated in a gene mutation assay at the *HPRT* locus in V79 cells of the Chinese hamster, with and without metabolic activation (S9 mix). The study was performed in two independent experiments using identical experimental procedures. In the first experiment, the treatment period was 4 hours with and without metabolic activation. The second experiment was performed with a treatment time of 4 hours with metabolic activation and 24 hours without metabolic activation. As no relevant cytotoxic effect occurred up to the maximum concentration in the preliminary cytotoxicity assay, the main assay was conducted at concentrations ranging from 109.4 to 3500 µg/mL with and without S9 mix. Precipitation of the test item at the end of treatment was observed by the unaided eye at 3500 µg/mL in both main experiments with and without metabolic activation. In experiment I, turbidity of the medium was noted at 875 and 1750 µg/mL with metabolic activation.

No relevant cytotoxic effect occurred up to the maximum concentration with metabolic activation. A moderate cytotoxic effect, indicated by a relative cloning efficiency I below 50%, was observed in the first experiment without metabolic activation at the highest soluble concentration of 1750 µg/mL. The relative cloning efficiency I was back above 50% at the next higher precipitating concentration of 3500 µg/mL. Following 24 hours of treatment in the second experiment without metabolic activation, relevant cytotoxic effects were observed at 1750 and 3500 µg/mL. Again, the 3500 µg/mL concentration exceeds the current OECD-recommended top concentration of 2000 µg/mL (OECD, 2014). No substantial or reproducible dose-dependent increase of the mutant frequencies exceeding the threshold of 3 times over the solvent control was observed at any concentration either with or without S9 mix or after 4- or 24-hour treatment. Appropriate reference mutagens were used as positive controls and showed a distinct increase in induced mutant colonies. 5-COOH-S-2200 did not induce gene mutations at the *HPRT* locus in V79 cells under the experimental conditions and is considered non-mutagenic in this assay (Wollny, 2011b).

2-CH_2OH-S-2200

2-CH_2OH-S-2200 ((2*RS*)-2-[2-(5-hydroxymethyl-2-methylphenoxymethyl)phenyl]-2-methoxy-*N*-methylacetamide) is a rat, hen and goat metabolite and is also present in crops (including rotational). Results of toxicity studies with this metabolite are summarized in Table 41.

In an acute oral toxicity study in rats, 2-CH_2OH-S-2200 (purity 100%) was administered, as a suspension in 0.5% aqueous methylcellulose solution, by gavage to two groups of three female rats in a single oral dose of 2000 mg/kg bw. Clinical and mortality observations were made at 10 and 30 minutes and 1, 2 and 4 hours after treatment and once daily for 14 days thereafter. Body weights were determined on the day of dosing (day 0), day 7 and day 14. On day 14, animals were euthanized, and all organs and tissues were examined macroscopically. All animals survived the observation period with

no clinical signs. All animals gained weight, and no pathological findings were noted during necropsy. Therefore, the oral LD_{50} of 2-CH_2OH-S-2200 in rats was greater than 2000 mg/kg bw (Asano, 2012c).

Table 41. Results of toxicity studies with the metabolite 2-CH_2OH-S-2200

Study	Species	Purity (%)	Results	Reference
Acute oral toxicity study	Rat (Slc:WistarHannover/Rcc)	100	LD_{50} > 2 000 mg/kg bw	Asano (2012c)
Ames test	*Salmonella typhimurium*, *Escherichia coli*	100	Not mutagenic	Kitamoto (2012e)

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

In a reverse gene mutation assay in bacteria (Ames test), 2-CH_2OH-S-2200 (purity 100%) was tested on four strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) and one strain of *Escherichia coli* (WP2*uvr*A) in the presence and absence of S9 mix. In a dose range–finding assay, precipitation was observed at and above a concentration of 1250 µg/plate. No cytotoxicity was observed. In the main assay, concentrations ranged from 156 to 5000 µg/plate with and without S9 mix. There was no statistically significant, dose-related increase in the number of revertant colonies, with or without S9 mix. Positive controls showed the appropriate increase in the number of revertant colonies. 2-CH_2OH-S-2200 was not mutagenic under these test conditions (Kitamoto, 2012e).

4-OH-S-2200

4-OH-S-2200 ((2*RS*)-2-[2-(4-hydroxy-2,5-dimethylphenoxymethyl)phenyl]-2-methoxy-*N*-methylacetamide) is a rat, hen and goat metabolite and is also present in crops (including rotational). Results of toxicity studies with this metabolite are summarized in Table 42.

Table 42. Results of toxicity studies with the metabolite 4-OH-S-2200

Study	Species	Purity (%)	Results	Reference
Acute oral toxicity study	Rat (Slc:WistarHannover/Rcc)	99.9	LD_{50} > 2 000 mg/kg bw	Asano (2012d)
Ames test	*Salmonella typhimurium*, *Escherichia coli*	99.9	Not mutagenic	Kitamoto (2012f)

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

In an acute oral toxicity study in rats, 4-OH-S-2200 (purity 99.9%) was administered, as a suspension in 0.5% aqueous methylcellulose solution, by gavage to two groups of three female rats in a single oral dose of 2000 mg/kg bw. Clinical and mortality observations were made at 10 and 30 minutes and 1, 2 and 4 hours after treatment and once daily for 14 days thereafter. Body weights were determined on the day of dosing (day 0), day 7 and day 14. On day 14, animals were euthanized, and all organs and tissues were examined macroscopically. All animals survived the observation period with no clinical signs. All animals gained weight, and no pathological findings were noted during necropsy. Therefore, the oral LD_{50} of 4-OH-S-2200 in rats was greater than 2000 mg/kg bw (Asano, 2012d).

In a reverse gene mutation assay in bacteria (Ames test), 4-OH-S-2200 (purity 99.9%) was tested on four strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) and one strain of *Escherichia coli* (WP2*uvr*A) in the presence and absence of S9. As no cytotoxicity was noted in the dose range–finding assay, concentrations ranged from 156 to 5000 µg/plate with and without S9 mix.

Precipitation was observed at and above 1250 µg/plate with and without S9 mix in both the dose range–finding and main assays. There was no statistically significant, dose-related increase in the number of revertant colonies, with or without S9 mix. Positive controls showed the appropriate increase in the number of revertant colonies. 4-OH-S-2200 was not mutagenic under these test conditions (Kitamoto, 2012f).

De-Xy-S-2200

De-Xy-S-2200 ((2*RS*)-2-(2-hydroxymethylphenyl)-2-methoxy-*N*-methylacetamide) is a rat, hen and goat metabolite and is also present in crops (including rotational). Results of toxicity studies with this metabolite are summarized in Table 43.

Table 43. Results of toxicity studies with the metabolite De-Xy-S-2200

Study	Species	Purity (%)	Results	Reference
Acute oral toxicity study	Rat (Slc:WistarHannover/Rcc)	100	LD_{50} > 2 000 mg/kg bw	Asano (2011)
Ames test	*Salmonella typhimurium*, *Escherichia coli*	100	Not mutagenic	Kitamoto (2011)

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

In an acute oral toxicity study in rats, De-Xy-S-2200 (purity 100%) was administered, as a suspension in 0.5% aqueous methylcellulose solution, by gavage to two groups of three female rats in a single oral dose of 2000 mg/kg bw. Clinical and mortality observations were made at 10 and 30 minutes and 1, 2 and 4 hours after treatment and once daily for 14 days thereafter. Body weights were determined on the day of dosing (day 0), day 7 and day 14. On day 14, animals were euthanized, and all organs and tissues were examined macroscopically. All animals survived the observation period. No mortality was observed. All animals showed a decrease of spontaneous activity from 30 minutes after administration. This finding disappeared on day 3. In four animals, ataxic gait from 1 to 4 hours after administration and prone position from 2 to 4 hours after administration were observed. In addition, abdominal stains and lateral position were observed in three and two animals, respectively. Both findings were noted from 4 hours after administration and disappeared on day 2. Furthermore, one animal showed lacrimation at 4 hours after administration and irregular respiration on day 2. All animals gained weight, and no pathological findings were noted during necropsy. Therefore, the oral LD_{50} of De-Xy-S-2200 in rats was greater than 2000 mg/kg bw (Asano, 2011).

In a reverse gene mutation assay in bacteria (Ames test), De-Xy-S-2200 (purity 100%) was tested on four strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) and one strain of *Escherichia coli* (WP2*uvr*A) in the presence and absence of S9. As no cytotoxicity and no precipitation were observed in a dose range–finding assay, concentrations ranged from 156 to 5000 µg/plate with and without S9 mix. There was no statistically significant, dose-related increase in the number of revertant colonies, with or without S9 mix. Positive controls showed the appropriate increase in the number of revertant colonies. De-Xy-S-2200 was not mutagenic under these test conditions (Kitamoto, 2011).

(e) Microbiological effects

The fungicide mandestrobin was evaluated at the 2018 JMPR to determine its impact on microbiota in the gastrointestinal tract. As no data were submitted by the sponsors, a literature search was performed using a number of search engines. These included BioOne (http://www.bioone.org/), Google (https://www.google.com/), Google Scholar (http://scholar.google.com/), PubMed

(http://www.ncbi.nlm.nih.gov/pubmed), ScienceDirect (http://www.sciencedirect.com/) and Web of Science (https://apps.webofknowledge.com).

The search strategy used the input keywords of the fungicide chemical name (mandestrobin), chemical structure, antimicrobial mode of action, antimicrobial spectrum of activity, antimicrobial resistance, resistance mechanisms and genetics, microbiome, microbiota, gut microbiota, gut microbiome, gastrointestinal microbiota, gastrointestinal microbiome, etc., as well as the Boolean operators AND, OR and NOT.

The extensive search and review of the scientific literature did not find any reports on the effects of mandestrobin on the intestinal microbiome to include in the toxicological risk assessment.

3. Observations in humans

A formal statement from the manufacturer noted that members of staff involved in the synthesis and development of mandestrobin are routinely monitored and that no indication of mandestrobin-related ill-health have been detected by, or reported to, medical staff (Nishioka, 2012).

In a recent report (Nishimoto, 2017), the health of 27 factory workers involved in the manufacturing of mandestrobin technical material was monitored for 2 years in accordance with Japanese law. At least once a year, every employee underwent a medical examination, which included body measurements, eyesight and audibility, blood pressure measurement, haematological examination, liver function test, blood lipid examination, blood sugar and haemoglobin A1c, urine analysis and renal function test. Since the production of mandestrobin started, there have been no findings to suggest adverse effects caused by mandestrobin. This is consistent with the fact that there have been no claims or reports of health problems, nor any incident of intoxication related to the manufacturing/packing operations.

Comments

Biochemical aspects

In metabolism studies in which rats were administered racemic [^{14}C]mandestrobin labelled at either the benzyl or phenoxy ring as a single dose of 5 or 1000 mg/kg bw, excretion was approximately 90% (>70% within 48 hours). The major routes of excretion were through the faeces (67–75%) and urine (16–22%). There were no significant sex-, dose- or radiolabel-related differences in either the rates or routes of excretion. In bile duct–cannulated rats, elimination of radioactivity after a single low dose of 5 mg/kg bw was rapid, with more than 95% of the dose eliminated within 24 hours. About 80% of the excreted radioactivity was eliminated via the bile, and the amount recovered in faeces of the cannulated rats accounted for less than 2% of the total radioactivity. Elimination via urine was comparable to that of intact animals. For both low and high doses, there were no significant sex differences in the pharmacokinetic parameters. Mean plasma elimination half-lives were approximately 20 and 27 hours at the low and high doses, respectively. C_{max} was observed within 3 and 10 hours post-dosing for the low and high doses, respectively. At the high dose, additional plasma concentration peaks were observed, which were particularly prominent during the first 12 hours post-dosing. The multiple peaks could possibly reflect enterohepatic recirculation, which is supported by the enhanced rapidity of elimination in bile duct–cannulated animals. At the high dose, systemic exposure (AUC) was sub-dose-proportional, indicating saturation of absorption processes following administration.

Following repeated administration of a low dose of radiolabelled compound at 5 mg/kg bw for up to 14 consecutive days, radioactivity was primarily excreted in the faeces (50–57%), with urine containing 13–15% of the administered dose. There were no significant sex differences. In a metabolism study conducted with the *R*- or *S*-isomer, after a single low dose of 5 mg/kg bw, the excretion of *R*-isomer (>95% at 72 hours) was more rapid than excretion of the *S*-isomer (>94% at 120 hours), likely due to enterohepatic recirculation. The majority of radioactivity was excreted in faeces (up to 73% *R*-

isomer; up to 77% *S*-isomer). Urinary excretion was higher in female rats (32% *R*-isomer; 25% *S*-isomer) than in male rats (22% *R*-isomer; 15% *S*-isomer).

Mandestrobin was widely distributed throughout the body. No major differences in distribution were observed, regardless of dose, sex or label position. The major tissue residues were seen in the gastrointestinal tract, liver, kidney, uterus and ovaries at 168 hours after dosing. A similar distribution of radioactivity into tissues was observed following repeated dosing. There was evidence of accumulation in tissues, but no persistence.

Mandestrobin was extensively metabolized to numerous metabolites. Unchanged parent was found in faeces at less than 0.2% and less than 6% of the administered dose after a single low and single high dose, respectively. The primary routes of metabolism were by oxidation and subsequent conjugation with glucuronic acid, demethylation with subsequent oxidation, or oxidation with subsequent demethylation. The metabolic profile was generally independent of sex, dose, label position or whether the animals were bile duct cannulated or not. Metabolite fractions in plasma, liver and kidney were identified. There was generally no discernible shift in metabolism at high or repeated doses except in the kidney. Metabolic profiles in the kidney exhibited differences in the type and number of metabolites observed depending on both sex and dosing regimen (single versus repeated dosing). In an investigation of the ADME of the isomers, 12 metabolites were identified and quantified; 5-CA-S-2200-NHM was the predominant metabolite of the *R*-isomer, and 4-OH-S-2200 followed by 5-COOH-S-2200 were the most abundant metabolites of the *S*-isomer. However, the same metabolites were identified for both the *R*-isomer and the *S*-isomer. 4-OH-S-2200-GlucA is likely to be subject to enterohepatic circulation. This metabolic pathway is likely to occur more commonly with the *S*-isomer, because the rate of radiolabel excretion was slower (delayed presumably by enterohepatic recirculation) than that for the *R*-isomer (Mikata, 2011; Kendrick & Farrell, 2012; Kendrick, Farrell & Murphy, 2012).

Toxicological data

In rats, the acute oral and dermal LD_{50} values were greater than 2000 mg/kg bw (Asano, 2010a,b), and the acute inhalation LC_{50} was greater than 4.96 mg/L (Deguchi, 2010). Mandestrobin was not irritating to the skin of rabbits (Ota, 2010a), but was mildly irritating to the eyes of rabbits (Ota, 2010b). Mandestrobin was not sensitizing to the skin of guinea-pigs (Ota, 2010c).

The short-term toxicity of mandestrobin was tested in mice, rats and dogs, and the long-term toxicity and carcinogenicity were tested in mice and rats. The target organ in all species was the liver, with the dog being the most sensitive species. In the rat, thyroid follicular hypertrophy was also observed, concurrently with or at higher doses than effects on the liver.

In a 90-day study in mice in which mandestrobin was administered at a dietary concentration of 0, 1750, 3500 or 7000 ppm (equal to 0, 204, 405 and 807 mg/kg bw per day for males and 0, 252, 529 and 1111 mg/kg bw per day for females, respectively), the NOAEL was 3500 ppm (equal to 529 mg/kg bw per day), based on reduced body weight gain in females at 7000 ppm (equal to 1111 mg/kg bw per day) (Beck, 2011a).

In a 90-day study in rats in which mandestrobin was administered at a dietary concentration of 0, 800, 4000, 10 000 or 20 000 ppm (equal to 0, 54, 283, 743 and 1545 mg/kg bw per day for males and 0, 62, 320, 788 and 1886 mg/kg bw per day for females, respectively), the NOAEL was 4000 ppm (equal to 283 mg/kg bw per day), based on increased liver weights accompanied by hepatocyte hypertrophy, increased total cholesterol and thyroid follicular hypertrophy in both sexes at 10 000 ppm (equal to 743 mg/kg bw per day) (Beck, 2011b).

In a 90-day study in dogs in which mandestrobin was administered at a dietary concentration of 0, 4000, 12 000 or 40 000 ppm (equal to 0, 90.9, 267.8 and 933.1 mg/kg bw per day for males and 0, 102.7, 304.4 and 820.4 mg/kg bw per day for females, respectively), the NOAEL was 4000 ppm (equal to 90.9 mg/kg bw per day), based on increased liver weights, histopathological changes in the liver and increased alkaline phosphatase activity in blood in both sexes at 12 000 ppm (equal to 267.8 mg/kg bw per day) (Beck, 2012a).

In a 1-year study in dogs in which mandestrobin was administered at a dietary concentration of 0, 200, 800, 4000 or 8000 ppm (equal to 0, 4.3, 19.2, 92.0 and 180.7 mg/kg bw per day for males and 0, 4.5, 20.4, 92.0 and 225.7 mg/kg bw per day for females, respectively), the NOAEL was 800 ppm (equal to 19.2 mg/kg bw per day), based on increased relative liver weights, hepatocyte hypertrophy and hepatocyte pigment and disturbances to clinical biochemistry parameters (increased ALP, GGT and triglycerides) in males at 4000 ppm (equal to 92.0 mg/kg bw per day) (Beck, 2012b).

In an 18-month toxicity and carcinogenicity study in mice, mandestrobin was administered at a dietary concentration of 0, 700, 2000 or 7000 ppm (equal to 0, 82.5, 238.8 and 823.9 mg/kg bw per day for males and 0, 99.2, 280.3 and 994.0 mg/kg bw per day for females, respectively). The NOAEL for toxicity was 7000 ppm (equal to 823.9 mg/kg bw per day), the highest dose tested. No treatment-related increases in tumour incidence were observed in this study (Beck, 2012c).

In a 2-year toxicity and carcinogenicity study in rats, mandestrobin was administered at a dietary concentration of 0, 400, 2000, 7000 or 15 000 ppm (equal to 0, 21.0, 105.1, 375.6 and 804.3 mg/kg bw per day for males and 0, 26.7, 135.2, 475.0 and 1016.2 mg/kg bw per day for females, respectively). The NOAEL for toxicity was 400 ppm (equal to 26.7 mg/kg bw per day), based on effects on body weight and liver (histopathological changes and increased liver weights) in females at 2000 ppm (equal to 135.2 mg/kg bw per day). The NOAEL for carcinogenicity was 7000 ppm (equal to 375.6 mg/kg bw per day), based on an equivocal increase in the incidence of tumours (ovarian sex cord stromal adenomas and testicular interstitial cell adenomas) at the highest dose (Beck, 2012d).

Mandestrobin was tested for genotoxicity in an adequate range of in vitro and in vivo assays. No evidence of genotoxicity was found (Kitamoto 2010a,b,c; Wollny, 2010).

The Meeting concluded that mandestrobin is unlikely to be genotoxic.

In view of the lack of genotoxicity, the absence of carcinogenicity in mice and the fact that only an equivocal increase in testicular interstitial cell and ovarian sex cord stromal adenomas was seen in rats at the highest dose tested, the Meeting concluded that mandestrobin is unlikely to pose a carcinogenic risk to humans from the diet.

In a two-generation reproductive toxicity study in which rats were given mandestrobin at a dietary concentration of 0, 1000, 3000 or 10 000 ppm (equal to 0, 47.77, 145.7 and 511.7 mg/kg bw per day for males and 0, 65.68, 200.3 and 672.0 mg/kg bw per day for females, respectively), the NOAEL for parental toxicity was 1000 ppm (equal to 47.77 mg/kg bw per day), based on increased liver weights, diffuse liver hypertrophy, brown pigment in the bile duct/periportal area in both sexes in the F_1 generation and periductular inflammatory cell infiltration in F_1 females at 3000 ppm (equal to 145.7 mg/kg bw per day). The NOAEL for offspring toxicity was 1000 ppm (equal to 47.77 mg/kg bw per day), based on decreased spleen weights in F_1 male and F_2 female pups. The NOAEL for reproductive toxicity was 10 000 ppm (equal to 511.7 mg/kg bw per day), the highest dose tested (Matsuura, 2012).

In a developmental toxicity study in rats given mandestrobin by gavage at 0, 100, 300 or 1000 mg/kg bw per day from GDs 6 to 19, the NOAEL for maternal toxicity was 300 mg/kg bw per day, based on reduced feed consumption at 1000 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 300 mg/kg bw per day, based on an increase in a limited number of visceral and skeletal malformations at 1000 mg/kg bw per day (Rhodes, 2012a).

In a developmental toxicity study in rabbits given mandestrobin by gavage at 0, 100, 300 or 1000 mg/kg bw per day from GDs 7 to 28, the NOAELs for both maternal and embryo/fetal toxicity were 1000 mg/kg bw per day, the highest dose tested (Rhodes, 2012b).

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

In an acute neurotoxicity study, mandestrobin was given to rats by gavage at a dose of 0, 500, 1000 or 2000 mg/kg bw. The NOAEL for neurotoxicity was 1000 mg/kg bw, based on decreased overall locomotor activity (total and/or ambulatory counts) at 2000 mg/kg bw. The NOAEL for systemic toxicity was 2000 mg/kg bw, the highest dose tested (Herberth, 2011b).

In a 90-day neurotoxicity study in rats given mandestrobin at a dietary concentration of 0, 1500, 5000 or 15 000 ppm (equal to 0, 99, 338 and 1024 mg/kg bw per day for males and 0, 122, 415 and 1223 mg/kg bw per day for females, respectively), the NOAEL for systemic toxicity was 5000 ppm (equal to 338 mg/kg bw per day), based on transient decreases in body weight, body weight gain and feed consumption in males at 15 000 ppm (equal to 1024 mg/kg bw per day). The NOAEL for neurotoxicity was 15 000 ppm (equal to 1024 mg/kg bw per day), the highest dose tested (Herberth, 2012).

Although there were no indications of neuropathological effects of mandestrobin, the Meeting concluded that mandestrobin may cause transient, acute neurobehavioural effects at high doses.

In a 28-day immunotoxicity study in female rats given mandestrobin at a dietary concentration of 0, 1500, 5000 or 15 000 ppm (equal to 0, 147, 471 and 1419 mg/kg bw per day, respectively), the NOAEL for immunotoxicity (splenic AFC response) was 15 000 ppm (equal to 1419 mg/kg bw per day), the highest dose tested (Hosako, 2011b).

Two in vivo studies (one in rats, one in mice) to gain insight into the mechanistic basis of the liver and thyroid effects observed in the main studies in rats were provided (Asano, 2012e; Yamada, 2012b). The effects observed indicated a constitutive androstane receptor (CAR)–mediated induction of liver enzymes and subsequent perturbations of thyroid hormones, similar to a phenobarbital-like mode of action (Capen et al., 1999; Meek et al., 2003; Cohen et al., 2004).

In an in vitro non-GLP-compliant human estrogen and androgen receptor transactivation assay, mandestrobin and its metabolites (5-COOH-S-2200, 4-OH-S-2200, 5-CH_2OH-S-2200 and 5-CA-S-2200-NHM) did not show agonistic or antagonistic effects on human estrogen receptor alpha or human androgen receptor (Suzuki, 2012). In a second in vitro non-GLP-compliant study, a steroidogenesis assay in NCI-H295R cells, mandestrobin did not influence testosterone or estradiol production (Kubo, 2012).

No information on the potential effects of mandestrobin on the microbiome of the human gastrointestinal tract is available.

Toxicological data on metabolites

Metabolite 2-CH_2OH-S-2200 (free and conjugated)

The free form of 2-CH_2OH-S-2200 ((2*RS*)-2-[2-(5-hydroxymethyl-2-methylphenoxymethyl)-phenyl]-2-methoxy-*N*-methylacetamide) is a rat (<1% of the applied dose in urine), hen, goat and plant metabolite. The conjugated form is also a plant and animal metabolite. The submitted studies were performed with the free form of the metabolite only.

The acute oral LD_{50} of metabolite 2-CH_2OH-S-2200 in rats was greater than 2000 mg/kg bw (Asano, 2012c).

Metabolite 2-CH_2OH-S-2200 was tested in a gene mutation assay in bacteria. There was no evidence of mutagenicity (Kitamoto, 2012e).

The acute toxicity of the free and conjugated 2-CH_2OH-S-2200 metabolite is considered to be similar to that of the parent compound. For chronic toxicity, the threshold of toxicological concern (TTC) approach could be applied using Cramer class III.

Metabolite 4-OH-S-2200 (free and conjugated)

Both the free and conjugated forms of 4-OH-S-2200 ((2*RS*)-2-[2-(4-hydroxy-2,5-dimethyl-phenoxymethyl)phenyl]-2-methoxy-*N*-methylacetamide) are rat (free form <1% of the applied dose in urine; conjugated form >30% of the applied dose in bile; present in plasma), hen, goat and plant metabolites. Submitted studies were performed with the free form of the metabolite only.

The acute oral LD_{50} of metabolite 4-OH-S-2200 in rats was greater than 2000 mg/kg bw (Asano, 2012d).

Metabolite 4-OH-S-2200 was tested in a gene mutation assay in bacteria. There was no evidence of mutagenicity (Kitamoto, 2012f).

Based on the high levels in bile, the toxicity of the free and conjugated 4-OH-S-2200 metabolite is considered to be covered by the parent compound.

Metabolite De-Xy-S-2200

De-Xy-S-2200 ((2*RS*)-2-(2-hydroxymethylphenyl)-2-methoxy-*N*-methylacetamide) is a rat (<1% of the applied dose in urine; present in liver and kidney), hen, goat and plant metabolite.

The acute oral LD_{50} of metabolite De-Xy-S-2200 in rats was greater than 2000 mg/kg bw (Asano, 2011).

Metabolite De-Xy-S-2200 was tested in a gene mutation assay in bacteria. There was no evidence of mutagenicity (Kitamoto, 2011).

The acute toxicity of metabolite De-Xy-S-2200 is considered to be similar to that of the parent compound. For chronic toxicity, the TTC approach could be applied using Cramer class III.

Metabolite 2-COOH-S-2200

2-COOH-S-2200 (2-({2-[(1*RS*)-1-methoxy-2-(methylamino)-2-oxoethyl]benzyl}oxy)-4-methylbenzoic acid) is a rat (<1% of the applied dose in urine and bile; present in plasma and kidney), hen and goat metabolite.

The acute oral LD_{50} of metabolite 2-COOH-S-2200 in rats was greater than 2000 mg/kg bw (Asano, 2012a).

Metabolite 2-COOH-S-2200 was tested in an adequate range of in vitro and in vivo genotoxicity assays. Negative results were obtained in gene mutation assays (Wollny, 2011a; Kitamoto, 2012a) and an in vivo micronucleus assay (Tanaka, 2012). A weak positive response was seen in an in vitro chromosomal aberration assay (Kitamoto, 2012b).

The acute toxicity of metabolite 2-COOH-S-2200 is considered to be similar to that of the parent compound. For chronic toxicity, the TTC approach could be applied using Cramer class III.

Metabolite 5-COOH-S-2200

5-COOH-S-2200 (3-({2-[(1*RS*)-1-methoxy-2-(methylamino)-2-oxoethyl]benzyl}oxy)-4-methylbenzoic acid) is a rat (1.3% of the applied dose in urine and faeces; present in plasma, liver and kidney), plant, hen and goat metabolite.

The acute oral LD_{50} of metabolite 5-COOH-S-2200 in rats was greater than 300 mg/kg bw and less than 2000 mg/kg bw (Asano, 2012b).

Metabolite 5-COOH-S-2200 was tested in an adequate range of in vitro genotoxicity assays. There was no evidence of genotoxicity (Wollny, 2011b; Kitamoto, 2012c,d).

The acute toxicity of metabolite 5-COOH-S-2200 is considered to be similar to that of the parent compound. For chronic toxicity, the TTC approach could be applied using Cramer class III.

Human data

In reports on manufacturing plant personnel, no adverse effects were noted (Nishioka, 2012; Nishimoto, 2017). No information on accidental or intentional poisoning in humans is available.

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

Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) for mandestrobin of 0–0.2 mg/kg bw, based on a NOAEL of 19.2 mg/kg bw per day for increased liver weights, histopathological changes in the liver and associated disturbance of blood biochemistry parameters in a 1-year dog study and using a safety factor of 100. This ADI provides a margin of about 4000 relative to the lowest-observed-adverse-effect level (LOAEL) for equivocal carcinogenic effects in rats.

The Meeting established an acute reference dose (ARfD) of 3 mg/kg bw, based on a NOAEL of 300 mg/kg bw per day for malformations observed in a developmental toxicity study in rats and using a safety factor of 100. This ARfD is applicable to women of childbearing age only.

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

Levels relevant to risk assessment of mandestrobin

Species	Study	Effect	NOAEL	LOAEL
Mouse	Seventy-eight-week study of toxicity and carcinogenicity[a]	Toxicity	7 000 ppm, equal to 823.9 mg/kg bw per day[b]	–
		Carcinogenicity	7 000 ppm, equal to 823.9 mg/kg bw per day[b]	–
Rat	Two-year study of toxicity and carcinogenicity[a]	Toxicity	400 ppm, equal to 26.7 mg/kg bw per day	2 000 ppm, equal to 135.2 mg/kg bw per day
		Carcinogenicity	7 000 ppm, equal to 375.6 mg/kg bw per day	15 000 ppm, equal to 804.3 mg/kg bw per day[c]
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	10 000 ppm, equal to 511.7 mg/kg bw per day[b]	–
		Parental toxicity	1 000 ppm, equal to 47.77 mg/kg bw per day	3 000 ppm, equal to 145.7 mg/kg bw per day
		Offspring toxicity	1 000 ppm, equal to 47.77 mg/kg bw per day	3 000 ppm, equal to 145.7 mg/kg bw per day
	Developmental toxicity study[d]	Maternal toxicity	300 mg/kg bw per day	1 000 mg/kg bw per day
		Embryo and fetal toxicity	300 mg/kg bw per day	1 000 mg/kg bw per day
	Acute neurotoxicity study[d]	Neurotoxicity	1 000 mg/kg bw	2 000 mg/kg bw
Rabbit	Developmental toxicity study[d]	Maternal toxicity	1 000 mg/kg bw per day[b]	–
		Embryo and fetal toxicity	1 000 mg/kg bw per day[b]	–
Dog	Ninety-day toxicity study[a]	Toxicity	4 000 ppm, equal to 90.9 mg/kg bw per day	12 000 ppm, equal to 267.8 mg/kg bw per day
	One-year toxicity study[a]	Toxicity	800 ppm, equal to 19.2 mg/kg bw per day	4 000 ppm, equal to 92.0 mg/kg bw per day

[a] Dietary administration.
[b] Highest dose tested.
[c] Based on an equivocal increase in the incidence of tumours.
[d] Gavage administration.

Acceptable daily intake (ADI)

0–0.2 mg/kg bw

Acute reference dose (ARfD)

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 mandestrobin

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	>95% based on urinary and biliary excretion within 24 hours
Dermal absorption	No information provided
Distribution	Extensively distributed throughout the body (mainly in gastrointestinal tract, liver and kidneys)
Potential for accumulation	Evidence of accumulation, but not persistence
Rate and extent of excretion	Faecal elimination, via the bile, was the primary route of elimination (~80%); urinary excretion up to ~20%
Metabolism in animals	Extensively metabolized to numerous metabolites; primary routes of metabolism are oxidation and subsequent conjugation with glucuronic acid, demethylation with subsequent oxidation, oxidation with subsequent demethylation
Toxicologically significant compounds in animals and plants	Mandestrobin
Acute toxicity	
Rat, LD_{50}, oral	>2 000 mg/kg bw
Rat, LD_{50}, dermal	>2 000 mg/kg bw
Rat, LC_{50}, inhalation	>4.96 mg/L air (maximal attainable concentration)
Rabbit, dermal irritation	Not irritating
Rabbit, ocular irritation	Mildly irritating
Guinea-pig, dermal sensitization	Not sensitizing (Magnusson & Kligman)
Short-term studies of toxicity	
Target/critical effect	Liver and clinical chemistry
Lowest relevant oral NOAEL	19.2 mg/kg bw per day (dog)
Lowest relevant dermal NOAEL	1 000 mg/kg bw per day, highest dose tested (rat)
Lowest relevant inhalation NOAEC	Not available
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Liver
Lowest relevant NOAEL	26.7 mg/kg bw per day (rat)

Carcinogenicity	Not carcinogenic in mice; equivocal increases in testicular interstitial cell and ovarian sex cord stromal adenomas in rats[a]
Genotoxicity	
	No evidence of genotoxicity[a]
Reproductive toxicity	
Target/critical effect	Parental: increased liver weights, diffuse liver hypertrophy, pigment in the bile duct and periductural inflammatory cell infiltration Offspring: lower spleen weights
Lowest relevant parental NOAEL	47.77 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	47.77 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	511.7 mg/kg bw per day, highest dose tested (rat)
Developmental toxicity	
Target/critical effect	Maternal effects: decreased feed consumption Developmental effects: visceral and skeletal malformations
Lowest relevant maternal NOAEL	300 mg/kg bw per day (rat)
Lowest relevant embryo/fetal NOAEL	300 mg/kg bw per day (rat)
Neurotoxicity	
Acute neurotoxicity NOAEL	1 000 mg/kg bw (rat)
Subchronic neurotoxicity NOAEL	1 024 mg/kg bw per day, highest dose tested (rat)
Developmental neurotoxicity NOAEL	Not available
Other toxicological studies	
Immunotoxicity NOAEL	1 419 mg/kg bw per day, highest dose tested (rat)
Studies on toxicologically relevant metabolites	
2-COOH-S-2200	Oral LD_{50} > 2 000 mg/kg bw Not genotoxic in vivo
5-COOH-S-2200	300 < LD_{50} < 2 000 mg/kg bw Not genotoxic in vitro
2-CH_2OH-S-2200	LD_{50} > 2 000 mg/kg bw Not genotoxic in vitro
4-OH-S-2200	LD_{50} > 2 000 mg/kg bw Not genotoxic in vitro
De-Xy-S-2200	LD_{50} > 2 000 mg/kg bw Not genotoxic in vitro
Human data	
	No detrimental health effects in manufacturing personnel

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

Summary

	Value	Study	Safety factor
ADI	0–0.2 mg/kg bw	One-year toxicity study in dogs	100
ARfD	3 mg/kg bw[a]	Developmental toxicity study in rats	100

[a] Applies to women of childbearing age only.

References

Asano H (2010a). Acute oral toxicity study of S-2200TG in rats. Unpublished report. Sumitomo Chemical Co., Ltd. Study No. 4161 (ROT-0010). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Asano H (2010b). Acute dermal toxicity study of S-2200TG in rats. Unpublished report. Sumitomo Chemical Co., Ltd. Study No. 4162 (ROT-0011). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Asano H (2011). Acute oral toxicity study of De-Xy-S-2200 in rats. Unpublished report. Sumitomo Chemical Co., Ltd. Study No. 4225 (ROT-0031). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Asano H (2012a). Acute oral toxicity study of 2-COOH-S-2200 in rats. Unpublished report. Sumitomo Chemical Co., Ltd. Study No. 4234 (ROT-0043). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Asano H (2012b). Acute oral toxicity study of 5-COOH-S-2200 in rats. Unpublished report. Sumitomo Chemical Co., Ltd. Study No. 4233 (ROT-0044). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Asano H (2012c). Acute oral toxicity study of 2-CH_2OH-S-2200 in rats. Unpublished report. Sumitomo Chemical Co., Ltd. Study No. 4242 (ROT-0053). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Asano H (2012d). Acute oral toxicity study of 4-OH-S-2200 in rats. Unpublished report. Sumitomo Chemical Co., Ltd. Study No. 4241 (ROT-0054). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Asano H (2012e). Short-term study for mode of action analysis for rat liver and thyroid findings by S-2200TG – dose response, time-course and reversibility. Unpublished report. Sumitomo Chemical Co., Ltd. Study No. S1560 (ROT-0067). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Beck W (2011a). Amended final report 1: S-2200 technical grade: 13 week oral (dietary) administration toxicity study in the mouse. Unpublished report. Covance Laboratories Ltd. Study No. 0333/291 (ROT-0023). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Beck W (2011b). S-2200 technical grade: 13 week oral (dietary) administration toxicity study in the rat. Unpublished report. Covance Laboratories Ltd. Study No. 0333/290 (ROT-0025). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Beck W (2012a). Amended final report 2: S-2200 technical grade: 13 week oral (dietary) administration toxicity study in the dog. Unpublished report. Covance Laboratories Ltd. Study No. 8211560 (ROT-0024). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Beck W (2012b). S-2200 technical grade: 52 week oral (dietary) administration toxicity study in the dog. Unpublished report. Covance Laboratories Ltd. Study No. 8211561 (ROT-0071). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Beck W (2012c). S-2200 technical grade: 78 week oral (dietary) administration carcinogenicity study in the mouse. Unpublished report. Covance Laboratories Ltd. Study No. 0333/293 (ROT-0073). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Beck W (2012d). S-2200 technical grade: 104 week oral (dietary) administration combined toxicity/ carcinogenicity study in the rat. Unpublished report. Covance Laboratories Ltd. Study No. 0333/292 (ROT-0072). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Capen CC, Dybing E, Rice JM, Wilbourn JD, editors (1999). Species differences in thyroid, kidney and urinary bladder carcinogenesis. Lyon: International Agency for Research on Cancer (IARC Scientific Publications No. 147).

Cohen SM, Klaunig J, Meek E, Hill RH, Pastoor T, McKeeman LL et al. (2004). Evaluating the human relevance of chemically induced animal tumors. Toxicol Sci. 78(2):181–6.

Deguchi Y (2010). Acute inhalation toxicity study of S-2200 TG in rats. Unpublished report. Sumitomo Chemical Co., Ltd. Study No. 4160 (ROT-0020). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Galloway S, Lorge E, Aardema MJ, Eastmond D, Fellows M, Heflich R et al. (2011). Workshop summary: Top concentration for in vitro mammalian cell genotoxicity assays; and report from working group on toxicity measures and top concentration for in vitro cytogenetics assays (chromosome aberrations and micronucleus). Mutat Res. 723:77–83.

Greaves P (2000). Histopathology of preclinical toxicity studies: interpretation and relevance in drug safety evaluation, second edition. Elsevier Science.

Herberth M (2011a). An oral (gavage) dose range–finding acute neurotoxicity study of S-2200TG in Wistar rats. Unpublished report. WIL Research Laboratories, LLC. Study No. WIL-118051 (ROT-0036). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Herberth M (2011b). An oral (gavage) acute neurotoxicity study of S-2200TG in Wistar rats. Unpublished report. WIL Research Laboratories, LLC. Study No. WIL-118052 (ROT-0037). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Herberth M (2012). A 90-day oral dietary neurotoxicity study of S-2200TG in Wistar rats. Unpublished report. WIL Research Laboratories, LLC. Study No. WIL-118053 (ROT-0050). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Hood RD (2005). Developmental and reproductive toxicology – a practical approach, second edition. Taylor and Francis.

Hosako H (2011a). S-2200TG – a 28-day dietary dose range–finding study in Wistar Han rats. Unpublished report. WIL Research Laboratories, LLC. Study No. WIL-118055 (ROT-0035). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Hosako H (2011b). S-2200TG – a 28-day oral (dietary) immunotoxicity study in female Wistar Han rats. Unpublished report. WIL Research Laboratories, LLC. Study No. WIL-118056 (ROT-0039). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Hoshino N (2010). Dose range–finding study for two-generation reproduction study of S-2200 TG in rats. Unpublished report. Mitsubishi Chemical Medience Corporation. Study No. B091037 (ROT-0018). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Kendrick J, Farrell C (2012). Amended final report 1: [^{14}C]S-2200: absorption, distribution, metabolism and excretion following repeat oral administration to the rat. Unpublished report. Covance Laboratories Ltd. Study No. 0333/298 (ROM-0036). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Kendrick J, Farrell C, Murphy B (2012). Amended final report 1: [^{14}C]S-2200: absorption, distribution, metabolism and excretion following single oral administration to the rat. Unpublished report. Covance Laboratories Ltd. Study No. 0333/297 (ROM-0033). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Kirkland D (1998). Chromosome aberration testing in genetic toxicology – past, present and future. Mutat Res. 404(1):173–85.

Kitamoto S (2010a). Reverse mutation test of S-2200TG in bacterial systems. Unpublished report. Sumitomo Chemical Co., Ltd. Study No. 4163 (ROT-0012). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Kitamoto S (2010b). In vitro chromosomal aberration test on S-2200TG in Chinese hamster lung cells (CHL/IU). Unpublished report. Sumitomo Chemical Co., Ltd. Study No. 4164 (ROT-0013). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Kitamoto S (2010c). Micronucleus test on S-2200TG in CD-1 mice. Unpublished report. Sumitomo Chemical Co., Ltd. Study No. 4154 (ROT-0014). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Kitamoto S (2011). Reverse mutation test of De-Xy-S-2200 in bacterial systems. Unpublished report. Sumitomo Chemical Co., Ltd. Study No. 4223 (ROT-0038). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Kitamoto S (2012a). Reverse mutation test of 2-COOH-S-2200 in bacterial systems. Unpublished report. Sumitomo Chemical Co., Ltd. Study No. 4221 (ROT-0041). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Kitamoto S (2012b). In vitro chromosomal aberration test on 2-COOH-S-2200 in Chinese hamster lung cells (CHL/IU). Unpublished report. Sumitomo Chemical Co., Ltd. Study No. 4219 (ROT-0046). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Kitamoto S (2012c). Reverse mutation test of 5-COOH-S-2200 in bacterial systems. Unpublished report. Sumitomo Chemical Co., Ltd. Study No. 4222 (ROT-0042). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Kitamoto S (2012d). In vitro chromosomal aberration test on 5-COOH-S-2200 in Chinese hamster lung cells (CHL/IU). Unpublished report. Sumitomo Chemical Co., Ltd. Study No. 4220 (ROT-0047). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Kitamoto S (2012e). Reverse mutation test of 2-CH_2OH-S-2200 in bacterial systems. Unpublished report. Sumitomo Chemical Co., Ltd. Study No. 4226 (ROT-0048). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Kitamoto S (2012f). Reverse mutation test of 4-OH-S-2200 in bacterial systems. Unpublished report. Sumitomo Chemical Co., Ltd. Study No 4227 (ROT-0049). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Kubo H (2012). In vitro steroidogenesis assay of S-2200TG in H295R cells. Unpublished report. Sumitomo Chemical Co., Ltd. Study No. HK001 (ROT-0065). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Matsuura I (2012). Two-generation reproduction toxicity study of S-2200TG in rats. Unpublished report. Mitsubishi Chemical Medience Corporation. Study No. B100842 (ROT-0064). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Meek E, Bucher JR, Cohen SM, Dellarco V, Hill RN, McKeeman L et al (2003). Framework for human relevance analysis of information on carcinogenic modes of action. Crit Rev Toxicol. 33(6):591–653.

Meuten DJ (1982). Relationship of serum total calcium to albumin and total protein in dogs. J Am Vet Med Assoc. 180(1):63–7.

Mikata K (2011). Metabolism of S-2200 *R*-isomer (S-2167) and S-2200 *S*-isomer (S-2354) in rats. Unpublished report. Sumitomo Chemical Co., Ltd. Study No. 4136 (No. ROM-0021). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Nishimoto Y (2017). Report on health status of factory workers engaged in manufacturing of mandestrobin technical material. Unpublished report no. ROT-0097. Sumitomo Chemical Co., Ltd. Submitted to WHO by Sumitomo Chemical Co., Ltd.

Nishioka K (2012). Statement from S-2200 manufacturer. Unpublished report no. ROT-0074. Sumitomo Chemical Co., Ltd. Submitted to WHO by Sumitomo Chemical Co., Ltd.

OECD (2014). Document supporting the Working Group of National Coordinators of the Test Guidelines Programme (WNT) decision to implement revised criteria for the selection of the top concentration in the in vitro mammalian cell assays on genotoxicity (Test Guidelines 473, 476 and 487). Paris: Organisation for Economic Co-operation and Development (ENV/JM/TG(2014)17).

Ogata H (2011). A 28-day repeated dose dermal toxicity study of S-2200 technical grade in rats. Unpublished report. Mitsubishi Chemical Medience Corporation. Study No. P100595 (ROT-0022). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Ota M (2010a). Primary skin irritation test of S-2200TG in rabbits. Unpublished report. Sumitomo Chemical Co., Ltd. Study No. 4152 (ROT-0015). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Ota M (2010b). Amended final report: Primary eye irritation test of S-2200TG in rabbits. Unpublished report. Sumitomo Chemical Co., Ltd. Study No. 4153 (ROT-0016). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Ota M (2010c). Skin sensitization test of S-2200TG in guinea pigs (maximization test). Unpublished report. Sumitomo Chemical Co., Ltd. Study No. 4165 (ROT-0019). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Rhodes J (2009a). S-2200 TG: oral (gavage) range-finding study of prenatal development in the rat. Unpublished report. Covance Laboratories Ltd. Study No. 8202048 (ROT-0009). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Rhodes J (2009b). S-2200 TG: oral (gavage) range-finding study of prenatal development in the rabbit. Unpublished report. Covance Laboratories Ltd. Study No. 8202040 (ROT-0008). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Rhodes J (2012a). S-2200 TG: oral (gavage) prenatal development toxicity study in the rat. Unpublished report. Covance Laboratories Ltd. Study No. 8202037 (ROT-0051). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Rhodes J (2012b). S-2200 TG: oral (gavage) prenatal development toxicity study in the rabbit. Unpublished report. Covance Laboratories Ltd. Study No. 8202046 (ROT-0052). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Shaw D (2008). S-2200: 7 days dietary study in the dog. Unpublished report. Covance Laboratories Ltd. Study No. 0333-285. Submitted to WHO by Sumitomo Chemical Co., Ltd.

Shaw D (2009a). S-2200: 14 day dietary administration range-finding study in the mouse. Unpublished report. Covance Laboratories Ltd. Study No. 0333-277. Submitted to WHO by Sumitomo Chemical Co., Ltd.

Shaw D (2009b). S-2200: 4 week dietary study in the dog. Unpublished report. Covance Laboratories Ltd. Study No. 0333-278. Submitted to WHO by Sumitomo Chemical Co., Ltd.

Shimoi A, Kuwayama C, Miyauchi M, Kakinuma C, Kamiya M, Harada T et al. (2001). Vacuolar change in the thyroid follicular cells in BrlHan: WIST@Jcl (GALAS) rats. J Toxicol Pathol. 14(3):253–7.

Suzuki N (2012). Evaluation of effects of S-2200TG and its metabolites on human estrogen receptor alpha and human androgen receptor using in vitro reporter gene assays. Unpublished report. Sumitomo Chemical Co., Ltd. Study No. RGA-130 (ROT-0066). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Tanaka J (2012). Micronucleus assay of 2-COOH-S-2200 in mice. Unpublished report. Biosafety Research Center, Foods, Drugs and Pesticides (BSRC). Study No. D624 (028-071) (ROT-0040). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Wollny H-E (2010). Gene mutation assay in Chinese hamster V79 cells in vitro (V79/HPRT) with S-2200TG. Unpublished report. Harlan Cytotest Cell Research GmbH. Study No. 1289600 (ROT-0021). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Wollny H-E (2011a). Gene mutation assay in Chinese hamster V79 cells in vitro (V79/HPRT) with 2-COOH-S-2200. Unpublished report. Harlan Cytotest Cell Research GmbH. Study No. 1394801 (ROT-0033). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Wollny H-E (2011b). Gene mutation assay in Chinese hamster V79 cells in vitro (V79/HPRT) with 5-COOH-S-2200. Unpublished report. Harlan Cytotest Cell Research GmbH. Study No. 1394802 (ROT-0034). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Yamada T (2012a). The toxicological relevance of the liver and thyroid alterations observed in rats treated with S-2200TG based on mode of action. Unpublished report no. ROT-0070. Sumitomo Chemical Co., Ltd. Submitted to WHO by Sumitomo Chemical Co., Ltd.

Yamada T (2012b). Short-term study for mode of action analysis for mouse liver findings by S-2200TG. Unpublished report. Sumitomo Chemical Co., Ltd. Study No. S1618 (ROT-0068). Submitted to WHO by Sumitomo Chemical Co., Ltd.

Yamada T, Miyata K (2012). Interpretation of higher incidence of ovarian sex-cord stromal tumour in female rats treated with S-2200TG in a 2-year carcinogenicity study. Unpublished report no. ROT-0069. Sumitomo Chemical Co., Ltd. Submitted to WHO by Sumitomo Chemical Co., Ltd.

MANDIPROPAMID (addendum)

First draft prepared by
Lars Niemann[1] and David A. Eastmond[2]

[1] Department of Pesticides Safety, German Federal Institute for Risk Assessment, Berlin, Germany
[2] Department of Molecular, Cell & Systems Biology, University of California, Riverside, California, United States of America (USA)

Explanation 407
Evaluation for acceptable intake 407
1. Toxicological studies with mandipropamid 407
1.1 Short-term studies of toxicity 407
1.2 Special studies 410
(a) Changes in the liver 410
(b) Endocrine disrupting potential 412
(c) Immunotoxicity 413
2. Toxicological studies with metabolite SYN500003 413
2.1 Acute toxicity 413
2.2 Genotoxicity 414
Comments 418
Toxicological evaluation 418
References 418

Explanation

Mandipropamid was reviewed by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) for the first time in 2008, when an acceptable daily intake (ADI) of 0–0.2 mg/kg body weight (bw) was established and it was concluded that an acute reference dose (ARfD) was not necessary.

Following a request for additional maximum residue levels by the Codex Committee on Pesticide Residues, mandipropamid was placed on the agenda of the present Meeting, which assessed additional toxicological information available since the last review.

A 90-day feeding study in mice, an immunotoxicity study in mice and several mechanistic studies performed with the parent compound were submitted. In addition, an acute toxicity study and a number of genotoxicity studies with the metabolite (4-chlorophenyl)-prop-2-ynyloxy-acetic acid (SYN500003) were provided.

Evaluation for acceptable intake

1. Toxicological studies with mandipropamid

1.1 Short-term studies of toxicity

Groups of 10 male and 10 female C57BL/10JfCD-1 mice per dose were fed diets containing mandipropamid (batch no. SEZ2BP007; purity 96.5%) at a concentration of 0, 300, 800, 2000 or 5000 parts per million (ppm) (equal to 0, 37.2, 98.0, 247.6 and 624.3 mg/kg bw per day for males and 0, 47.3, 128.9, 315.8 and 800.5 mg/kg bw per day for females, respectively) for 90 days. The main purpose of the study was to identify appropriate doses for a subsequent carcinogenicity study, which was evaluated by JMPR in 2008. Over the course of the 90-day study, the animals were monitored for clinical signs and weighed regularly, and feed consumption was measured. At scheduled termination, cardiac blood samples were taken for haematology (clinical chemistry parameters were not measured). The mice were killed and grossly examined. Selected organs were weighed, and a

representative selection of organs and tissues was taken for histopathology. Microscopic examination of tissues and organs was confined to the control and high-dose groups, except for the livers, which were also examined in the other treated groups.

No premature mortality or clinical signs of toxicity were noted. The in-life findings attributed to treatment comprised slight, but dose related, reductions in body weight and body weight gain in male mice, which were statistically significant at the two highest doses. A statistically significantly lower mean terminal body weight in low-dose males was not part of a dose–response relationship and thus was most likely not related to treatment (see Table 2 below). In females, an initial (day 2) weight loss was seen in the two highest dose groups, whereas the only statistically significant effect at later stages of the study was a reduced body weight at the highest dose at termination (see Table 2 below). Lower feed consumption compared with the control groups was occasionally observed in both sexes at 5000 ppm and in males at 2000 ppm.

At termination, microcytic and hypochromic anaemia was apparent in both sexes at 5000 ppm and in females also at 2000 ppm, as haematocrit, mean cell volume and haemoglobin were statistically significantly reduced. Red blood cell count was not statistically significantly lower in males and not affected in females at all, suggesting a possible iron or cobalamin (B_{12}) deficiency behind the anaemia. In line with that, statistically significantly lower mean cell haemoglobin and lower erythrocyte size were noted in males of the 2000 and 5000 ppm groups and in females in all treatment groups (Table 1). However, the effects in females at 300 and 800 ppm, even though statistically significant, were minor. As the differences relative to the control values were less than 5%, these effects were considered to be of uncertain toxicological relevance. Furthermore, haematocrit and total haemoglobin were not affected in these female groups.

Table 1. Haematological parameters[a] in the 90-day study with mandipropamid in mice

Parameter	0 ppm	300 ppm	800 ppm	2 000 ppm	5 000 ppm
Males					
Number examined	10	9	10	10	9
Red blood cell count ($\times 10^{12}$/L)	9.43 ± 0.20	9.57 ± 0.37	9.48 ± 0.25	9.45 ± 0.31	9.24 ± 0.30
Mean cell volume (fL)	50.1 ± 0.7	49.6 ± 0.6	49.6 ± 0.5	48.9** ± 1.0	49.0** ± 0.5
Haematocrit (%)	47.3 ± 1.3	47.4 ± 2.1	47.0 ± 1.4	46.2 ± 1.6	45.3** ± 1.3
Haemoglobin (g/dL)	14.4 ± 0.4	14.5 ± 0.6	14.3 ± 0.4	14.1 ± 0.5	13.7** ± 0.4
Mean cell haemoglobin (pg)	15.2 ± 0.1	15.1 ± 0.2	15.1 ± 0.2	14.9** ± 0.2	14.8** ± 0.3
Females					
Number examined	8	10	10	9	10
Red blood cell count ($\times 10^{12}$/L)	9.41 ± 0.29	9.67 ± 0.31	9.54 ± 0.32	9.29 ± 0.34	9.46 ± 0.23
Mean cell volume (fL)	50.2 ± 0.6	49.4* ± 0.5	48.4** ± 0.8	48.1** ± 0.8	47.8** ± 0.7
Haematocrit (%)	47.2 ± 1.5	47.7 ± 1.6	46.2 ± 1.9	44.7** ± 1.9	45.2** ± 1.1
Haemoglobin (g/dL)	14.5 ± 0.4	14.5 ± 0.5	14.1 ± 0.6	13.6** ± 0.6	13.8** ± 0.4
Mean cell haemoglobin (pg)	15.4 ± 0.2	15.0** ± 0.1	14.8** ± 0.2	14.6** ± 0.2	14.6** ± 0.2

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (analysis of variance)
[a] Mean ± standard deviation.
Source: Milburn (2005)

There were no gross findings that could be attributed to treatment in any group. Dose-related increases in absolute liver weight were seen in both sexes at the two highest doses (Table 2). "Adjusted" organ weights, rather than relative organ weights, were the subject of statistical analysis

by the sponsor; this approach is based on a publication by Shirley (1977). As the increase in adjusted organ weights was statistically significant from 800 ppm onwards and, at 2000 and 5000 ppm, exceeded 10%, it is considered adverse. The liver weight increase was accompanied by cytoplasmic eosinophilia of periportal hepatocytes in most female mice in the 2000 and 5000 ppm groups and in half of the males at 5000 ppm. Surprisingly, no hypertrophy was reported.

Table 2. Organ weights[a] in the 90-day study with mandipropamid in mice

Parameter	0 ppm	300 ppm	800 ppm	2 000 ppm	5 000 ppm
Males					
Number examined	10	10	10	10	10
Terminal body weight (g)	32.0 ± 2.8	29.9** ± 0.8	31.0 ± 1.4	30.5** ± 2.0	30.2** ± 1.3
Absolute liver weight (g)	1.35 ± 0.15	1.29 ± 0.08	1.40 ± 0.13	1.55** ± 0.17	1.71** ± 0.19
Relative liver weight (%)	4.22 ± 0.21	4.32 ± 0.23	4.51 ± 0.25	5.07 ± 0.35	5.66 ± 0.41
Liver weight adjusted for body weight (g)	1.27	1.34	1.38*	1.56**	1.75**
Absolute spleen weight (mg)	75 ± 6	69 ± 7	77 ± 11	73 ± 7	71 ± 9
Relative spleen weight (%)	0.235 ± 0.014	0.232 ± 0.021	0.248 ± 0.031	0.238 ± 0.020	0.235 ± 0.022
Spleen weight adjusted for body weight (mg)	72	72	76	73	73
Females					
Number examined	10	10	10	10	10
Terminal body weight (g)	24.4 ± 1.3	25.0 ± 1.0	24.5 ± 0.9	24.4 ± 1.3	23.4** ± 0.8
Absolute liver weight (g)	1.04 ± 0.10	1.09 ± 0.08	1.15 ± 0.15	1.21** ± 0.14	1.28** ± 0.10
Relative liver weight (%)	4.27 ± 0.26	4.36 ± 0.26	4.67 ± 0.48	4.95 ± 0.44	5.47 ± 0.31
Liver weight adjusted for body weight (g)	1.04	1.03	1.13*	1.20**	1.36**
Absolute spleen weight (mg)	93 ± 23	85 ± 7	82* ± 10	78** ± 7	74** ± 8
Relative spleen weight (%)	0.381 ± 0.091	0.338 ± 0.019	0.334 ± 0.037	0.319 ± 0.024	0.316 ± 0.034
Spleen weight adjusted for body weight (mg)	93	83	82*	78**	76**

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (analysis of variance and/or covariance, as proposed by the sponsor; based on Shirley, 1977); relative organ weights not statistically analysed

[a] Mean ± standard deviation.

Source: Milburn (2005)

It is worth noting that a statistically significant, dose-related decrease in spleen weight was observed only in females from 800 ppm onwards (Table 2). The lower spleen weight was not in parallel to body weight, which was only marginally affected. However, a high variability in the control group was also noticed. No conclusion can be drawn if this organ weight change was

somehow related to the haematological findings. No evidence of extramedullary haematopoiesis in the liver or in the spleen was found. In one female at 5000 ppm, an increased amount of haemosiderin was detected in the spleen, whereas histopathological spleen findings were not reported for males. On balance, the toxicological relevance of the decrease in spleen weight is equivocal, and this finding is not considered a reliable basis for identifying the no-observed-adverse-effect level (NOAEL).

Based on haematological and liver weight effects at 2000 ppm (equal to 247.6 mg/kg bw per day), the NOAEL was 800 ppm (equal to 98.0 mg/kg bw per day). This NOAEL is higher than those identified in the short-term studies in dogs and rats that were previously reviewed by JMPR. Target organs in this study were the liver and the erythropoietic system, with possible involvement of the spleen. In addition, there were rather weak effects on body weight at higher doses. The liver findings were well in line with what was seen in rats and dogs. Effects on red blood cells resembled those noted in rats, also with regard to the doses at which they were observed (Milburn, 2005).

1.2 Special studies

(a) Changes in the liver

Biochemical and pathological changes in the liver subsequent to dietary administration of mandipropamid for up to 28 days were investigated in mice and rats.

In the first study, 20 male and 20 female C57BL/10Jf/Alpk mice (5 weeks old at the start of the study) received mandipropamid via their diet at a concentration of 0, 800, 2000 or 5000 ppm (equivalent to 0, 120, 300 and 750 mg/kg bw per day, respectively) for up to 28 days. Five males and five females per dose were killed following 7, 14 or 21 days of treatment or at study termination after 28 days. Three days before the respective scheduled terminations, the animals were surgically implanted with Alzet osmotic mini-pumps containing 5-bromo-2′-deoxyuridine (BrdU) at a concentration of 15 or 30 mg/mL (contradictory information given in the report) in 0.9% saline to facilitate assessment of possible hepatic proliferation.

Once a day, the animals were monitored for clinical signs. Body weights and feed consumption were recorded on a weekly basis. Urine was collected from the same five males and five females scheduled for termination on days 7, 14, 21 and 28. At interim or terminal kill, blood was taken by cardiac puncture, and a number of clinical chemistry parameters were determined. The liver was removed, weighed and subjected to histopathology. The number of apoptotic bodies per 1000 hepatocytes was measured by means of a histochemistry technique (terminal deoxynucleotidyl transferase deoxyuridine triphosphate [dUTP] nick end labelling [TUNEL]). Subcellular liver fractions were prepared, and total protein and cytochrome P450 (CYP) content, activities of cytochrome P450–mediated monooxygenases (ethoxyresorufin *O*-deethylase [EROD] and 7-pentoxyresorufin *O*-depentylase [PROD]) and glutathione *S*-transferases (GSTs), non-protein sulfhydryl levels and the extent of testosterone hydroxylation were determined in these samples. Apparently, most of these biochemical investigations (for total cytochrome P450 content, PROD, EROD and GST activities) were also performed on liver samples from another study. In this range-finding experiment, mandipropamid had been administered to mice (five of each sex per group) for 28 days at a dietary concentration of 700, 2100 or 7000 ppm.

There were no clinical signs of toxicity, but body weight tended to be lower at the highest dose from day 15 onwards. A liver weight increase was seen after 7 days of treatment in high-dose males and in females at the intermediate and high doses. In the animals killed at later time points, liver weight was consistently increased in the groups receiving 2000 or 5000 ppm. Clinical chemistry analysis in plasma revealed increases in cholesterol, gamma-glutamyl transferase (GGT) and aspartate aminotransferase (AST) at the high dose, but, surprisingly, decreases in alanine aminotransferase (ALT) and alkaline phosphatase (ALP). However, these findings were not consistent with regard to sex or duration of treatment. The most noteworthy histological finding in the liver was eosinophilia of the periportal region in both sexes at the intermediate and high doses, which was noted after 7 days of substance administration. After 28 days, but not before, apoptosis was reduced in females receiving 5000 ppm. Increases in EROD, PROD and GST activities in both sexes at 2000 and 5000 ppm

suggested an induction of xenobiotic metabolizing enzymes in the mouse liver. However, there was no evidence of increased liver cell proliferation or cytotoxicity. In the animals that had been treated for 28 days in the second study, increases in PROD and GST activities were observed in both sexes at 2100 and 7000 ppm, whereas EROD activity was lower in females at 700 and 2100 ppm but was not different from the control value at 7000 ppm (Dow, 2007a).

The same methods as in the study in mice (Dow, 2007a) were applied in two studies in Alpk:APfSD (Wistar-derived) rats, the results of which were reported together. In the first study, 20 male and 20 female rats were administered mandipropamid at a dietary concentration of 0, 100, 500 or 3000 ppm (equivalent to 0, 10, 50 and 300 mg/kg bw per day, respectively). Five males and five females from each dose group were killed after 7, 14, 21 or 28 days of treatment. In the second study, the only dietary concentration was 5000 ppm (equivalent to 500 mg/kg bw per day), and only male rats were employed. Ten animals from this treatment group and the control group were killed after 3, 7 or 14 days of treatment.

In addition, in vitro metabolism of mandipropamid was investigated in rat liver microsomes, partly following exposure to the cytochrome P450 inhibitors α-naphthaflavone, proadifen and furafylline.

There were no clinical signs of toxicity, and only a marginal decrease in body weight was noted in male rats receiving the test substance at 3000 ppm for 28 days. Liver weight was increased at 3000 and 5000 ppm, and this effect became more pronounced with duration of treatment. Liver cell hypertrophy was noted in the group receiving 3000 ppm after a minimum exposure of 21 days. This finding was more common and occurred earlier in females. No evidence of increased cell proliferation, cytotoxicity or an impact on apoptosis frequency was observed. Alterations in blood clinical chemistry parameters comprised increases in cholesterol, total protein and albumin in plasma at 3000 ppm, whereas triglycerides, AST and ALP were decreased. In the group receiving 5000 ppm, a small, transient increase in plasma glutamate dehydrogenase activity was observed after 3 and 7 days, whereas triglycerides were marginally decreased. Changes were generally small and not entirely consistent with regard to sex and time points of measurement.

PROD and GST activities in plasma were increased at 3000 and 5000 ppm. Higher GST activities were also noted in males at 500 ppm. In males, non-protein sulfhydryl levels were increased at 500 and 3000 ppm after 28 days of treatment. A rather strong increase in the activity of GGT in liver homogenates was observed in females at 3000 ppm. Thus, as in the mouse, there is good evidence for an induction of liver enzymes in the rat.

In vitro experiments revealed the abundance of at least four distinct metabolites, two of which could be identified and were the same as or similar to metabolites found in vivo. CYP2B and/or CYP1A2 were at least partially responsible for biotransformation in rat microsomes (Dow, 2007b).

In another study, liver cell proliferation was investigated in female Alpk:APfSD (Wistar-derived) rats. Groups of 30 animals either were fed a diet containing 5000 ppm mandipropamid (equal to 424 mg/kg bw per day) or were kept on basal diet for up to 15 days. Ten females per group were terminated on day 4, 8 or 15. Three days prior to first kill on day 4 or 7 days prior to termination on day 8 or 15, mini-pumps releasing BrdU at a rate of 10 µL/hour were surgically implanted.

Throughout the study, the animals were monitored for clinical signs, body weight development and feed consumption. At termination, cardiac blood samples were taken for clinical chemistry measurements. Liver weight was determined, and liver samples were taken for histopathology, examination of BrdU incorporation or, by means of the TUNEL technique, determination of apoptosis frequency.

Apart from slight initial reductions in body weight and feed intake, treatment was well tolerated. The only alteration in clinical chemistry parameters that could be attributed to treatment was an increase in cholesterol levels in the mandipropamid-treated group from day 8 onwards, confirming

previous findings in male rats (Dow, 2007b) and in mice (Dow, 2007a). As was occasionally seen in these other studies, activities of ALT and AST were decreased after 2 weeks of feeding, but such an effect, even if treatment related, would not be considered adverse.

There were no gross lesions, apart from an enlarged liver in one animal in the 5000 ppm group. Mean liver weight (when adjusted for body weight) was 7%, 22% or 17% higher than in the control animals at termination on days 4, 8 and 15, respectively. However, histopathology of the liver did not reveal any findings that could be attributed to treatment. There was no impact on BrdU labelling index or on the number of apoptotic bodies. On balance, the results suggested that the test compound did not induce or stimulate liver cell proliferation, did not affect the cell cycle and had no effect on apoptosis (Lees, 2006).

(b) Endocrine disrupting potential

The endocrine disrupting potential of mandipropamid was examined in vitro and in vivo.

Estrogenic, anti-estrogenic, androgenic and anti-androgenic properties were investigated in recombinant *Saccharomyces cerevisiae* strains by testing the ability of mandipropamid to interact with the human estrogen receptor (ER) or androgen receptor (AR), which had been integrated in the yeast. 17β-Estradiol (ER agonist), hydroxytamoxifen (ER antagonist), dihydrotestosterone (AR agonist) and flutamide (AR antagonist) were used as positive control substances; they all produced the expected effects, with which the activity of mandipropamid was compared. Activation of ER or AR would have become apparent by the stimulation of β-galactosidase owing to concomitant expression of the reporter gene *LacZ*, eventually resulting in a colour reaction that can be measured by absorbance. Antagonist activity was determined by the inhibition of agonistic effects of 17β-estradiol and dihydrotestosterone.

Mandipropamid did not exhibit estrogenic, androgenic or anti-estrogenic activity at concentrations up to 1 mmol/L. It is not clear why testing was not performed at higher concentrations, as concentrations above 10 mmol/L were included for investigation of anti-androgenic properties, revealing equivocal responses. For clarification, an AR competitive binding assay was performed in which 17α-methyltrienolone was used as the positive control substance. No evidence of AR binding was obtained, but the highest concentration was 0.5 mmol/L. These data are not considered suitable to confirm or dismiss the possible anti-androgenic effect at very high concentrations. On balance, this poorly reported study is considered unreliable and inconclusive (Moffat, 2001).

A uterotrophic assay was performed using six groups of 10 immature female Alpk:APfSD (Wistar-derived) rats. Two groups received mandipropamid by gavage at a dose of 500 or 1000 mg/kg bw per day for 3 days. In two additional groups, estradiol benzoate at a dose of 0.4 mg/kg bw per day was given together with the mandipropamid doses. The positive control group received only estradiol benzoate at 0.4 mg/kg bw per day for 3 days, and the negative control group was administered the vehicle (i.e. 1% carboxymethylcellulose in water) for 3 days.

The animals were observed for clinical signs, and body weight was recorded daily. Approximately 24 hours after the final dose, the rats were killed and grossly examined. The uteri were removed, and their wet weights were recorded.

No signs of toxicity were noted, and body weight was not affected. There were no gross findings at necropsy. Uterus weights were not increased over the vehicle control values when only mandipropamid had been given. Parallel administration of estradiol benzoate, in contrast, resulted in a marked increase in uterine weight that was similar to that seen in the positive control group.

To conclude, mandipropamid proved negative in this assay. The results suggest that the substance is neither estrogenic nor anti-estrogenic (Twomey, 2001).

(c) Immunotoxicity

An immunotoxicity study was conducted in which groups of 10 female CD-1 mice were fed mandipropamid for 28 days at a concentration of 0, 300, 1000 or 3000 ppm (equal to 0, 56, 187 and 649 mg/kg bw per day, respectively). A positive control group received cyclophosphamide by oral gavage at a dose of 10 mg/kg bw per day for 28 days. On day 25 of treatment, all animals received a single intravenous injection of 2×10^8 sheep red blood cells as the antigen. Blood samples were collected on day 29 for antibody detection. At termination on the same day, liver with gall bladder, spleen and thymus were taken and weighed.

Treatment with mandipropamid was well tolerated. There were no unscheduled deaths, clinical signs or effects on body weight, feed consumption or organ weights in any dose group. No remarkable findings were noted at necropsy. Specific immunoglobulin M (IgM) levels in serum were not different from those in the negative control group.

In contrast, an immunosuppressive effect of the positive control substance was indicated by a statistically significant decrease in serum antibodies (IgM) to the sheep red blood cells. In addition, mean absolute spleen weight was reduced, but this finding was most likely related to a somewhat lower body weight in this group, which might be treatment related. One animal from this group died on test.

On balance, there was no evidence that mandipropamid might affect the T cell–mediated immunological response in mice (Donald & Marr, 2011).

2. Toxicological studies with metabolite SYN500003

The chemical structure of metabolite SYN500003 is shown in Fig. 1.

Fig. 1. Chemical structure of SYN500003

2.1 Acute toxicity

An acute oral toxicity study using the "up-and-down procedure" was performed with female CD (CrL:CD (SD) IGS BR) rats. In total, 11 nulliparous animals 8–12 weeks of age at the start of the study were used. The test substance SYN500003 (batch no. KI-6311/5; purity not given) was suspended in arachis oil (as it did not dissolve in distilled water) and administered to fasted rats by oral gavage at a dosing volume of 10 mL/kg bw. Treatment was conducted in sequence, with at least a 48-hour interval between dosing of individual animals. Because no toxicity was expected, as suggested by available information, a limit test was first performed in five rats that received an oral dose of 2000 mg/kg bw. Based on unexpected deaths in this part of the study, three additional females were administered 2000 mg/kg bw, and three more received a dose of only 550 mg/kg bw. This latter dose was selected because it had been recommended by the statistical program used.

Rats were monitored for mortality and clinical signs, weighed and, after death on test or scheduled termination after the 2-week post-observation period, subjected to gross necropsy.

Three of eight rats receiving the high dose (2000 mg/kg bw) died within 1 day post-dosing, and three more females had to be killed for humane reasons on day 1 or 3. Signs of intoxication comprised hunched posture, lethargy, piloerection, diarrhoea, diuresis, dehydration, ataxia or tiptoe

gait, laboured respiration or a slower respiration rate, distended abdomen and pallor of the extremities. In the two surviving high-dose animals, signs had disappeared by day 4 or 5, with hunched posture being the most persistent. No deaths or clinical signs were noted in the animals receiving the low dose (550 mg/kg bw).

All animals that died on test or had to be killed prematurely lost weight, whereas all survivors gained weight over the post-observation period. In one of the two females surviving high-dose administration, body weight gain was observed only in the second week, but it was the highest among all animals.

In the premature decedents, common gross pathological findings comprised haemorrhagic or at least abnormally red lungs, dark liver and kidney. Gaseous distention of the stomach was also observed in one animal, whereas the non-glandular region of the stomach was haemorrhagic in another one. No abnormalities were seen in the survivors, irrespective of the dose administered.

A median lethal dose (LD_{50}) of 1049 mg/kg bw was calculated for female rats, with 95% confidence limits of 550 and 2000 mg/kg bw (Pooles, 2006). Based on this study, the metabolite is of moderate acute oral toxicity, but is more toxic than the parent mandipropamid, which has an LD_{50} of greater than 5000 mg/kg bw (Moore, 2004).

2.2 Genotoxicity

SYN50003 was evaluated in three genotoxicity studies in vitro and one study in vivo. These studies are summarized in Table 3, and additional details are given below.

Table 3. Summary of genotoxicity studies with SYN500003

Type of study	Test system	Concentration/ dose range tested	Metabolic activation	Batch/ purity (%)	Result	Reference
In vitro						
Reverse gene mutation test in bacteria (Ames test)	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537 and *Escherichia coli* WP2 (pKM101) and WP2*uvr*A (pKM101)	100–5 000 µg/plate	±S9	KI-631/5, 99	Negative	Callander (2006)
Gene mutation in mammalian cells (mouse lymphoma assay)	Mouse lymphoma L5178Y cells ($TK^{+/-}$)	140.4–2 246.0 µg/mL	±S9	AMS 1201/2, 99.4	Negative, cytotoxic at maximum concentrations	Wollny (2013)
Chromosomal aberration	Human lymphocytes	14.6–2 250 µg/mL	±S9	AMS 1201/2, 99.4	Positive without activation, negative with activation	Bohnenberger (2013)
In vivo						
Micronucleus assay	Bone marrow cells of male NMRI mice	500–2 000 mg/kg bw (single dose)		AMS 1201/2, 99.4	Negative	Dony (2013)

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

No evidence of genotoxicity was obtained in the bacterial reverse gene mutation (Ames) assay in four *Salmonella typhimurium* and two *Escherichia coli* strains when tested according to the standard plate incorporation or the preincubation test protocols, with and without metabolic activation. The incubation period for all experiments was 3 days. All the experiments, with and without metabolic activation, were negative. In contrast, appropriate positive controls caused a distinct increase in mutation frequency (Callander, 2006).

Likewise, no increase in gene mutation frequency was observed in the in vitro thymidine kinase assay in L5178Y mouse lymphoma cells, either with or without metabolic activation. An exposure time of 4 hours was chosen for all experiments, followed by a 48-hour incubation of the cultures for expression and exponential growth. The positive control substances (methyl methane sulfonate in the absence of S9 mix and cyclophosphamide in the presence of S9 mix) caused genotoxicity, proving the correct performance of the method and the test system. As no increase in small colonies of $TK^{-/-}$ cells was noted with the test compound, no evidence of chromosomal alterations was found in this in vitro cell system. In contrast, both positive control substances produced an increase not only in general mutation frequency, but also in small colonies (Wollny, 2013).

In contrast, SYN500003 caused structural chromosomal aberrations in human lymphocytes in the absence of metabolic activation in one of two experiments. This finding achieved statistical significance from 734.2 µg/mL onwards and was dose related. The response was nearly equivalent to that of the positive control (Table 4). Substantial cytotoxicity, as evidenced by a more than 50% reduction in mitotic index, was confined to the highest concentration and thus cannot explain the positive response pattern. Moreover, there is no well-established link between mitotic index and frequency of aberrant cells. Some precipitation of the test item in the culture medium was observed only in the second experiment with activation at the maximum concentration. It should be noted that in the positive study, exposure time was longer (22 hours) and cells were immediately harvested, in contrast to the other experiments, with an exposure time of only 4 hours followed by an 18-hour recovery period before harvest and slide preparation. Another difference was that blood had been taken from a 29-year-old male donor for the first (negative) experiment, but from a 36-year-old female donor for the second (positive) one.

Correct performance of the test method and system was shown by clear increases produced by the positive control substances ethyl methane sulfonate and cyclophosphamide. No evidence of numerical aberrations (polyploidy) was observed in any experiment. The positive finding in vitro must be further investigated in vivo before a final conclusion on the clastogenic potential of this metabolite may be drawn (Bohnenberger, 2013).

Table 4. Structural chromosomal aberrations in the second experiment with SYN500003

	Without activation (22-hour exposure, immediate harvest)			With activation (4-hour exposure, 18-hour recovery)		
Test item (concentration in µg/mL)	**Mitotic index (%)**	**Aberrant cells including gaps (%)**	**Aberrant cells excluding gaps (%)**	**Mitotic index (%)**	**Aberrant cells including gaps (%)**	**Aberrant cells excluding gaps (%)**
Solvent control (DMSO)	100	0.5	0.5	100	1.0	1.0
EMS (770)[a]	29.4	53.0	51.0**	–	–	–
CPA (7.5)	–	–	–	52.9	13.0	12.5**
SYN500003 (419.8)	84.7	2.0	2.0	–	–	–
SYN500003	62.5	5.0	5.0*	88.6	1.5	1.5

Test item (concentration in µg/mL)	Without activation (22-hour exposure, immediate harvest)			With activation (4-hour exposure, 18-hour recovery)		
	Mitotic index (%)	Aberrant cells including gaps (%)	Aberrant cells excluding gaps (%)	Mitotic index (%)	Aberrant cells including gaps (%)	Aberrant cells excluding gaps (%)
(734.2)						
SYN500003 (1285.7)	53.3	8.0	7.5**	84.4	1.0	1.0
SYN500003 (2 250.0)[a]	33.1	38.0	38.0**	68.5	0.0	0.0

CPA: cyclophosphamide; DMSO: dimethyl sulfoxide; EMS: ethyl methane sulfonate; *: $P < 0.005$; **: $P < 0.001$ (Fisher's exact test)

[a] In the absence of activation, only 50 instead of 100 metaphases per culture were evaluated, due to the strong clastogenic effect.

Source: Bohnenberger (2013)

In an in vivo micronucleus assay in mouse bone marrow, SYN500003 proved negative. The test item was suspended in 1% carboxymethylcellulose and, in the main study, administered once by oral gavage at a dosing volume of 10 mL/kg bw to seven male NMRI mice per dose and sampling time. Animals were killed, and bone marrow cells were collected at either 24 or 48 hours post-dosing. The doses were 500, 1000 and 2000 mg/kg bw for the 24-hour preparation interval, whereas the animals to be killed after 48 hours received only the limit dose of 2000 mg/kg bw. The negative (vehicle) and positive control groups consisted of five animals. The positive control substance was cyclophosphamide, applied at a dose of 40 mg/kg bw. Two negative control groups (killed 24 or 48 hours after treatment) but only one positive control group (killed at 24 hours post-dosing) were included.

Animals were monitored for mortality and the occurrence of clinical signs. Bone marrow was sampled, and slides were prepared for microscopic evaluation for signs of cytotoxicity and micronucleus formation. In 2000 erythrocytes per slide, the ratio between normochromatic erythrocytes (NCEs) and polychromatic erythrocytes (PCEs) was determined. Two thousand PCEs per animal were analysed for the presence of micronuclei, using the same slides.

In the high-dose group, one male mouse was found dead 24 hours after treatment with SYN500003. This single death may have been treatment related. The animal was discarded and not used for evaluation of clastogenicity. Signs of toxicity in the high-dose group comprised ruffled fur and a reduction in spontaneous activity. In a pretest, hunched back and eyelid closure had also been observed at the same dose. No evidence of systemic toxicity was obtained in the low- and mid-dose groups.

The micronucleus incidence in all the groups receiving SYN500003 was not different from the frequency noted in the negative control groups. In contrast, a clear increase in micronuclei was observed in the positive control group. The range of micronucleus occurrence in test group animals was 0–6 per 2000 PCEs, compared with 1–4 in the negative control animals but 26–53 in the positive control animals. Thus, the outcome of this in vivo assay was negative. However, the PCE:NCE ratio was not skewed in any group. Thus, there was no evidence of bone marrow toxicity, and, in the absence of absorption, distribution, metabolism and excretion data for this metabolite, it could not be proven that the bone marrow had, in fact, been exposed to the test item (Dony, 2013).

For clarification on bone marrow exposure, additional studies were performed to demonstrate the presence of SYN500003 in mouse plasma following oral administration (Britton, 2018; Kosar, 2018).

An analytical method for the determination of SYN500003 in mouse plasma was validated. The metabolite was recovered from plasma by protein precipitation, and the processed samples were analysed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) detection. Validation was performed for a wide range of concentrations (10–4000 ng/mL). Sufficient precision, accuracy and specificity could be shown. Stability of the metabolite in mouse plasma was also demonstrated (Kosar, 2018).

Three male Crl:CD-1 (ICR) mice were dosed with SYN500003 (batch no. AMS 1201/2; purity 99.4%) in 1% carboxymethylcellulose by gavage at a dose of 1000 mg/kg bw and a dosing volume of 10 mL/kg bw. All animals were observed for any visible signs of reaction to treatment, and body weights were recorded. Blood samples were taken at 0.5, 1, 4 and 24 hours after dosing, either from the tail vein or, at termination, from the orbital sinus. Animals were killed following their final blood sample and discarded without necropsy.

There were no deaths, and no clinical signs were observed during the study. Systemic exposure to SYN500003 was confirmed in all three animals by detectable circulating concentrations of SYN500003 in plasma at 0.5, 1, 4 and 24 hours after dosing (Table 5). As validation of the analytical method had been performed up to a maximum concentration of 4000 ng/mL, the samples collected at 0.5, 1 and 4 hours were diluted, as indicated in the table notes.

Table 5. Plasma concentrations of SYN500003 in male mice following oral dosing at 1000 mg/kg bw

Animal number	Hours after application	Plasma concentration (ng/mL)
1	0.5	386 000[a]
	1	438 000[a]
	4	51 800[b]
	24	2 850
2	0.5	363 000[a]
	1	380 000[a]
	4	127 000[b]
	24	442
3	0.5	415 000[a]
	1	583 000[a]
	4	299 000[b]
	24	285

[a] Diluted to below 4000 ng/mL: 1:200 dilution.
[b] Diluted to below 4000 ng/mL: 1:100 dilution.
Source: Britton (2018)

As rather high concentrations of the metabolite were detected in plasma and as bone marrow is well perfused, it can be considered as proven that the bone marrow was adequately exposed in the micronucleus test (Dony, 2013), in which an even higher oral dose of 2000 mg/kg bw had been applied. It is not clear why a different mouse strain than the one used in the original micronucleus assay was used, but this difference is not considered to alter the conclusion (Britton, 2018).

Comments

Toxicological data

In a 90-day feeding study in mice, target organs were the liver and the erythropoietic system, with possible involvement of the spleen. The NOAEL was 800 ppm (equal to 98.0 mg/kg bw per day), based on evidence of slight anaemia, higher liver weight and cytoplasmic eosinophilia of hepatocytes at 2000 ppm (equal to 247.6 mg/kg bw per day). A lower spleen weight in females from 800 ppm onwards was of equivocal toxicological relevance (Milburn, 2005). The effects resembled those observed in other species, and the NOAEL was higher than those identified in short-term studies in rats and dogs (Annex 1, reference *115*).

In a 28-day study in female mice, the T cell–mediated immunological response was not affected by administration of mandipropamid up to the highest dose of 3000 ppm (equal to 649 mg/kg bw per day) (Donald & Marr, 2011).

The Meeting considered that the mechanistic studies submitted did not provide any information relevant to the evaluation of mandipropamid.

Toxicological data on metabolites and/or degradates

The potato metabolite of mandipropamid, SYN500003, was more toxic than the parent compound in an acute oral toxicity study in rats, with an LD_{50} of 1049 mg/kg bw (Pooles, 2006), compared with greater than 5000 mg/kg bw for the parent compound (Moore, 2004).

SYN500003 gave negative results in gene mutation tests in bacteria (Callander, 2006) as well as in mammalian cells (Wollny, 2013). However, it gave a positive response in a chromosomal aberration study in human lymphocytes (Bohnenberger, 2013). A negative result was seen in a micronucleus assay in mouse bone marrow in which 2000 mg/kg bw, the highest dose tested, produced clinical signs of toxicity; one possibly treatment-related death was also noted (Dony, 2013). In a “proof of exposure” study, high concentrations of the metabolite were detected in plasma following oral administration of 1000 mg/kg bw to rats (Britton, 2018). The abundance of SYN500003 in plasma can be considered as evidence that a sufficient amount had reached the bone marrow in the micronucleus test.

The Meeting concluded that SYN500003 is unlikely to be genotoxic in vivo.

Toxicological evaluation

The Meeting concluded that no revision of the ADI for mandipropamid was necessary.

For the metabolite SYN500003, the ADI for mandipropamid is not applicable, as the metabolite is structurally different from the parent compound and is more acutely toxic. No separate ADI or ARfD can be established for the metabolite, as the database is not sufficient for this purpose.

For chronic toxicity, the threshold of toxicological concern (TTC) concept for non-genotoxic substances (Cramer class III) is applicable to this metabolite.

References

Bohnenberger S (2013). SYN 500003 – chromosome aberration test in human lymphocytes in vitro. Unpublished report no. 1555801. Harlan CCR on behalf of Syngenta Ltd. Submitted to WHO by Syngenta Ltd.

Britton L (2018). SYN500003 – oral (gavage) proof of exposure study in the mouse. Unpublished report no. BFI0741. Sequani Ltd on behalf of Syngenta Ltd. Submitted to WHO by Syngenta Ltd.

Callander RD (2006). SYN 500003: bacterial mutation assay in *S. typhimurium* and *E. coli*. Unpublished report no. YV7195-REG (Syngenta no. T003954-05). Syngenta Central Toxicology Laboratory. Submitted to WHO by Syngenta Ltd.

Donald L, Marr C (2011). Mandipropamid – a 28 day immunotoxicity study by oral (dietary) administration in mice using sheep red blood cells as the antigen. Unpublished report no. 32617. Charles River Laboratories. Submitted to WHO by Syngenta Ltd.

Dony E (2013). SYN 500003 – micronucleus assay in the bone marrow cells of the mouse. Unpublished report no. 1555800. Harlan CCR on behalf of Syngenta Ltd. Submitted to WHO by Syngenta Ltd.

Dow J (2007a). NOA446510: effects in the liver following dietary administration in the mouse. Unpublished report no. CTL/026543/TEC/RE-001. Syngenta Central Toxicology Laboratory. Submitted to WHO by Syngenta Ltd.

Dow J (2007b). NOA446510: effects on the liver following dietary administration in the rat. Unpublished report no. CTL/026544/TEC/RE-001. Syngenta Central Toxicology Laboratory. Submitted to WHO by Syngenta Ltd.

Kosar F (2018). SYN500003 – validation of a bioanalytical method for the determination of SYN500003 in mouse plasma. Unpublished report no. BFI0743. Sequani Ltd on behalf of Syngenta Ltd. Submitted to WHO by Syngenta Ltd.

Lees D (2006). Mandipropamid – investigation of cell proliferation in the liver in female rats. Unpublished report no. XR7561-TEC. Syngenta Central Toxicology Laboratory. Submitted to WHO by Syngenta Ltd.

Milburn G (2005). NOA446510: 90 day dietary toxicity study in the mouse. Unpublished report no. PM1265-REG. Syngenta Central Toxicology Laboratory. Submitted to WHO by Syngenta Ltd.

Moffat GJ (2001). AMS (NOA446510): assessment of intrinsic estrogenic/androgenic activity in vitro. Unpublished report (report number not provided). Syngenta Central Toxicology Laboratory. Submitted to WHO by Syngenta Ltd.

Moore GE (2004). NOA446510 tech. (design code NOA446510A): acute oral toxicity up and down procedure in rats. Unpublished report no. 14702. Product Safety Laboratories, Dayton, NJ, USA. Submitted to WHO by Syngenta Crop Protection Inc. [cited in Annex 1, reference *115*].

Pooles A (2006). SYN 500003: acute oral toxicity in the rat – up and down procedure. Unpublished report (report number not provided). SafePharm Laboratories. Project no. 0006/0737. Submitted to WHO by Syngenta Ltd.

Shirley E (1977). The analysis of organ weight data. Toxicology. 8(1):13–22.

Twomey K (2001). NOA446510: uterotrophic assay in immature rats for PDT. Unpublished report no. CTL/ZR1602. Syngenta Central Toxicology Laboratory. Submitted to WHO by Syngenta Ltd.

Wollny H-E (2013). SYN 500003 – cell mutation assay at the thymidine kinase locus ($TK^{+/-}$) in mouse lymphoma L5178Y cells. Unpublished report no. 1555802. Harlan CCR on behalf of Syngenta Ltd. Submitted to WHO by Syngenta Ltd.

NORFLURAZON

First draft prepared by
Lars Niemann[1] and Alan Boobis[2]

[1] Department of Pesticides Safety, German Federal Institute for Risk Assessment, Berlin, Germany
[2] Department of Medicine, Imperial College London, London, United Kingdom

Explanation 421
Evaluation for acceptable intake 422
1. Biochemical aspects 422
1.1 Absorption, distribution and excretion 422
1.2 Biotransformation 423
2. Toxicological studies 424
2.1 Acute toxicity 424
(a) Lethal doses 425
(b) Dermal irritation 427
(c) Ocular irritation 427
(d) Dermal sensitization 427
2.2 Short-term studies of toxicity 428
(a) Oral administration 428
(b) Dermal application 437
(c) Exposure by inhalation 437
2.3 Long-term studies of toxicity and carcinogenicity 437
2.4 Genotoxicity 449
2.5 Reproductive and developmental toxicity 450
(a) Single-generation and multigeneration studies 450
(b) Developmental toxicity 458
2.6 Special studies 461
(a) Neurotoxicity 461
(b) Immunotoxicity 461
(c) Mechanistic studies 461
(d) Studies on endocrine properties 461
3. Observations in humans 463
Comments 463
Toxicological evaluation 467
References 470

Explanation

Norflurazon (Fig. 1) is the International Organization for Standardization–approved common name for 4-chloro-5-(methylamino)-2-(α,α,α-trifluoro-*m*-tolyl)-3-(2*H*)-pyridazinone (International Union of Pure and Applied Chemistry), with the Chemical Abstracts Service number 27314-13-2.

Norflurazon is a fluorinated pyridazinone compound that is applied as a pre-emergence herbicide for selective control of annual grasses and broadleaf weeds in a number of crops, mostly in fruits, but also in cotton, hops, almonds and walnuts. In susceptible plant species, it inhibits carotenoid biosynthesis by inhibition of phytoene desaturase, resulting in chlorophyll photodegradation and eventually chlorosis (Saunders et al., 1985; USEPA, 2001; Lamberth, 2017).

Norflurazon 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.

Even though all relevant toxicological end-points are covered by submitted studies, it should be noted that most studies are very old, were not performed under good laboratory practice (GLP) and

did not follow current test guidelines, except where otherwise specified. Published literature has been taken into consideration, but is generally very limited.

Fig. 1. Chemical structure of norflurazon

F F F N N H_3C NH O Cl

Evaluation for acceptable intake

1. Biochemical aspects

1.1 Absorption, distribution and excretion

The absorption, distribution and excretion of ^{14}C-labelled norflurazon (purity 98.8%; radiolabelled in the trifluoromethyl moiety) were investigated in female Sprague Dawley rats (five per dose) following a single oral gavage administration of 2 or 110 mg/kg body weight (bw) in corn oil. A third group of the same size was fed the non-radiolabelled test substance for 14 days at a dietary dose of 2 mg/kg bw per day followed by a radiolabelled dose of 2 mg/kg bw administered 24 hours later by oral gavage. In addition, two females received 2 mg/kg bw of radiolabelled norflurazon as an intravenous injection in physiological saline and ethanol (1:1). Following treatment, rats were caged in all-glass metabolism chambers to collect urine and faeces separately. One of the orally dosed rats in the low-dose group was additionally monitored for expired radiolabelled volatiles. Ninety-six hours after administration of the single or the final repeated dose, the rats were killed, and selected tissues were removed for subsequent analysis for radioactivity.

No analysis of blood for pharmacokinetic parameters was performed.

As no bile duct cannulation group was included, oral absorption could be estimated either on the basis of urinary excretion only or by comparison of faecal elimination following oral and intravenous administration, even though only two animals were in the intravenous group. Based on urinary excretion only, absorption appeared to be as low as 20%. However, faecal excretion accounted for more than 70% of the intravenous dose and was similar to that after oral dosing. Accordingly, oral absorption of norflurazon appeared to be quantitative, at least above 90%. Extensive metabolism (see section 1.2) also suggested high oral absorption. Most of the applied radioactivity was eliminated via the faeces, but urinary excretion was also significant. About 80% of the low dose was eliminated within 24 hours; in contrast, excretion of the high dose was slower, but virtually complete after 96 hours (see Table 1). Previous dietary administration of the non-radiolabelled material had no impact on this pattern.

Exhalation was negligible (0.1% of the applied radioactivity), but investigation was confined to a single animal.

Tissue residues were generally low, with the highest relative radioactivity observed in liver and kidneys (Saunders et al., 1985).

Table 1. Elimination and recovery of ^{14}C-labelled norflurazon in female rats over 96 hours

Matrix (time point)	Mean % of administered radioactivity			
	Oral, low dose	**Intravenous, low dose**	**Oral, high dose**	**Repeated oral, low dose**
Urine (24 hours)	18.4	24.8	8.1	24.7
Urine (96 hours)	21.3	28.4	18.5	27.1
Faeces (24 hours)	58.3	57.0	40.1	54.7
Faeces (96 hours)	77.8	70.7	79.5	65.3
Selected tissues	0.5	0.4	0.2	0.3
Carcass	0.4	0.5	0.4	0.5
Total recovery	100.0	100.0	98.6	93.2

Source: Saunders et al. (1985)

In an older study, excretion of norflurazon labelled with both ^{3}H and ^{14}C was studied in 10 male Wistar rats following daily oral gavage administration of 10 mg of the test substance (a 1% suspension in 0.5% aqueous carboxymethylcellulose) for 15 days. Urine and faecal samples were collected and pooled either for the entire study duration (five animals) or for the individual days (five animals). Twenty-four hours after the final dose, rats were killed, and blood, liver, spleen, kidney, testes, brain, muscle and fat were taken for subsequent analysis for radioactivity.

Total recovery of radioactivity was remarkably variable in the individual rats. Elimination in both urine and faeces accounted for 60% to nearly 100% when the entire treatment period was considered. In the rats for which data from individual days were available, a mean of about 75% of the administered radioactivity was excreted. The fate of the remaining portion is unknown, as only traces were found in the tissues, but no precise measurements were reported. The data obtained with either the ^{3}H or the ^{14}C label demonstrated sufficient parallelism. The predominant excretion route was via the faeces. In the five individual animals observed for the entire study period, faecal excretion accounted for 45–74% of the radiolabels, whereas 13–26% of the radiolabels were found in urine. In the group of five male rats for which daily collections were pooled, an average of 56–59% of the radiolabels were found in faeces and 17–19% in urine, depending on the radiolabel.

This study does not comply with modern standards, and the information provided is limited. However, the study may be considered complementary to the more comprehensive and reliable study by Saunders et al. (1985) reported above and allows the conclusion that excretion in male and female rats is similar (Karapally, 1974).

In the report, reference is made to even older studies that were not available to the current Meeting.

1.2 Biotransformation

In the study described in section 1.1 in which female Sprague Dawley rats were administered ^{14}C-labelled norflurazon by gavage, biotransformation was extensive, as only 2% or less of the administered doses were excreted as unchanged parent. The main metabolic pathways were *N*-demethylation and glutathione conjugation. Replacement of the chlorine in norflurazon by hydrogen was characterized as an additional, but minor, metabolic pathway.

Numerous metabolites, which were analysed by liquid and thin-layer chromatography and mass spectrometry, were found in urine and faeces, of which nine could be identified. Only one of them (metabolite 5, a sulfoxide) accounted for more than 10% of the applied dose (up to 39% in urine,

following intravenous administration), whereas the others represented only 1–2% (Saunders et al., 1985).

The results of the above study were also published in one of the very few articles on the toxicology of norflurazon that were retrieved from the open literature. No new information was given, but the (most abundant in urine) sulfoxide metabolite was designated as metabolite 3 (Quistad et al., 1989).

In the older study by Karapally (1974) in which male Wistar rats were administered norflurazon labelled with both ^{3}H and ^{14}C by gavage (see section 1.1), metabolites were detected and identified by thin-layer and column chromatography and by gas chromatography–mass spectrometry, following amberlite purification and several derivatization steps (methylation, acetylation and trimethylsilylation). Metabolism of norflurazon was extensive, with hydroxylation, demethylation and sulfur conjugation being the main pathways. Only low amounts of the parent compound were found in urine (0.1%) and faeces (5.4%). Metabolites were not fully characterized, and their number cannot be determined from the report.

Consideration of the two studies together allows the conclusion that metabolism in male and female rats is similar (Karapally, 1974; Saunders et al., 1985).

2. Toxicological studies

2.1 Acute toxicity

A summary of acute toxicity, skin and eye irritation, and skin sensitization studies with the active ingredient norflurazon is given in Table 2.

Table 2. Acute toxicity studies (including skin and eye irritation and skin sensitization) with norflurazon (active ingredient)

Study type	Species	Batch and purity	Result	Reference
Oral toxicity	Rat (males only)	Not available	LD_{50} = 9 300 mg/kg bw	Bagdon (1972)
Dermal toxicity	Rabbit (males only)	Not available	LD_{50} > 20 000 mg/kg bw	Bukva (1970)
Inhalation toxicity (4-hour nose-only exposure)	Rat	Lot no. 0920376; purity 98.3%	LC_{50} > 2.4 mg/L air	Hoffman (1996)
Skin irritation	Rabbit	Not available	Not irritating	Bagdon (1972)
Eye irritation	Rabbit	Not available	Not irritating	Bagdon (1972)
Skin sensitization	Guinea-pig	Not available	Inconclusive	Bagdon (1973)

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

In addition, the sponsor submitted a number of (more recent) acute toxicity studies in which formulations containing norflurazon were tested. Even though such studies are normally not taken into consideration by JMPR, it was decided to report those with the plant protection product Norflurazon 80 DF, which contains 80% active ingredient, because of the limited database available for norflurazon. An overview of these studies is given in Table 3. Studies with another formulation containing less than 5% norflurazon were submitted by the sponsor but were excluded from the evaluation.

Table 3. Acute toxicity studies (including skin and eye irritation and skin sensitization) with the formulation Norflurazon 80 DF[a]

Study type	Species	Result	Reference
Oral toxicity	Rat	LD_{50} = 1 210 mg/kg bw (males) and 1 080 mg/kg bw (females)	Gardner (1987a)
Dermal toxicity	Rat	LD_{50} > 2 000 mg/kg bw	Gardner (1987b)
Skin irritation	Rabbit	Very slightly irritating	Liggett & Smith (1988)
Eye irritation	Rabbit	Slightly irritating	Liggett & Smith (1987)
Skin sensitization (Buehler method)	Guinea-pig	Not sensitizing	Kynoch & Parcell (1987)

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

[a] Batch no. 1130377; 80% norflurazon content.

The studies summarized in the above two tables are described in more detail below.

(a) Lethal doses

Oral administration

In an acute toxicity study in rats, a herbicide with the designation “9789 technical grade” was the test item. The sponsor confirmed that the technical active ingredient was norflurazon. The test compound was suspended in 1% carboxymethylcellulose with 0.2% Tween 80 and administered to 10 male Sprague Dawley rats per group at a dose of 5000, 10 000 or 15 000 mg/kg bw following a 16-hour fast. Following treatment, the survivors were observed for 7 days and then killed. At 5000 mg/kg bw, two deaths occurred within 24 or 72 hours post-dosing, respectively. At 10 000 mg/kg bw, five rats died, all within the first 24 hours. At 15 000 mg/kg bw, nine rats died on the first day, whereas the remaining animal survived. In all groups, toxic signs consisted of decreased locomotor activity, ptosis, sedation, muscle tremors and clonic or tonic convulsions, which became more pronounced with increasing dose. All these signs occurred within 4 hours after application and had resolved in the survivors after 1 or 2 days. Based on the mortality pattern, a median lethal dose (LD_{50}) of 9300 mg/kg bw (standard deviation ± 1358) was calculated (Bagdon, 1972).

In another acute toxicity study, the formulation Norflurazon 80 DF (Zorial Rapid 80 DF; batch no. 1130377; purity 80%) was dissolved in 1% aqueous methylcellulose and administered at a volume of 10 mL/kg bw to male and female CD rats. Subsequent to a preliminary test in which two male and two female rats had been dosed with 1000 or 2500 mg/kg bw, a limit test with a dose of 5000 mg/kg bw was carried out in five rats of each sex. As a next step, further groups of five males and five females each received a dose of 800, 1260 or 2000 mg/kg bw. This last experiment was considered the main study and was reported in the most detail. All surviving animals were killed 15 days post-dosing. All animals (including those that died on test) were necropsied and grossly examined.

The number of presumably treatment-related deaths in all groups is summarized in Table 4.

The mortality data appear somewhat contradictory, in particular when the data from the first and second phases of the study are compared. Apparently based on the main study only, LD_{50} values of 1210 mg/kg bw for males and 1080 mg/kg bw for females were calculated by probit analysis. The combined value for both sexes was 1140 mg/kg bw, with a confidence interval ranging from 900 to 1420 mg/kg bw. Clinical signs were observed in all animals from all groups, occurred within 5 hours after dosing and comprised piloerection, ptosis, hunched posture, lethargy, abnormal gait, decreased respiratory rate, pallor of the extremities and increased salivation. Straub tail and jumping movements were noticed from 1260 mg/kg bw onwards, and collapse or clonic convulsions at 2000 mg/kg bw. In the survivors, recovery was complete by day 6 or 7 at the latest. Body weight development appeared

to be impaired in the survivors during the first week after treatment, but subsequently normalized. Severe body weight losses were noted prior to death, whereas the survivors gained weight. Terminal necropsy did not reveal remarkable gross changes that could be attributed to treatment or could explain the mortalities (Gardner, 1987a).

Table 4. Mortality in an acute oral toxicity study with Norflurazon 80 DF

Dose (mg/kg bw)	Animals on study (M/F)	Males		Females	
		No. of deaths	Days of deaths	No. of deaths	Days of deaths
800	5/5	0	–	2	2
1 000	2/2	1	1	0	–
1 260	5/5	3	2, 4	2	2
2 000	5/5	5	1, 2	5	2
2 500	2/2	0	–	2	1
5 000	5/5	5	1	4	1

bw: body weight; F: female; M: male
Source: Gardner (1987a)

Based on this study, and taking into account the rather high concentration of the active ingredient in this product, it may be concluded that norflurazon is of moderate acute oral toxicity. This result is somewhat contrary to the result of the older study by Bagdon (1972), which suggests either a different impurity profile of the active ingredient or a strong impact of co-formulants in the tested product. As the sponsor denied the possibility that co-formulants might have enhanced the toxicity of norflurazon, the more recent, higher-quality study with the formulation is considered the more reliable study with which to assess the acute oral toxicity of norflurazon.

Dermal application

In an acute dermal toxicity study, SAN 9789 (norflurazon, batch and purity not given) was applied for 24 hours to the shaved backs of male New Zealand white rabbits (three per dose group) at a dose of 5000, 10 000 or 20 000 mg/kg bw. No deaths, clinical signs of toxicity or gross abnormalities at necropsy after 7 days were observed in any dose group. The LD_{50} was in excess of 20 000 mg/kg bw. The occurrence or absence of local effects (skin irritation) was not reported (Bukva, 1970).

In a dermal limit test, the formulation Norflurazon 80 DF (Zorial Rapid 80 DF; batch no. 1130377; purity 80%) was prepared as a 100% (weight/volume) paste in distilled water and administered to the dorsolumbar region of five male and five female CD rats at a volume of 2 mL/kg bw. The applied dose was 2000 mg/kg bw. Exposure time was 24 hours, and the animals were observed for 14 days before being killed. All rats survived, remained healthy and gained weight. No signs of irritation or necropsy findings were noted. The LD_{50} in this study was greater than 2000 mg/kg bw (Gardner, 1987b). This study with a formulation was regarded as better suited for the assessment of the acute dermal toxicity of norflurazon than the outdated study with the active ingredient itself.

Exposure by inhalation

In one of the few studies with norflurazon that have been performed according to current standards, acute inhalation toxicity was examined in five male and five female Crl:CD (SD)BR rats exposed (nose-only) for 4 hours to norflurazon (lot no. 0920376; purity 98.3%) generated as a dust.

The intended concentration was 2 mg/L; the analytically verified mean actual concentration was 2.4 mg/L. A mass median aerodynamic diameter of 2.9 µm was determined, with a geometric standard deviation of 2.2. Animals were monitored for mortality and any signs of toxicity or distress during exposure and for 14 days following exposure. No mortalities were observed. During or immediately after exposure, clinical signs (in 1–5 rats of each sex) were excessive salivation, chromodacryorrhoea, red nasal discharge, dried red or brown material on the facial area, laboured breathing and, occasionally, rales. All animals appeared normal by day 3. No systemic effects were noted, all animals gained weight and no gross lesions were observed at necropsy. The inhalation median lethal concentration (LC_{50}) in rats was greater than 2.4 mg/L (Hoffman, 1996).

(b) Dermal irritation

Application of norflurazon (500 mg) to the shaved and partly abraded skin of three male and three female rabbits for 24 hours under occlusion did not cause skin erythema or oedema (Bagdon, 1972). Furthermore, it was reported in the skin sensitization study (see below) that no signs of irritation were observed in guinea-pigs (Bagdon, 1973).

In a guideline-compliant study with the formulation Norflurazon 80 DF in male New Zealand white rabbits, only marginal evidence of skin irritation was obtained. Following a 4-hour exposure under occlusion, very slight erythema was noted in three out of six animals on day 1 and was still visible in two of them on day 2. No signs of skin irritation were apparent in the remaining rabbits (Liggett & Smith, 1988). This study appears more reliable than the much older study reported above.

(c) Ocular irritation

In a study with norflurazon technical active ingredient in three male and three female rabbits, no signs of eye irritation were observed (Bagdon, 1972).

In a guideline-compliant study, the formulation Norflurazon 80 DF proved slightly irritating to the eyes of New Zealand white rabbits, as conjunctival redness, chemosis and discharge were observed in all animals 1 hour after instillation and afterwards. All these signs had disappeared within 2–3 days. No damage to the cornea or iris was noted in any animal (Liggett & Smith, 1987). This study appears more reliable than the much older study reported above.

(d) Dermal sensitization

The skin sensitization potential of norflurazon was evaluated in groups of six male Hartley strain guinea-pigs following intradermal or topical induction. For intradermal induction, a 0.1% solution of the test compound was prepared in 1% carboxymethylcellulose and 0.2% Tween 80 and injected into the upper dorsal quadrant of the back 3 times per week until 10 injections had been given. For challenge, a further intradermal dose was applied 2 weeks after the final induction. The same treatment regimen was performed in the negative control group receiving only the vehicle and in a positive control group of the same size. The positive control substance was 0.1% phenylenediamine hydrochloride dissolved in 0.9% saline. For topical induction, guinea-pigs were administered either the test compound 10 times (every 2 or 3 days) as an aqueous paste or 2% phenylenediamine hydrochloride in 0.9% saline. Following each treatment, the respective sites were occluded for 6 hours. Topical challenge was performed 2 weeks after the final induction.

In the intradermal induction group, guinea-pigs exhibited slight erythema and in two cases also slight oedema due to injection of norflurazon. However, following challenge, no erythema was seen; although oedema was noted in one animal only, it disappeared by 48 or 72 hours. After topical induction and challenge, no evidence of irritation or sensitization was noted. These findings cannot be taken as convincing evidence that norflurazon is not sensitizing, as the positive control substance also did not cause a positive response (Bagdon, 1973).

A product study with Norflurazon 80 DF was performed in female guinea-pigs of the Hartley/Dunkin strain by means of a modified Buehler test with nine inductions. For induction and challenge, 60% dilutions of the test item were prepared in distilled water. Both the test group and the negative control group comprised 10 animals. None of the test or control animals exhibited any dermal reaction following induction or challenge (Kynoch & Parcell, 1987). However, no positive control group was included, so the reliability of the test cannot be proven. In addition, the Buehler test is now considered less predictive than the local lymph node or the maximization assay.

2.2 Short-term studies of toxicity

(a) Oral administration

Mice

The only available short-term toxicity study in mice was a range-finding experiment performed in preparation for a long-term toxicity and carcinogenicity study (see section 2.3). Groups of five weanling male and female CD-1 (HaM/ICR Swiss) mice were administered the test substance (batch no. CH0004; purity 98.8%) via their diet for 28 days. The nominal dietary concentrations were 0, 70, 210, 420 and 2520 parts per million (ppm) (equivalent to 0, 10.5, 31.5, 63 and 378 mg/kg bw per day, respectively). The animals were monitored for mortality, clinical signs, abnormal behaviour, body weight and feed consumption. At necropsy, the mice were examined for gross changes, and weights of liver and kidneys were determined.

No changes in mortality, clinical signs, behaviour, body weight or feed consumption or remarkable findings were noted that could be attributed to treatment. In males, dose-related increases in absolute and relative liver weights were observed at 210 ppm and above, whereas a similar effect on liver weight in females was confined to the highest-dose group. Absolute kidney weights were not altered, but there was an increase in relative kidney weight in male mice at 210 ppm and above. In females, in contrast, relative kidney weight was lower at the top dose level. The only gross lesions were noted in males and females at the highest dose, as livers in some, but not all, animals had granular or diffuse smooth granular appearance that was interpreted to indicate fatty degeneration. No recommendation for dose selection in the long-term toxicity and carcinogenicity study was given in the report (Tisdel, 1971).

Rats

In a 28-day feeding study, norflurazon (SAN9789; batch and purity not given) was administered to groups of 10 male and 10 female CFE strain rats per group at a dietary concentration of 0, 500, 1000 or 5000 ppm (equal to 0, 52, 105 and 517 mg/kg bw for males and 0, 70, 140 and 717 mg/kg bw for females, respectively). The end-points evaluated in this study included body weight, feed consumption, clinical signs, haematology, clinical chemistry and urine analysis parameters, gross pathology and organ weights (liver, kidneys, adrenals, brain and testes). Histopathological examination comprised a representative range of organs and tissues, but, with the exception of liver and kidney, was confined to the control and high-dose groups.

There were no test substance–related deaths. Clinical signs were confined to excessive shedding of hair, rough fur and mild alopecia in high-dose males and females. Slight to moderate rales were noted in all groups, including the controls, but were a bit more pronounced at the high dose. This sign might indicate infection, which could call the study quality into question.

High-dose males exhibited a lower mean body weight from the beginning of the study, with the difference relative to the control group achieving statistical significance at study termination in week 4. This effect was clearly not due to lower feed intake, as mean feed consumption was not different among the groups. No effects on body weight were noted in low- or mid-dose males or in females in any treatment group.

Haematology, clinical chemistry and urine analysis did not reveal any findings that could be attributed to substance administration. It must be emphasized, however, that the range of examined haematological and clinical chemistry parameters was quite limited and did not comply with current standards.

No treatment-related gross findings were observed at necropsy in any group. No absolute organ weights were reported. Dose-related, statistically significant increases in relative liver weight were seen in the mid- and high-dose groups in males and in all treatment groups in females. Dose-related, statistically significant increases in relative kidney weight were seen in males in all treated groups, whereas relative kidney weight was increased in high-dose females only. Relative testis weight was higher at the high dose, but this finding was presumably due to lower mean body weight. Relative adrenal weights exhibited a very unique pattern that cannot be explained (Table 5). However, it must be noted that adrenal weights generally may be subject to wide variation.

Table 5. Terminal body weight and relative organ weights in the 28-day feeding study in rats

	Relative organ weight (%)							
	Males				**Females**			
Organ	**0 ppm**	**500 ppm**	**1 000 ppm**	**5 000 ppm**	**0 ppm**	**500 ppm**	**1 000 ppm**	**5 000 ppm**
Terminal body weight (g)	*217.1*	*208.8*	*210.4*	*172.7**	*157.1*	*156.9*	*155.8*	*150.9*
Liver	4.68	5.12	5.77*	7.64*	4.58	5.28*	5.53*	7.77*
Kidneys[a]	0.82	0.90*	0.91*	0.98*	0.87	0.92	1.03	1.12*
Adrenals[a]	0.024	0.032*	0.030	0.024	0.043	0.047	0.053*	0.036**
Testes	1.36	1.52	1.46	1.68*	–	–	–	–

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (analysis of variance)
[a] The left and right kidney weights and the left and right adrenal weights appear to have been combined.
Source: Fogleman (1970)

Histopathology revealed hypertrophy and hyperplasia of liver cells in male and female rats receiving 1000 or 5000 ppm. Cloudy swelling, enlargement of nuclei, karyolysis and karyorrhexis, and necrosis were sometimes described in liver samples taken from these groups. In females, cloudy swelling and vacuolization of the renal tubules and slight tubular calcinosis were observed in a few animals at the intermediate and high doses.

This study does not comply with current standards and is poorly reported. Based on the relatively limited information reported, the no-observed-adverse-effect level (NOAEL) was identified as 500 ppm (equal to 52 mg/kg bw per day), based on relative liver and kidney weight increases, accompanied by histopathological lesions, at 1000 ppm (equal to 105 mg/kg bw per day) (Fogleman, 1970).

In a 90-day feeding study, norflurazon (SAN9789; batch and purity not given) was administered to groups of 20 male and 20 female CFE strain rats per group at a dietary concentration of 0, 250, 500 or 2500 ppm (equal to 0, 24, 45 and 248 mg/kg bw per day for males and 0, 26, 52 and 275 mg/kg bw per day for females, respectively). In addition to mortality and the occurrence of clinical signs, the end-points evaluated in this study included body weight (determined once a week), feed consumption, gross pathology, and weights of liver, kidneys, adrenals, heart, brain, thyroid and testes. Clinical pathological examinations were performed at scheduled termination. Haematological parameters included erythrocyte and leukocyte counts, haematocrit, haemoglobin and differential white blood cell count. The clinical chemistry parameters comprised glucose, urea nitrogen and uric

acid, total serum protein and albumin, aspartate aminotransferase, alkaline phosphatase, lactate dehydrogenase, cholesterol, bilirubin, calcium and phosphate. During urine analysis, pH and urinary sediment were examined, along with tests for sugar, ketone bodies, albumin and occult blood. Histopathological examination at scheduled termination was performed on a representative range of organs and tissues from five males and five females from each group. Five male and five female rats were randomly selected from each group and killed after 4 weeks of feeding. Clinical chemistry parameters were measured, animals were necropsied and organs (except thyroid) were weighed. Histopathology was apparently not performed in these rats.

There were four unscheduled deaths during the study, all of them close to termination. A low-dose female had a liver tumour (diagnosed as reticulum cell sarcoma). Two mid-dose males died of a respiratory infection. (Signs of respiratory disease occurred in all groups, in particular during weeks 4 through 6.) The cause of death of a high-dose female could not be determined as a result of autolysis. In the absence of clinical signs that could be attributed to treatment in any group and without a dose-related pattern, these fatalities were most likely not related to treatment; however, the respiratory infection may raise concerns about the hygienic conditions and quality of this old study.

Effects on body weight and feed consumption were confined to the high-dose group and were relatively minor. In high-dose males, a transient reduction in mean body weight was noted at the beginning of the study. The difference relative to the control group was statistically significant from weeks 2 through 4, but was not greater than approximately 10%. During the second half of the study, the mean body weight approached the control value. It is not clear if this "normalization" might have been partly due to the interim killing of five animals in this group (the mean body weight of the five prematurely killed males in week 4 was 170.2 g, whereas the mean body weight of the 15 males remaining on study at the same time point was 189 g). In high-dose females, a statistically significantly lower body weight relative to the control group was observed after the first week of treatment and again in weeks 8, 10 and 12 (Table 6). These body weight findings were clearly not due to a lower feed intake, as feed consumption, even though extremely variable throughout the individual study weeks, did not differ very much between the groups, and no consistent trend could be observed. The substantial body weight gain from week 0 to week 4 finds its explanation in the fact that treatment of the animals commenced just after weaning.

Table 6. Mean body weights in selected weeks in the 90-day study in rats

	Mean body weight (g)							
	Males				**Females**			
Week (no. per group)	**0 ppm**	**250 ppm**	**500 ppm**	**2 500 ppm**	**0 ppm**	**250 ppm**	**500 ppm**	**2 500 ppm**
Prior to treatment (*n* = 20)	60.1	60.1	59.8	59.8	61.9	61.0	61.7	61.4
1 (*n* = 20)	97.3	89.7	100.6	91.6*	92.6	89.6	92.2	88.3*
4 (*n* = 20)	206.2	206.2	209.3	184.3**	157.2	156.1	152.2	150.2
8 (*n* = 15)	291.9	297.8	296.5	276.4	203.9	195.3	195.6	191.3*
12 (*n* = 15)	345.0	352.4	346.1	337.3	223.4	218.7	218.9	205.0*

n: number; no.: number; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Student's *t*-test)
Source: Fogleman (1971a)

Haematology, clinical chemistry and urine analysis did not reveal statistically significant or dose-related differences among the groups, either at 28 days or at 90 days. No gross abnormalities were noted at necropsy, apart from some lung findings indicative of respiratory infection in all groups, which were apparently not related to treatment. As in the 28-day study described above (Fogleman,

1970), there were changes in relative organ weights. In the animals from the interim groups (killed at week 4), relative liver weights were increased at the high dose in both sexes. In addition, relative kidney weight was higher in high-dose females. These findings were confirmed in the animals that were killed after 90 days. Again, relative liver and kidney weights were elevated in males and females receiving the high dose. In females, a statistically significantly higher relative liver weight was also noted at the intermediate dose. In addition, mean relative thyroid weight was increased in high-dose males (Table 7).

Table 7. Relative organ weights in the 90-day feeding study in rats at termination

	Relative organ weight (%)							
	Males (*n* = 15)				**Females (*n* = 15)**			
Organ	**0 ppm**	**250 ppm**	**500 ppm**	**2 500 ppm**	**0 ppm**	**250 ppm**	**500 ppm**	**2 500 ppm**
Liver	3.86	3.77	4.33	4.89*	3.96	4.01	4.17*	5.00*
Kidneys	0.76	0.83	0.78	0.94*	0.74	0.75	0.74	0.81
Thyroid	0.005	0.006	0.005	0.01**	0.006	0.006	0.008	0.008

n: number; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Student's *t*-test)
Source: Fogleman (1971a)

The only remarkable histopathological findings were moderate follicular cell hypertrophy, moderate depletion of colloid and a slight to moderate increase in interstitial vascularity in the thyroids of high-dose males, suggesting some stimulation of thyroid activity. It is worth noting that the relative liver and kidney weight increases were not accompanied by microscopic lesions, suggesting that they either were due to lower body weight or reflect an adaptive response rather than an adverse effect.

Based on effects on body weight in both sexes and on the relative thyroid weight increase and histopathological findings in the thyroid of males at 2500 ppm (equal to 248 mg/kg bw per day), the NOAEL was identified as 500 ppm (equal to 45 mg/kg bw per day) (Fogleman, 1971a).

It must be noted that this study does not comply with current standards and is poorly reported. There are only a few summary tables, statistical analysis of absolute organ weights was lacking and much of the text and many tables in the submitted electronic version were difficult to read.

In a 9-month feeding study, norflurazon (SAN9789; batch no. 004; purity 98.8%) was administered to 40 male and 40 female Sprague Dawley rats per group at a dietary concentration of 0, 125, 250 or 500 ppm (equivalent to 0, 6.25, 12.5 and 25 mg/kg bw per day, respectively). The control group consisted of 80 rats of each sex receiving basal diet only. Treatment commenced 2 weeks after weaning. Apparently, the animals were mated and produced a litter as part of the study, but no information regarding this reproduction part is given in the report. The animals were monitored for mortality and the occurrence of clinical signs and were weighed weekly. Feed consumption was determined weekly, except during the mating period for males and females and during lactation for females, and feed efficiency was calculated. Haematological and clinical chemistry parameters were measured in five males and five females from the control and high-dose groups at 17 weeks on test and in 10 males and 10 females from the same groups at termination. Haematology included erythrocyte and leukocyte counts, haematocrit, haemoglobin and differential white blood cell count. The clinical chemistry parameters comprised glucose, blood urea nitrogen, total serum protein, aspartate aminotransferase, alkaline phosphatase and lactate dehydrogenase. During urine analysis in the 10 control and high-dose animals of each sex at termination, pH, glucose, ketone bodies, albumin and occurrence of occult blood were examined. All animals surviving to scheduled termination as

well as those that died on test were subjected to gross necropsy. At termination, weights of liver, kidneys, heart, spleen, thyroid and gonads were determined, and relative organ weights were calculated. Histopathological examination was confined to liver, kidneys and thyroid (even though many more organs and tissues had been collected) and was performed in 40 animals of each sex from the control groups and in all high-dose rats as well as in those that died on test. In addition, neoplasms were examined histologically.

Twelve animals died during the study, five of them in the control group. Nephritis was the most common cause of death. None of these deaths could be attributed to treatment. A low-dose male had a kidney tumour (adenocarcinoma), and a subcutaneous tumour (adenofibroma) was noted in a control female. These findings are considered incidental.

There were no clinical signs that could be attributed to substance administration. Body weight, body weight gain, feed consumption and feed efficiency were not affected by treatment. Haematological, clinical chemistry and urine analysis parameters were also not affected, but the number of animals under examination for these end-points was rather limited. No gross abnormalities were noted at necropsy. In female rats, there was evidence of higher absolute and relative liver weights, but the figures are poorly depicted in the submitted copy of the study, and statistical analysis was not performed. Histopathology did not reveal alterations in the liver or thyroid, but some kidney lesions were more frequent in the animals at the high dose. At 500 ppm, the incidence of tubular degeneration was more than doubled in males (20/40 compared with 8/40 in controls) and was also increased in females (9/40 compared with 1/40 in controls). In addition, in females, kidney pigmentation was increased (18/40 in the high-dose group compared with 4/40 in controls), and hyaline tubules were observed more frequently (22/40 in the high-dose group compared with 8/40 in controls). An increase in hyaline tubules was also noted in high-dose male rats, but the difference relative to the control group (30/40 compared with 19/40) was less pronounced than in females.

Although there were indications of toxicity at least at 500 ppm (equivalent to 25 mg/kg bw per day), with the kidney identified as the main target organ, a NOAEL cannot be identified owing to the deficiencies of this poor quality study (Harris, 1972).

Dogs

The oldest study in beagle dogs (four of each sex per dose) was of limited quality, as the duration of treatment in the different groups varied (18 versus 10 weeks), doses were increased twice during the study in the low-dose (25–150 ppm) group and dietary administration was discontinued at the intermediate and high doses (1000 and 5000 ppm) and changed to daily capsule administration of 25 or 125 mg/kg bw for the duration of the study. The study author reported mainly gastrointestinal signs and poor palatability (Fogleman, 1971b).

Owing to the deficiencies of this study, the results are not reliable. This study may be considered fully replaced by the 6-month study and the modern 1-year study in beagle dogs reported below.

In a 6-month feeding study, norflurazon (batch and purity not given) was administered to beagle dogs (four of each sex per group) at a dietary concentration of 0, 50, 150 or 450 ppm (equal to 0, 1.5, 5.0 and 14.3 mg/kg bw per day for males and 0, 1.6, 4.8 and 17.8 mg/kg bw per day for females, respectively). Parameters under investigation were body weight, body weight gain, feed consumption, clinical signs (including neurological examination), haematology, urine analysis and clinical chemistry parameters (including determination of microsomal liver enzymes at termination), ophthalmology, organ weights, and gross and microscopic pathology. In contrast to other, older studies with norflurazon, histopathology of a wide range of organs was performed in all animals.

There were no unscheduled deaths during the study, and no clinical (including neurological or ophthalmological) signs could be attributed to treatment. Body weight and body weight gain were not affected by treatment, and there were no significant differences in feed consumption. Although the

total feed intake in high-dose females was, on average, higher than in the control group (59.0 kg versus 51.3 kg), even though the mean body weight gain of 0.5 kg was the same, this was mainly due to a single female with an extraordinarily high feed intake of 85.6 kg over the study period. In males, the feed intake in the mid- and high-dose groups was also higher than in controls, but this finding was not dose related and resulted in a slightly higher body weight gain.

Red blood cell count tended to decrease in high-dose females after 3 and 6 months of treatment (Table 8). However, the mean values were still within the reference range of 5.5–8.5 × 10^6/µL for healthy dogs (Moritze, Schwendenwein & Kraft, 2014), suggesting that the effect might be treatment related, but was not necessarily adverse. One of the females in this group had a value of only 5.13 × 10^6/µL after 6 months, the lowest individual value of all females on study, but the pretest value of 5.66 × 10^6/µL in the same animal was also the lowest at this time point. The haematocrit was lower in high-dose females as well, and the difference was even more impressive when compared with the pretest mean in the same group. There was no concomitant impact on haemoglobin. No similar haematological changes were observed in males, in which consistent increases in red blood cell count and haematocrit over the course of the study were apparent in all groups, including the controls.

Table 8. Alterations in selected haematological and clinical chemistry parameters in the 6-month feeding study in beagle dogs (group mean values, with standard deviation if available)

	Males (*n* = 4)				**Females (*n* = 4)**			
Parameter	**0 ppm**	**50 ppm**	**150 ppm**	**450 ppm**	**0 ppm**	**50 ppm**	**150 ppm**	**450 ppm**
RBC count (× 10^6/µL)								
Pretest[a]	5.96	5.78	5.88	6.46	6.85	6.95	6.26	6.53
3 months	7.18	7.11	6.86	7.03	7.74	6.71	6.78	6.12
6 months	6.72	7.02	7.02	6.79	6.87	6.79	6.50	5.85
Haematocrit (%)								
Pretest	42.25	40.75	41.25	44.75	49.50	50.25	43.30	45.00
6 months	47.50	49.75	48.25	46.75	48.50	48.00	45.00	40.75
Cholesterol (mg/100 mL)								
Pretest	198 ± 31	209 ± 10	221 ± 22	209 ± 28	189 ± 11	187 ± 7	214 ± 21	201 ± 7
3 months	206 ± 74	198 ± 23	288 ± 14	283 ± 30	190 ± 14	228 ± 48	255 ± 80	283 ± 22
6 months	197 ± 48	196 ± 12	253 ± 38	268 ± 13	247 ± 63	225 ± 40	261 ± 15	254 ± 55
ALP (mU/mL)								
Pretest	60.2 ± 14.5	53.1 ± 4.8	64.8 ± 22.5	53.8 ± 11.2	70.8 ± 29.1	49.6 ± 10.1	78.8 ± 18.5	61.4 ± 20.6
6 months	24.2 ± 9.9	32.7 ± 15.8	57.3 ± 21.6	54.8 ± 16.4	45.7 ± 31.9	37.3 ± 7.9	76.4 ± 44.0	65.8 ± 49.0

ALP: alkaline phosphatase; ppm: parts per million; RBC: red blood cells; U: units
[a] "Pretest": 2 weeks before commencement of study.
Source: Klotzsche & Carpy (1973)

Clinical chemistry revealed an increase in mean cholesterol concentrations in mid- and high-dose males after 3 and 6 months and at least in high-dose females after 3 months (Table 8). With

regard to alkaline phosphatase, a decrease in activity was apparent in both sexes with ongoing duration of the study in the control and low-dose groups. However, such a reduction was not observed in the mid- and high-dose groups, which might point to liver toxicity. For both parameters, large interindividual variability in some groups, including the controls, must be noted. All the other parameters measured in blood or urine did not show any changes that could be attributed to treatment. Even though liver weight was increased (see below), no induction of liver enzymes was observed.

Necropsy did not reveal any gross pathological findings. Absolute and relative liver weights were increased at the middle and high doses in both males and females; although there was not always a clear dose–response relationship and the differences were not always statistically significant (Table 9), this is of limited relevance in a study with such a small number of animals. Extremely high absolute liver weights of 459 or 407 g were noted in two high-dose females, in contrast to rather low liver weights (234 and 249 g) in the two other females in the same group. The higher absolute liver weight in low-dose males was not regarded as potentially adverse, as it was not accompanied by clinical chemistry findings suggesting liver toxicity (see Table 8) or by histopathological lesions (see below).

Table 9. Mean absolute and relative liver weights (with standard deviation) in the 6-month feeding study in beagle dogs

	Males (*n* = 4)				**Females (*n* = 4)**			
Parameter	**0 ppm**	**50 ppm**	**150 ppm**	**450 ppm**	**0 ppm**	**50 ppm**	**150 ppm**	**450 ppm**
Absolute liver weight (g)	245.82 ± 24.18	320.52* ± 34.05	340.65** ± 25.88	347.50** ± 27.24	234.50 ± 33.43	235.22 ± 26.99	289.80* ± 14.51	337.20 ± 112.71
Relative liver weight (%)	2.58 ± 0.38	3.02 ± 0.70	3.28 ± 0.46	3.26* ± 0.14	2.82 ± 0.29	2.77 ± 0.32	3.19 ± 0.52	4.20* ± 0.79

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (statistical test not provided)
Source: Klotzsche & Carpy (1973)

There were no changes in other organ weights that might be related to treatment.

Clear treatment-related histopathological findings were confined to the highest dose. The liver and the thyroid were mainly affected, but the effects were rather minor in nature. Liver findings comprised congestion in five out of eight high-dose dogs and hypertrophy in one of them. Regarding the thyroid, the number of animals exhibiting an increase in colloid vacuoles was increased at the high dose. The study authors also mentioned an increase in fatty changes in the myocardium and in epithelial vesiculation in the mammary gland of high-dose dogs, along with some evidence of pneumonia; they interpreted these latter findings as signs of “diminished resistance” and indicative of an unspecific toxic effect of the high dose. It must be noted that the information on histopathological examination is based on a summary table only, as the quality of the copy does not allow meaningful assessment of the raw data.

The NOAEL was 50 ppm (equal to 1.5 mg/kg bw per day), based on liver weight increases, histopathological findings in liver and thyroid and alterations in clinical chemistry parameters at 150 ppm (equal to 4.8 mg/kg bw per day) (Klotzsche & Carpy, 1973).

In one of the very few available guideline-compliant toxicological studies performed under GLP conditions, norflurazon (batch no. P.148/86/31; purity 98.2%) was administered to groups of four male and four female beagle dogs at a dietary concentration of 0, 50, 200 or 800 ppm (equal to 0, 1.7, 6.2 and 27 mg/kg bw per day for males and 0, 1.5, 6.3 and 23 mg/kg bw per day for females, respectively) for 1 year. The animals were monitored for clinical signs and weighed weekly. Feed intake was determined on a weekly basis. Comprehensive haematological and clinical chemistry examinations, including urine analysis, were performed at 3-month intervals. At scheduled

termination, the dogs were killed and necropsied. Organ weights were measured, and histopathological examination of a wide range of organs and tissues was conducted in all groups.

There were no unscheduled deaths during the study, but one high-dose female showed subdued behaviour and weight loss. This was apparently due to severe reduction in feed intake during the early part of the study. There is an indication that a palatability problem might have been behind this, as moistening of the diet with water resulted in normalization of feed intake and regaining of body weight and condition. No clinical signs that could be attributed to treatment were noted in other animals.

In general, body weight (gain), feed consumption and feed efficiency were impaired by norflurazon treatment (Table 10). All treated dogs gained less weight than the control animals over the course of the study. This effect was most pronounced in high-dose males and mid- and high-dose females and more equivocal in low- and mid-dose males and low-dose females, as the difference relative to the respective control group was small and, in males, there was no clear dose–response relationship. A marked decrease in feed consumption over the whole study period was observed in both sexes at the high dose, but a weak effect in the low- and mid-dose groups cannot be excluded, suggesting a palatability problem. Based on feed conversion ratios (i.e. the amount of feed consumed divided by body weight gain), feed efficiency appeared to be compromised in high-dose males and mid- and high-dose females over the first 16 weeks of the study. For the subsequent part of the study, this calculation was not performed. This finding might suggest that the lower body weight gain did not result only from lower feed intake, but could also indicate a toxic or metabolic effect. It is not surprising that the mean total body weight was lowest at study termination in the high-dose group in both sexes (see Table 12 below).

Table 10. Mean weekly body weight gain and daily feed consumption in the 1-year study in beagle dogs

	Males				**Females**			
Parameter	**0 ppm**	**50 ppm**	**200 ppm**	**800 ppm**	**0 ppm**	**50 ppm**	**200 ppm**	**800 ppm**
Body weight gain (kg/week)								
Weeks 0–9	0.21	0.14	0.18	0.08	0.17	0.16	0.10	−0.01
Weeks 0–28	0.10	0.08	0.09	0.04*	0.08	0.08	0.06	0.02
Weeks 0–52	0.06	0.05	0.05	0.03	0.06	0.05	0.04	0.03
Feed consumption (g/day)								
Weeks 0–9	362	359	354	306	338	325	311	252
Weeks 0–52	356	346	325	314	322	293	301	272

ppm: parts per million; *: $P < 0.05$ (analysis of variance, followed by Dunnett's test)
Source: Warren et al. (1990)

Haematology revealed a reduction in red cell parameters (haematocrit, haemoglobin and red blood cell count) in both sexes at the high dose. These findings, suggestive of slight anaemia, were most pronounced at week 12. In high-dose males, platelet count was increased. Clinical chemistry analysis pointed to liver toxicity, with elevated cholesterol levels (confirming the results of the older 6-month study reported above; Klotzsche & Carpy, 1973), at least in mid- and high-dose males, and, occasionally, activities of some enzymes. All these effects were confined to the high-dose or the mid- and high-dose groups (Table 11). The only urine analysis finding that might be related to treatment was a slightly higher specific gravity of urine in males and females receiving 800 ppm, which is normally not considered a pathological effect.

Table 11. Selected haematological, blood clinical chemistry and urine analysis findings from the 1-year study in beagle dogs (group mean values)

Parameter	Males				Females			
	0 ppm	50 ppm	200 ppm	800 ppm	0 ppm	50 ppm	200 ppm	800 ppm
RBC count (10^6/mm^3)								
Pretest	6.515	6.625	6.688	6.178	6.698	6.635	6.643	6.330
Week 12	7.075	7.038	6.843	6.133*	7.380	7.140	7.198	6.113*
Week 52	7.808	7.873	8.035	7.275	7.718	7.768	7.620	7.055
Platelet count (10^3/mm^3), week 52	286.50	310.25	333.50	399.25	334.75	317.75	337.25	351.0
Cholesterol (mmol/L), week 52	3.44	3.98	5.04	4.78	4.61	3.87	4.82	4.90

ppm: parts per million; RBC: red blood cells; *: $P < 0.05$ (analysis of variance, followed by Dunnett's test)
Source: Warren et al. (1990)

Only a few gross pathological changes could be attributed to treatment. According to the pathology report, these were a discoloured liver with granular or nodular capsule in one high-dose female and gall bladders with black or brown foci in the mucosa in two males and one female from the high-dose group. However, similar gall bladder findings were noted in one low-dose female and one mid-dose male. Dark brown discolouration of the kidney was observed in one high-dose male.

The only treatment-related organ weight change was an increase in (absolute and relative) liver weights in high-dose males and females and in mid-dose males (Table 12), confirming the results from the older 6-month study by Klotzsche & Carpy (1973). No statistical significance was achieved, but such an analysis is of low value in a study with only four animals of each sex per dose group. A substance-related effect is not excluded.

Table 12. Mean absolute and relative liver weights (with standard deviation) in the 1-year feeding study in beagle dogs

Parameter	Males (*n* = 4)				Females (*n* = 4)			
	0 ppm	50 ppm	200 ppm	800 ppm	0 ppm	50 ppm	200 ppm	800 ppm
Mean body weight (kg)	10.88 ± 2.80	10.55 ± 1.24	10.78 ± 1.40	9.27 ± 0.33	10.98 ± 0.78	10.20 ± 1.15	10.02 ± 1.33	9.77 ± 0.67
Absolute liver weight (g)	281.3 ± 56.0	272.2 ± 40.2	310.0 ± 9.7	321.9 ± 61.0	292.5 ± 32.4	228.5 ± 14.7	247.7 ± 61.2	329.4 ± 44.8
Relative liver weight (%)	2.625 ± 0.360	2.577 ± 0.204	2.909 ± 0.330	3.462 ± 0.573	2.675 ± 0.332	2.262 ± 0.297	2.468 ± 0.506	3.361 ± 0.281

n: number; ppm: parts per million
Source: Warren et al. (1990)

Histopathological examination revealed hepatitis in two high-dose males. Incidences and degree of biliary cholestasis and mucosal hyperplasia were seen only in the high-dose group, even though only one female and one male, respectively, were affected. Male dogs also had an increased incidence of renal cortical tubular pigmentation at 200 and 800 ppm, suggesting that the kidney might also be a target organ. It is noted that this information from the text is not fully supported by the tables.

The NOAEL was 50 ppm (equal to 1.5 mg/kg bw per day), based on impairment of nutritional parameters, such as body weight (gain), feed consumption and feed efficiency, in females and alterations in clinical chemistry suggestive of liver toxicity and increased liver weights in both sexes at 200 ppm (equal to 6.2 mg/kg bw per day). Kidney findings of equivocal toxicological significance, including renal tubular pigmentation, were noted in males at 200 ppm and above (Warren et al., 1990).

The outcome of this study is very much in line with the results of an older 6-month study (Klotzsche & Carpy, 1973). Taken together, these studies suggest that the dog might be more sensitive than the rat.

(b) Dermal application

No studies were submitted.

(c) Exposure by inhalation

No studies were submitted.

2.3 Long-term studies of toxicity and carcinogenicity

Mice

In a combined chronic toxicity and carcinogenicity study, norflurazon (described as "technical formulation of product 9789"; batch no. 004; purity 98.8%) was administered to groups of 125 male and 125 female CD-1 (HaM/ICR Swiss) mice at a dietary concentration of 85, 340 or 1360 ppm (equivalent to 13, 51 and 200 mg/kg bw per day, respectively) for up to 2 years. Two control groups of the same size were included and fed on untreated basal diet. The design of this very old study is unusual and does not comply with current standards or any guideline, as treatment was commenced in weanling mice that had been selected from the F_{1A} litters of a two-generation reproduction study (reported in section 2.5). As their parents had received the test substance (apparently at the same dietary concentrations) over an 18-week premating period and throughout gestation and lactation, preceding prenatal or postnatal exposure of the animals in the treatment groups is likely and could have, at least theoretically, contributed to the findings.

A clear deficiency of this study was the loss of 56 male mice from one of the two control groups and of 56 low-dose males after 20 weeks on study as a result of an accident. To replace these lost animals, a "small group" of new F_0 animals was purchased, placed on either control or low-dose diets and mated to produce new F_{1A} litters, from which 56 males per group were randomly selected and, after weaning, treated for 104 weeks as in the original study. Thus, part of the treatment and investigation took part at a later time. This unusual approach would normally be considered to have severely disturbed the integrity of the study, making it unacceptable according to current guidelines, even though some of the data were reported separately.

The animals were monitored for mortality and clinical signs, body weight, body weight gain and feed consumption. Satellite groups of 10 males and 10 females each were fed either control diet or the high dose of norflurazon for up to 26 weeks. At 10 or 26 weeks, five satellite animals of each sex per group were killed, and blood samples were taken for haematology and clinical chemistry, but the range of clinical chemistry parameters was small and did not comply with current standards.

All surviving animals were killed between weeks 100 and 104 and, together with those animals that died on test, were necropsied and grossly examined. At scheduled termination, the following organs were weighed: liver, heart, spleen, kidneys, pituitary, brain, gonads, thyroid and adrenals. A range of organs and tissues was taken and preserved for histopathology, but microscopic evaluation was in fact confined to the control and high-dose animals, apart from major lesions, which

were examined from all test groups. Because of tumour findings, the livers of all animals were examined histologically.

There were no clinical signs that could be attributed to treatment. In both sexes, survival was sufficient, at greater than 50% overall and greater than 60% in most groups at week 100. A common cause of premature deaths, but apparent in all groups, including the controls, was lymphosarcoma.

A slight reduction in body weight in high-dose males was likely treatment related. During the second year of the study, mean body weight of high-dose males was 37–39 g on average over the individual weeks, compared with 40–42 g in the control, low-dose and mid-dose groups. No statistically significant differences were observed in females, and there were no alterations in feed consumption in either sex. It is questionable whether the rather weak effect on body weight in one sex may be considered sufficient to conclude that the maximum tolerated dose had been reached. However, this conclusion could be drawn because of liver tumours in male mice (see below).

Haematology and clinical chemistry in a small number of mice did not reveal any changes that could be attributed to administration of the test substance for either 10 or 26 weeks.

Gross examination of mice at scheduled termination and of premature decedents suggested an impact at the high dose on the liver and perhaps on the kidneys in both sexes and on the adrenals in males only. In particular, when analysis was made on the basis of “all animals on study”, there was a higher number of mice exhibiting macroscopic liver changes and evidence of nephritis, such as pitted surface (Table 13). Even though statistical analysis of these findings was not performed, effects at the high dose and some effects at the intermediate dose appear outside the normal biological variance, as demonstrated by the large differences between the two control groups. With regard to nephritis in males, an impact of the intermediate dose cannot be excluded.

Table 13. Selected gross pathological findings in male and female mice at termination after 2 years and in those animals that died on test (combined incidence)

	Combined incidence of pathological finding				
Parameter	**0 ppm**	**0 ppm**	**85 ppm**	**340 ppm**	**1 360 ppm**
Males					
Number examined	*125*	*124*	*124*	*125*	*125*
Liver: enlarged	2	8	5	7	15
Liver: dark, congested, haemorrhagic	1	2	1	6	15
Kidneys: nephritis	4	8	3	12	14
Adrenals: enlarged[a]	0	2	2	1	9
Females					
Number examined	*125*	*126*[b]	*126*[b]	*125*	*125*
Liver: enlarged	4	9	9	8	15
Liver: dark, congested, haemorrhagic	1	7	7	8	18
Kidneys: nephritis	5	5	6	7	12
Adrenals: enlarged[a]	5	0	11	0	7

ppm: parts per million

[a] Reported only for animals killed at scheduled termination.

[b] Apparently, in both groups, one female had been erroneously taken for a male when the very young animals were allocated to the different groups.

Source: Tisdel (1975a)

Higher absolute and relative liver weights were noted after 24 months in males in all treated groups, revealing a dose-related pattern (Table 14). The slightly lower mean absolute and relative kidney weights in high-dose males, in contrast, reflect the lower terminal body weight. In females, absolute and relative liver weights were also increased at least at the intermediate and high doses, whereas kidney weight was not affected. Occasional changes in other organ weights did not follow a pattern and were considered incidental.

Table 14. Organ weight changes in male and female mice at termination after 2 years

Parameter	0 ppm	0 ppm	85 ppm	340 ppm	1 360 ppm
Males					
Number examined	71	81	85	65	74
Mean body weight (g)	37	35	36	36	33
Mean absolute liver weight (g)	1.87	1.92	2.05*	2.18*	2.41*
Mean relative liver weight (% of control)	5.14	5.57	5.69*	6.14*	7.38*
Mean absolute kidney weight (g), left / right	0.393 / 0.394	0.373 / 0.389	0.376 / 0.388	0.385 / 0.401	0.338* / 0.361*
Mean relative kidney weight (% of control), left / right	1.032 / 1.006	1.072 / 1.117	1.047 / 1.081	1.072 / 1.118	1.029 / 1.103
Females					
Number examined	64	70	77	59	71
Mean body weight (g)	31	31	32	32	31
Mean absolute liver weight (g)	1.69	1.30	1.87	1.90*	2.12*
Mean relative liver weight (% of control)	5.43	5.79	5.88	6.02*	6.93*
Mean absolute kidney weight (g), left / right	0.285 / 0.299	0.277 / 0.290	0.277 / 0.294	0.277 / 0.291	0.290 / 0.298
Mean relative kidney weight (% of control), left / right	0.920 / 0.963	0.889 / 0.936	0.872 / 0.926	0.876 0.922	0.955 / 0.978

ppm: parts per million; *: statistically significantly different from at least one control group
Source: Tisdel (1975a)

Histopathology of control and high-dose animals at scheduled termination revealed an increase in liver lesions in male but not in female animals (Table 15). There was a dose-related increase in liver cell adenoma in all male dose groups. This was accompanied by a higher incidence of hepatocellular hypertrophy, at least at the high dose. A similar effect in the low- and mid-dose groups is likely when the low number of animals under examination is taken into account. Hypertrophy often precedes adenoma development. Taken together, these findings are well in line with the increase in liver weight and the gross liver changes and are certainly treatment related. It is surprising that similar gross observations in females did not seem to find a histopathological correlate. Even though carcinoma incidence was increased among mid-dose males at scheduled termination, this finding did not provide convincing evidence of a treatment-related increase in malignant liver tumours in general. There was no dose–response relationship, as the incidence at the high dose was similar to that of the control groups. A few more liver tumours were detected when the premature deaths were taken into consideration (Table 16), but the general pattern did not change. An increase in tumours other than in the liver was not observed in either males or females.

Table 15. Selected histopathological findings in the 2-year feeding study in mice at termination

Pathological finding	0 ppm	0 ppm	85 ppm[a]	340 ppm[a]	1 360 ppm
Males					
Number examined	71	81	37	33	74
Liver: hypertrophy	5	7	6	2	31
Liver: adenoma	3	2	7	10	16
Liver: carcinoma	3	1	–	4	1
Spleen: pigmentation	6	11	–	–	19
Kidneys: pyelonephritis	12	7	3	4	10
Intestines: amyloidosis	5	9	–	–	18
Females					
Number examined	64	70	32	15	71
Liver: hypertrophy	13	4	5	2	13
Liver: adenoma	1	1	2		2
Liver: carcinoma	0	0	1		1
Spleen: pigmentation	13	11	–	–	33
Pancreas: hyperplasia	0	0	–	–	8
Kidneys: pyelonephritis	11	13	2	–	28
Ovaries: cystic	28	35	16	5	48
Bone marrow: pigmentation	0	5	4	–	15

–: organ not examined; ppm: parts per million
[a] Histological examination only of animals with gross lesions.
Source: Tisdel (1975a)

Table 16. Overview of liver cell tumours in the 2-year feeding study in male mice, based on additional examination of the livers of all animals

Group and tumour	0 ppm	0 ppm	85 ppm	340 ppm	1 360 ppm
Males, died on test	54	43	39	60	51
Liver: adenoma	0	1	1	1	0
Liver: carcinoma	1	1	1	1	3
Males, killed at termination	71	81	85	65	74
Liver: adenoma	3	2	7	10	16
Liver: carcinoma	3	1	0	4	1
Males on study	125	124	124	125	125
Liver: adenoma	3	3	8	11	16
Liver: carcinoma	4	2	1	5	4

ppm: parts per million
Source: Tisdel (1975a)

The most prominent and presumably treatment-related non-neoplastic kidney lesion was pyelonephritis, which was much more common in high-dose females than in the controls (Table 15). Gross kidney lesions in mid- and high-dose male mice had no morphological correlate at termination.

However, in premature deaths, nephritis was more often diagnosed in male decedents (17/44 compared with 10/47 and 7/39 in the two control groups). Another adverse effect in female mice was pancreatic hyperplasia, which was noted only at the high dose. Cystic degeneration of the ovaries was more often observed in the high-dose group than in control females. This finding might point to exacerbation of age-related changes and could be hormone mediated. The same assumption with regard to ageing might hold true for the observed increase in intestinal amyloidosis in high-dose males. The toxicological significance of spleen pigmentation in high-dose males and of bone marrow pigmentation in high-dose females is equivocal.

In addition to the original study report, further evaluations of the liver pathology in male mice were performed. In the introductory part of the study report, reference is made to a "second opinion" of an external expert (Ward Richter, A.J. Carlson Animal Research Facility, The University of Chicago), who had concluded, following his review of the histological slides from the terminal males only, that the increase in hypertrophy and adenoma was confined to the high-dose group.[1] In addition, he found no evidence of malignant tumours and considered norflurazon to be non-carcinogenic. A letter to Sandoz Pharmaceutical Research Laboratories dated 9 July 1975 and signed by Professor Richter was submitted by the sponsor in copy, along with some notes, partly handwritten. Different diagnostic criteria and a partly different nomenclature of pathological liver findings were apparently applied for this second evaluation of the slides. The two control groups were combined, and the control and low-dose animals that were recruited late for the study due to the accident described above were also included in Professor Richter's analysis (Richter, 1975). The outcome is summarized in Table 17.

Table 17. Histopathological liver findings in male mice according to Professor Richter

Finding	Control	85 ppm	340 ppm	1 360 ppm
No. ("listed")	152	82	64	74
No. (examined)	144	19	19	74
Hypertrophy, multifocal	5	2	2	11
Hyperplasia, nodular	1	4	2	0
Adenoma	8	8	7	16
Cholangioma	2	0	0	0
Carcinoma	4	1	1	0
Neoplasia, total	14	9	8	16

ppm: parts per million
Source: Richter (1975)

The number of animals "listed" is the same as or at least similar to the number of animals killed at scheduled termination. The evaluation of livers from the low- and mid-dose groups is far from being comprehensive, as only a few animals were included in the review, and no clear justification for the selection was given. Based on what can usually be expected in ageing mice, the incidence of hypertrophy in all groups appears remarkably low. However, the author mentioned that the diagnosis "hypertrophy" was made only when its degree was higher than "normally found". Even then, the difference compared with the previous examination is striking. Statistical analysis of the results was not performed. Based on the results shown in Table 17, the conclusion that norflurazon was not carcinogenic cannot be accepted, as there was a clear increase in adenoma at the high dose compared with the control group (approximately 22% versus 5.6%). The total liver tumour incidence

[1] Even though the pathologist in the first sentence of his letter explained that rat liver tissues had been shipped to him, it can be reasonably assumed that these were, in fact, the mouse liver tissue slides obtained from the long-term study under evaluation.

was also higher (approximately 22% versus 9.7%). The analysis is flawed by ignoring the animals that died on study. This failure results in underestimation of the total tumour incidence, in particular with regard to carcinoma in the mid- and high-dose groups. One might get the wrong impression that the carcinoma incidence even decreased. Meaningful comparison is confined to the control and high-dose groups because of the low number of animals from the low- and mid-dose groups. Therefore, this second histopathological evaluation does not contribute to the identification of a NOAEL for this study.

A third histopathological evaluation of the liver slides from this study was performed by a consultant pathologist (Dr Rust), with its outcome being subject to statistical analysis. This re-evaluation was apparently performed in 1980 on behalf of the company Sandoz in response to questions raised by the United States Environmental Protection Agency (USEPA) in 1979. It was submitted by the sponsor to JMPR as part of the report on the long-term toxicity and carcinogenicity study in mice, even though it is not possible that it was part of the original study when the timelines are taken into consideration. Therefore, it is regarded as a separate document for the current evaluation and is referenced as such (Rust, 1980).

The outcome of this third histopathological evaluation of liver tissues from male mice that either had died on test or were killed at scheduled termination is summarized in Table 18. This time, the incidences of the different histopathological diagnoses were reported separately for the two control groups. The discrepancies with the total number of male mice on study were explained by absent tissues (either from the liver or "any tissue"), which were simply not available for some animals when the re-evaluation was performed, or, more rarely, severe autolysis or incomplete necropsy record. The pathologist also re-evaluated lung tissues, mainly from the control and high-dose groups, but confirmed that there was no evidence of a higher incidence of benign or malignant lung tumours in the high-dose group.

***Table 18**. Summary of histopathological liver findings in male mice according to Rust (1980)*

Finding	**0 ppm**	**0 ppm**	**85 ppm**	**340 ppm**	**1 360 ppm**
Tissues provided: males died on test	43	35	32	54	42
Tissues provided: scheduled termination	71	82	82	64	73
Tissues examined	114	117	114	117	115
Nodular hyperplasia/hypertrophy	7	1	6	5	10
Hepatocellular adenoma	0	4 (3[a])	5	1	11
Hepatocellular carcinoma	7	5 (6[a])	3	4	6

ppm: parts per million

[a] According to the pathology report, one animal had both an adenoma and a carcinoma. According to current guidelines, the more severe diagnosis should be used. This would decrease the adenoma incidence and increase the carcinoma incidence in control group 2.

Source: Rust (1980)

Lung metastases of liver cell carcinoma were found in one control group 2 animal and in one mouse of the mid-dose group. The histopathological re-evaluation also revealed amyloidosis, focal necrosis due to acute hepatitis or lymphoid pericholangitis and confirmed the presence of lymphoreticular tumours in some animals. All these findings were not dose related and thus were not attributed to test substance administration.

There are many striking differences between the histopathological evaluation by Rust (1980) and the evaluation in the original study report as well as the reanalysis by Richter (1975). Most likely they are due to the use of different diagnostic criteria, but, after decades, it is difficult to decide which evaluation was most reliable.

The company Sandoz performed a statistical analysis of these results, and this report was also submitted to JMPR as part of the study report. It is based on the additional histopathological evaluation by Rust (1980). For liver cell adenoma, a positive trend was seen, and a pairwise comparison revealed that the increase at the high dose was statistically significant. This statistical analysis can be considered to be replaced by that of Andriano (1981), reported below.

Apparently on the request of the USEPA, Andriano (1981) performed a series of statistical analyses on the liver tumour findings in male mice. For this statistical evaluation, the findings from the re-evaluation by Rust (1980) were apparently used, as the figures were the same. However, the possible impact of the replacement of a large number of animals from one of the control groups and the low-dose group during the study (see above) was taken into consideration by deleting the "accident-involved" treatment groups. Therefore, only data from the second control, mid-dose and high-dose groups were included in the new analysis, making it of little use for identifying the NOAEL. Both tests for trend (Cox's exact test version of Cochran-Armitage's test) and pairwise comparisons (by Fisher's exact test) were applied for analysis of liver lesions and were always one-tailed. Information on the statistical significance of the tumour and hypertrophy findings is taken from this report and summarized here.

For hepatocellular carcinoma, the incidence as determined by Andriano (1981) was as follows: control group: 5/117 (4%); mid-dose group (340 ppm): 4/117 (3%); and high-dose group (1360 ppm): 6/115 (5%). Neither the trend test nor the pairwise comparison revealed a statistically significant difference. This is in line with the previous interpretation of the results. A slightly higher incidence of carcinoma in the control group (6 instead of 5, see Table 18 above) would not alter the outcome.

For hepatocellular adenoma, the incidence was as follows: control group: 4/117 (3%); mid-dose group (340 ppm): 1/117 (1%); and high-dose group (1360 ppm): 11/115 (10%). Statistical analysis gave a positive trend ($P = 0.004$) and a positive result for the pairwise comparison of the high-dose group with the control group ($P = 0.002$). A lower number of adenomas in the control group (3 instead of 4, see Table 18 above) would not change the outcome that much. The adenoma incidence in the mid-dose group was, of course, not statistically significantly different from the control incidence (in fact, it was lower), but, according to the original report, 16 adenomas had been found in 125 animals. In the high-dose group, five adenoma cases were "lost", most likely due to the application of different diagnostic criteria.

For "nodular hypertrophy or hyperplasia", Andriano (1981) came to the following conclusion: control group: 1/117 (1%); mid-dose group (340 ppm): 5/117 (4%); and high-dose group (1360 ppm): 10/115 (9%). Again, there was a positive trend ($P = 0.004$), and the pairwise comparison revealed statistical significance for the increase at the high dose ($P = 0.005$).

For additional analyses, the author compiled all the animals with any hepatic proliferative change or excluded those with tumours. In all cases, the trend was positive, and so was the pairwise comparison for the high-dose group.

The evaluation of liver tumours in this long-term study obviously depends on the histopathological examination that is relied on. The histopathological re-evaluation by Rust (1980) seems the most appropriate, as the number of liver slides was nearly identical for all groups, and potentially preneoplastic liver findings were also considered. However, control group 1 and the low-dose group must be deleted and should not be considered further; these findings are not reliable, as they lack comparability with the other groups and as not all animals were treated in parallel. The same approach was taken by Andriano (1981) in the statistical re-evaluation.

To conclude, the carcinogenic effect on the liver in male mice was confined to the high-dose group (1360 ppm, equivalent to 200 mg/kg bw per day). The NOAEL for carcinogenicity was therefore 340 ppm (equivalent to 51 mg/kg bw per day). No progression to malignancy was observed, and no evidence of a neoplastic effect was seen in females. However, no information on the mode of action for tumour formation is available; accordingly, human relevance cannot be excluded.

For toxicity, a NOAEL could not be identified, as the low-dose group and one of the control groups were excluded, and there were treatment-related and adverse effects at the middle dose (340 ppm): an increase in nodular hyperplasia or hypertrophy in the liver of male mice, an increase in absolute and relative liver weights in both sexes, and a higher frequency of gross kidney changes (nephritis) in males. In addition, no comprehensive histopathological examination for non-neoplastic findings in organs other than the liver was performed at the middle dose. Therefore, no-effect levels for several histopathological findings for which the incidence at the high dose was higher than that in the control group could not be determined. These findings comprised pancreatic hyperplasia, pyelonephritis, splenic and bone marrow pigmentation and cystic ovaries in females as well as pigmentation of the spleen and intestinal amyloidosis in males. Thus, the lowest-observed-adverse-effect level (LOAEL) is 340 ppm (equivalent to 51 mg/kg bw per day), the lowest dose for which reliable data were available (Richter, 1975; Tisdel, 1975a; Rust, 1980; Andriano, 1981).

Rats

In a combined chronic toxicity and carcinogenicity study, norflurazon (described as "technical formulation of product 9789"; batch no. 004; purity 98.8%) was administered to groups of 85 male and 85 female Sprague Dawley rats at a dietary concentration of 125, 375 or 1025 ppm (equivalent to 6.25, 18.9 and 51.3 mg/kg bw per day, respectively). Two control groups of the same size were included and fed on untreated basal diet. Ten animals of each sex from one of the control and each of the treatment groups were randomly selected, killed after 52 weeks and subjected to pathological examinations. A separate report (Tisdel, 1973) on the first year of the study, including haematological, clinical chemistry and organ weight data and results of gross and histopathological examinations of these satellite group rats, was submitted and is referenced here where appropriate. The data obtained from haematological, clinical chemistry and urine analyses have been included and considered in the full report on the 2-year study as well.

The design of this very old study is unusual and does not comply with current standards or any guideline. It can be described as follows: Initially, male and female rats were fed the test substance for 9 months at a dietary concentration of 0, 125, 250 or 500 ppm. This part of the study is reported as a short-term experiment in section 2.2 above (Harris, 1972).

During the treatment period, these animals were mated. The reproduction part of the study is briefly reported in section 2.5 below (Tisdel, 1975c).

For the chronic toxicity and carcinogenicity study, weanling rats (weighing 45–55 g at the commencement of this long-term study) were selected from the F_{1A} litters. It must be kept in mind that their parents had received the test substance via their diet over an 18-week premating period and throughout gestation and lactation at doses that were partly different from those administered in the subsequent chronic toxicity and carcinogenicity study. Preceding prenatal or postnatal exposure of the animals in the treatment groups could have contributed to the findings in the long-term study.

It is not clear how the weanling rats for the chronic toxicity and carcinogenicity study were selected. A further clear deficiency of this study is the fact that the animals were moved, after 43 weeks on test, to a new animal facility.

End-points investigated included body weight, body weight gain, feed consumption, feed efficiency, clinical signs, ophthalmology, organ weights, and gross and microscopic pathology. Histopathology of a representative range of organs and tissues in all animals was confined to the control and high-dose groups. At the low and middle dose levels, only major lesions and kidneys were examined microscopically. A severe deficiency was the limited number of animals from which haematological, clinical chemistry and urine analysis data were obtained. Only 10 animals of each sex from one of the control groups and the high-dose group were included in this analysis, which was performed at study weeks 3, 13, 26, 52 and 100. Accordingly, no baseline analysis prior to commencement of treatment via the diet was performed. In addition, the range of blood clinical chemistry parameters was limited (e.g. thrombocytes and other blood coagulation parameters were not

measured). Taking into account that the kidney is a target organ of norflurazon, the absence of creatinine measurements is of particular concern.

The difference in survival between male and female rats was remarkably high. In males, survival was below 50% in all groups from week 96 onwards and at the high dose from week 92. Towards the end of the study, the decline appeared steepest in the high-dose group. Therefore, this group was terminated after week 102, instead of week 103. A lower number of survivors was also observed at the intermediate dose (Table 19). However, if the death rate between, for example, weeks 96 and 100 or from then until termination is compared, there is no evidence of a dose-related effect. It is not clear if the cause of death in the treated groups was treatment related, although the incidence of nephritis appeared higher in high-dose male decedents than in the control groups. In females, there was no impact of treatment on the mortality rate at any dose, and survival at scheduled termination was around or even above 50% in all groups.

Table 19. Survival in male rats from week 80 until termination

	Number of survivors (out of 85 rats)				
Week	**0 ppm**	**0 ppm[a]**	**125 ppm**	**375 ppm**	**1 025 ppm**
80	59	66	64	58	56
88	45	53	52	43	44
92	42	45	46	41	37
96	33	40	39	32	25
100	27	30	28	22	19
Terminal	23	24	23	16	14

ppm: parts per million
[a] No interim killing after 52 weeks had taken place.
Source: Tisdel (1975b)

No clinical signs could be attributed to treatment in male rats. In females, there was a higher number of rats at the high dose exhibiting spasms at least once during the study. The significance of the higher number of affected animals (15 in the group receiving 1025 ppm compared with 3 or 5 in the two female control groups) is equivocal. Although this finding was not accompanied by any pathological changes, peripheral nerves were not examined histologically. Ophthalmoscopy did not reveal differences between the control and treatment groups.

No significant dose-related or time-consistent effects on body weight or body weight gain were noted in male rats. In high-dose females, in contrast, mean body weight tended to become lower with longer duration of treatment. At termination, the difference (about 297 g compared with 331 or 329 g in the two control groups) was statistically significant. This finding was not due to lower feed consumption or feed efficiency, as both parameters were not affected in either sex or at any dose (although feed efficiency is depicted in tables for the first 13 weeks of the study only). It is also worth noting that body weight gain was not affected consistently in female rats throughout the study. There is no explanation for the adverse effect on body weight in females at the high dose.

Red blood cell parameters, leukocyte number and differential white blood cell count did not exhibit consistent differences between control and high-dose groups, in either the main study or the satellite groups kept for only 52 weeks.

Clinical chemistry parameters sometimes varied between groups, but most of the differences were not consistent over the course of the study. Variations within the same group at different time points probably reflected age-related changes. In the summary tables, no standard deviation is given for any parameter, and significant changes were not indicated, but had to be retrieved from the text. Only two findings might be attributed to treatment and were of potential concern. Blood urea nitrogen

was doubled in high-dose males at termination, compared with the controls. At earlier time points, no difference was noted. This effect could be indicative of the beginning of kidney damage, which was more pronounced in male rats. Also, at termination, 2,3-diphosphoglyceric acid was significantly decreased in high-dose males. The latter is an intermediate in glycolysis that is found mainly in erythrocytes (Juel & Milam, 1979; Rapoport, 1983), but it is an outdated parameter in toxicological studies. It was speculated that a decrease might suggest hypoxia or acidosis and could somehow be related to higher blood urea levels. Charache et al. (1970) reported that increased 2,3-diphosphoglyceric acid concentrations in red blood cells were related to a lower oxygen affinity in humans and that red blood cell–bound 2,3-diphosphoglyceric acid was increased in uraemic patients.

Urine analysis did not reveal meaningful differences between control and high-dose groups.

At interim necropsy after 1 year, higher incidences of nephritis, hydronephrosis and pyelonephritis were seen in treated animals, confirming the kidney as a target organ of norflurazon in rats. In the high-dose satellite male group, 6/10 animals exhibited gross kidney lesions of different stages. In high-dose females, three animals were similarly affected. At the low or intermediate dose, but not in the control groups, diffuse mottled appearance of the kidney surface was occasionally noted in both sexes. Thickened uterine walls and/or endometrial hyperplasia were also noted in the majority of females in all treated groups, whereas no such finding was reported for the controls. In addition, cystic degeneration of the ovaries was observed in three high-dose females. Mean absolute and relative liver weights were increased in high-dose males, and at least absolute liver weight was also higher in the mid-dose group. Kidney weights also tended to be higher in males at the high dose. In females, absolute and relative liver and kidney weights were increased in the high-dose group.

Histopathological examination of the satellite group animals revealed a number of renal findings, such as hyaline casts, tubular and glomerular degeneration and inflammatory changes, but no clear dose–response relationship was apparent, and not all gross lesions were confirmed microscopically. For instance, hydronephrosis was a necropsy finding in five high-dose males, but became a histological diagnosis in only three of them. Nephritis was more common in low-dose males and mid-dose females than in rats at the high dose. Regarding the uterus, histopathology confirmed endometrial hyperplasia in seven females in each of the treated groups, compared with a zero incidence in the control group. There were no other histological findings that could be attributed to treatment. A rather unusual (but clearly not treatment related) finding was the identification of a pancreatic tumour in one male and one female control rat.

At scheduled termination after 2 years, as well as in the decedents that died during the second year of treatment, macroscopic examination revealed some gross alterations, but mostly there were no differences between control and treated groups. At least in high-dose males, the number of animals with rough, pitted kidney surface was increased. In addition, dilated pelvis and hydronephrosis were more frequently observed in high-dose males. In females, incidences of all findings were similar in control and treated groups (Table 20).

Higher absolute and relative liver weights were noted at scheduled termination in high-dose males; a similar increase in low-dose males was not confirmed at the middle dose and, in the absence of a dose–response relationship, most likely occurred by chance. In females, there was a significant increase in relative liver weight at the high dose. This could be due to the lower mean body weight, but might also be indicative of an adaptive response. Kidney weights were markedly increased in male rats receiving the high dose as well as in mid- and high-dose females. In addition, ovary weight was significantly increased in high-dose females. In males, higher mean absolute and relative thyroid weights were noted in the high-dose group (Table 21). Even though the difference relative to the controls was not statistically significant, this effect would be in line with the outcome of the 90-day study in rats by Fogleman (1971a), in which the thyroid was identified as an additional target organ (see section 2.2 above).

Table 20. Selected gross pathological findings in male and female rats at termination after 2 years and in those animals that died on test

Parameter	0 ppm	0 ppm	125 ppm	375 ppm	1 025 ppm
Males					
Number examined	75	85	75	75	75
Kidneys: dilated pelvis	0	0	1	1	6
Kidneys: hydronephrosis	2	1	5	6	7
Kidneys: congestion, haemorrhages, dark	5	10	15	12	12
Kidneys: rough, pitted surface	29	30	25	32	42
Females					
Number examined	75	85	75	76 (?)	75
Adrenals: enlarged	17	14	9	15	22
Uterus: fluid filled	0	6	2	13	8
Uterus: enlarged, distended	15	14	8	13	15
Kidneys: dilated pelvis	0	3	0	2	5

ppm: parts per million
Source: Tisdel (1975b)

Table 21. Organ weight changes in male and female rats at termination after 2 years

Parameter	0 ppm	0 ppm	125 ppm	375 ppm	1 025 ppm
Males					
Number examined	23	24	23	16	14
Mean body weight (g)	484	485	490	493	474
Mean absolute liver weight (g)	21.7	22.5	24.5*	21.9	25.2*
Mean relative liver weight (% of control)	4.48	4.67	5.02*	4.77	5.32*
Mean absolute kidney weight (g), left / right	2.65 / 2.63	2.77 / 2.78	2.81 / 2.80	2.88 / 2.86	3.76** / 3.69**
Mean relative kidney weight (% of control), left / right	0.556 / 0.554	0.584 / 0.586	0.577 / 0.575	0.639 / 0.636	0.798** / 0.784**
Mean absolute thyroid weight (mg)	50.9	51.4	50.3	50.1	65.4
Mean relative thyroid weight (% of control)	0.011	0.010	0.010	0.011	0.014
Females					
Number examined	43	39	42	52	52
Mean body weight (g)	330	322	328	321	302
Mean absolute liver weight (g)	13.9	12.9	14.1	14.4	15.0
Mean relative liver weight (% of control)	4.24	4.05	4.38	4.54	5.02**
Mean absolute kidney weight (g), left / right	1.55 / 1.48	1.45 / 1.43	1.61 / 1.57*	1.67* / 1.66**	1.82** / 1.87**

Parameter	0 ppm	0 ppm	125 ppm	375 ppm	1 025 ppm
Mean relative kidney weight (% of control), left / right	0.484 / 0.461	0.460 / 0.455	0.517 / 0.500	0.534* / 0.533**	0.614** / 0.629**
Mean absolute ovary weight (mg), left / right	61.0 / 61.1	72.3 / 64.5	71.3 / 57.4	69.7 / 74.5	93.5** / 96.4**
Mean relative ovary weight (% of control), left / right	0.019 / 0.019	0.023 / 0.020	0.022 / 0.018	0.022 / 0.024	0.032** / 0.032**

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Student’s *t*-test)
Source: Tisdel (1975b)

Histopathology of control and high-dose animals at scheduled termination revealed increases in renal hyaline casts and nephritis in females, along with higher incidences of endometritis and squamous metaplasia of the uterus. The latter might be a tumour precursor. However, tumour incidences were not increased in females. In contrast to the histological findings in the satellite animals after 52 weeks of treatment, endometrial hyperplasia was visible in the control group females at termination, with an incidence comparable to that in the high-dose group. In male rats, an increase in nephritis was observed at the high dose, with 100% of the animals affected, compared with less than 50% of the controls. In addition, nodular cortical hypertrophy of the adrenals was observed only in treated males, although the total incidence was low. A single benign tumour type (i.e. pituitary adenoma) was seen more frequently in high-dose males. However, four control animals (two from each of the male control groups) that had died on test also had such a tumour, which is common in ageing rats. Thus, it is not likely that this increase can be attributed to treatment (Table 22).

Table 22. Selected histopathological findings in the 2-year feeding study in rats at termination

	Incidence of finding				
Pathological finding	**0 ppm**	**0 ppm**	**125 ppm[a]**	**375 ppm[a]**	**1 025 ppm**
Males					
Number examined	*23*	*24*	*23*	*16*	*14*
Kidneys: nephritis	10	7	10	8	14
Pituitary: (chromophobe) adenoma	4	3	–	–	9
Adrenals: nodular cortical hypertrophy	0	0	–	–	4
Females					
Number examined	*43*	*39*	*42*	*52*	*52*
Kidneys: nephritis	1	1	1	3	4
Kidneys: hyaline casts	22	24	28	43	41
Uterus: endometrial hyperplasia	27	21	–	–	31
Uterus: endometritis	4	2	–	–	9
Uterus: squamous metaplasia	3	0	–	–	9

ppm: parts per million
[a] Histological examination only of kidneys and gross lesions.
Source: Tisdel (1975b)

The NOAEL in this old study of questionable reliability was 125 ppm (equivalent to 6.25 mg/kg bw per day), based on effects on the kidney at 375 ppm (equivalent to 18.9 mg/kg bw per day). No convincing evidence of carcinogenicity was observed (Tisdel, 1973, 1975b).

2.4 Genotoxicity

Norflurazon was evaluated in a few genotoxicity studies, but only in vitro. These studies are summarized in Table 23.

Table 23. Summary of in vitro genotoxicity studies with norflurazon

Type of study	Test system	Concentration range tested	S9	Batch/purity	Result	Reference
Reverse gene mutation test in bacteria (Ames test) and yeast	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and 1538 and *Saccharomyces cerevisiae* D4	0.1–500 µg/plate (or 1 000 µg/plate with TA1537)	±	No information provided	Negative	Brusick (1977)
Reverse gene mutation test in bacteria (Ames test)	*Salmonella typhimurium* TA98, TA100, TA1535 and 1537 and *Escherichia coli* (hcr strain)	5–5 000 µg/plate	±	Batch not given; purity 99.8%	Negative	Anonymous (1980)
Rec assay	*Bacillus subtilis* strains H17 and M45	500–10 000 µg/disc	−	Batch not given; purity 99.8%	Negative	Anonymous (1980)
Chromosomal aberration	Chinese hamster ovary cells (12-hour exposure; in addition, 16-hour exposure without activation at highest dose)	32–500 µg/mL (without activation); 63–1 000 µg/mL (with activation)	±	Batch no. 1174/85; purity 97.9%	Negative	Putman (1985)
Unscheduled DNA synthesis and repair	Primary rat hepatocytes	1–1 000 µg/mL	Not needed	Batch no. 1174/85; purity 97.9%	Negative	Curren (1985)

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

No evidence of genotoxicity was obtained in the bacterial reverse gene mutation (Ames) assay in five *Salmonella typhimurium* strains and in a yeast system (i.e. *Saccharomyces cerevisiae* strain D4). All the tests, with and without metabolic activation, were negative. In contrast, appropriate positive controls caused a distinct increase in mutation frequency. However, the assay was poorly reported. In particular, it was not actually justified why 500 or (in the case of TA1537) 1000 µg/plate was chosen as the highest tested concentration. Moreover, *Saccharomyces cerevisiae* strain D4 is now considered an outdated test system for this study type. A main deficiency of the study is the absence of any information on batch and purity of the test compound (Brusick, 1977).

In a study from Japan (not signed or dated, but presumably reported in 1980), norflurazon (under designation NP-52; purity 99.8%) was tested in the same five *Salmonella typhimurium* strains as above and also in the *Escherichia coli* hcr strain, with and without metabolic activation. This time, higher concentrations were applied, and the results were again negative. Appropriate positive controls were included and caused a distinct increase in mutation frequency (Anonymous, 1980).

When both Ames tests are considered together, it may be concluded that norflurazon did not induce gene mutations in bacteria.

In the same very brief report, a rec assay in *Bacillus subtilis* was reported that was also negative. It is not clear how many replicates were used, and the description of the test method is very poor. Thus, the scientific validity of this experiment is limited (Anonymous, 1980).

In a valid in vitro chromosomal aberration assay in Chinese hamster ovary cells with norflurazon (purity 97.9%), no evidence of clastogenicity was obtained, either with or without activation. For one of the concentrations (500 µg/mL in the absence of metabolic activation), a cell cycle delay was reported. Positive control substances gave the expected increase in structural aberrations (Putman, 1985).

In an unscheduled DNA synthesis and repair assay, norflurazon (purity 97.9%), in contrast to the positive control compound, did not cause a significant increase in mean net nuclear counts and thus proved negative for direct interaction with DNA. However, it must be taken into account that the relevance of this method is now considered equivocal (Curren, 1985).

In an article from the open literature, induction of primary DNA damage in cells of the planarian *Polycelis felina*, a freshwater aquatic bioindicator organism, was reported. Norflurazon was used as the test substance because of its potential to contaminate groundwater owing to its long half-life. In an in vitro comet assay, tail length, percentage of DNA in the tails and tail moment were significantly increased at norflurazon concentrations of 0.2 and 2 µmol/L from day 1 of treatment onwards. At least with the high concentration, a peak was observed on day 4, whereas some recovery was apparent on day 7. No clear differences with regard to time course were seen when the lower concentration was applied. It must be taken into account that norflurazon also proved directly toxic to the planarians, as demonstrated by damage to the outer mucous layer, epidermis and cell parenchyma. No conclusion regarding effects on intact mammalian organisms may be drawn from this publication (Horvat et al., 2005).

To conclude, norflurazon proved negative for genotoxicity in standard test systems in vitro, but the database is poor. A genotoxicity test in mammalian cells is normally required, but is not available for norflurazon. Furthermore, no in vivo study on genotoxicity was submitted.

2.5 Reproductive and developmental toxicity

(a) Single-generation and multigeneration studies

Mice

A one-generation study was performed in CD-1 (HaM/ICR Swiss) mice. In the F_0 generation, groups of 50 male and 50 female mice received norflurazon at a dietary concentration of 85, 170 or 340 ppm (equivalent to 13, 26 and 200 mg/kg bw per day, respectively). Two control groups of the same size were kept in parallel and fed untreated basal diet. The parental animals were mated after 18 weeks on test. Only one litter was produced. Selected F_{1A} pups (125 males and 125 females from each group) were employed in a subsequent long-term toxicity study (Tisdel, 1975a) that is reported above in section 2.3. However, for this purpose, the 170 ppm dietary concentration was increased to 340 ppm, and the 340 ppm dietary concentration was increased to 1360 ppm.

The reproductive portion of the study did not reveal any indications of adverse effects of the test substance on fertility, reproductive parameters or pup development (Tisdel, 1975d).

Rats

In an old reproductive toxicity study, groups of 40 male and 40 female Sprague Dawley rats in the F_0 generation received the test compound (product 9789; lot no. 004; purity 98.8%) via their diet at a concentration of 125, 250 or 500 ppm (equivalent to 6.25, 12.5 and 25 mg/kg bw per day, respectively). Two control groups of the same size were fed on untreated basal diet. Following an 18-week premating period, females were mated one to one with males from the same group to produce the F_{1A} litter. From these litters, weanling animals were moved to a subsequent combined chronic

toxicity and carcinogenicity study (Tisdel, 1973, 1975b), which is reported in section 2.3 above. The reproduction study was continued 1 week after weaning of the F_{1A} pups, with a second mating to produce the F_{1B} litter. Following weaning of the second litter and a total treatment time of 9 months, the F_0 generation parental animals were killed. This part of the study was considered a separate subchronic study, which is also reported above (see section 2.2) (Harris, 1972). From the F_{1B} litters, male and female pups were randomly selected to become parental animals for the F_2 generation. Group size was the same, but the dietary concentrations were increased from 250 ppm to 375 ppm and from 500 ppm to 1025 ppm. The same concentrations were applied in the chronic toxicity and carcinogenicity study. The F_{2A} litter was employed to produce the F_{3A} and F_{3B} litters, the latter of which was used for teratological examinations of the fetuses after all presumably pregnant F_{2A} dams had been killed on day 20 of gestation.

Toxicity in adult parental rats was not separately reported. Either they were not fully examined for potential adverse effects or these were reported, if occurring, in the short- or long-term toxicity studies. Over all generations and litters, fertility, gestation, pup viability and lactation indices, litter size and pup body weight were not affected by treatment. Time to pregnancy, gestation length and sex ratio in pups also did not differ among the groups. The only remarkable finding was a very low number of pregnancies after the F_{2A} dams had been mated for the second time to produce the F_{3B} litters. In the two control groups, only 16 or 14 dams out of 40 actually produced a litter, and this rate was even lower in the high-dose group (7/40). The study author speculated that the dams might have been disturbed by noise due to construction work in the animal facility building, which would call the study quality into question. With regard to the teratological part of the study, resorption rate and fetal weight were similar across the groups, and there was no evidence of morphological abnormalities (Tisdel, 1975c).

In a two-generation reproduction study, norflurazon (batch no. P. 148/86/31; purity 98.1%) was administered in the diet to Wistar Crl:(WI) rats (25 of each sex per dose in both the F_0 and F_1 generations). Dietary concentrations were 0, 150, 750 and 1500 ppm (equal to 0, 10.2, 51.0 and 102.5 mg/kg bw per day, respectively, based on calculated substance intakes in the premating phases in both generations and sexes, with lowest intakes consistently seen in F_0 generation males). The F_0 generation rats were at least 8 weeks old at first dosing and were administered the test substance for 70 days prior to first mating (ratio of one male to one female) to produce the F_{1A} litters. Following weaning of these litters and a minimum premating interval of 10 additional days, the F_0 generation adults were bred again within their respective treatment groups to produce F_{1B} litters. Following weaning on day 21 postpartum, offspring from the F_{1B} litters were selected (one rat of each sex per litter, when possible, avoiding pairing of siblings) to become F_1 adults and, after a premating period of at least 84 days, parents of the F_{2A} and later of the F_{2B} generations. Thus, there were two litters per generation available for toxicological assessment.

All F_0 and F_1 generation parent animals were killed when no longer needed for the assessment of reproductive effects. Pregnant females that failed to deliver until day 24 of presumed gestation were also killed and necropsied. All litters in both generations were culled to four pups of each sex per litter on postnatal day 4. All the pups kept after culling but not needed for further breeding were terminated at weaning. End-points under evaluation included premature deaths, clinical signs, body weight and body weight gain, feed consumption, reproductive parameters (mating, fertility and gestation indices), litter parameters (livebirth, viability and lactation indices) and pup parameters. At scheduled termination, parental adults were necropsied, and liver and kidney weights were determined. Histopathology of the liver and kidneys was examined in all parental animals. Histopathological examinations of other organs were confined to parental animals from the control and high-dose groups in both generations and comprised the following organs: pituitary, ovaries, uterus and cervix, vagina, prostate, seminal vesicles with coagulating gland, and testes with epididymides. The pups were sexed and weighed on days 0, 4 (before and after culling), 7, 14 and 21. At their respective termination, all pups were grossly examined. Liver and kidney weights were determined in randomly selected pups at weaning. Although one would have expected the same

number of male and female pups from the same litter, this does not appear to have been the case (see Tables 24, 26 and 27 below). There was no explanation for this in the study report.

Among parental animals from the F_0 and F_1 generations, the total incidences of premature death were as follows: 1, 0, 2 and 2 in control, low-dose, mid-dose and high-dose males; and 0, 1, 0 and 1 in control, low-dose, mid-dose and high-dose females. As the few parental animals that either were found prematurely dead or had to be killed for humane reasons were nearly equally distributed among the groups and generations, the deaths could not be attributed to treatment. The same was true for the rarely occurring clinical signs, which did not exhibit any dose-related pattern.

There were occasional effects of treatment on body weight and body weight gain. These findings are considered treatment related but were confined to the high dose and were not consistently observed in both sexes or generations. In the F_0 generation, females receiving the high dose exhibited a statistically lower body weight gain ($P < 0.05$; Dunnett's test) in the second half of the initial premating interval (i.e. between weeks 5 and 10). During this period, body weight gain was about 34% lower than in the control group. A much less pronounced decrease of about 14% was also noted for the same period at the intermediate dose, but did not gain statistical significance. Despite these findings, mean body weight was not affected in females, and no effects at all were observed in the F_1 generation. In high-dose males, in contrast, there were no changes in the F_0 generation. However, a decrement in body weight gain by 11% in total was noted in the F_1 generation, achieving statistical significance for weeks 20 through 31, during which body weight gain was decreased by more than one third. The resulting mean body weight in these animals was the lowest among all groups from week 1 onwards, and the difference became statistically significant from week 18 throughout terminal week 31 ($P < 0.05$ or, in weeks 29–31, $P < 0.01$). At termination, the mean body weight was about 9% lower than in the control group.

During the gestation and lactation periods, there were only occasional findings regarding maternal body weights. During the first gestation of F_0 generation dams, mean body weight was significantly lower in the high-dose group on days 0, 7 and 14. Following the second mating, such a difference became apparent only on day 14, following a lower body weight gain in all treated groups from days 7 to 14. During lactation, slightly (but sometimes statistically significantly) lower mean body weight and body weight gain were noted in high-dose F_0 generation females. No such effects were ever seen in the F_1 generation, confirming that body weight parameters in females were affected only in the first generation, and not in the second.

Feed consumption was not affected in any segment of the study, suggesting that the rather weak effects on body weight cannot be attributed to a lower feed intake.

Gross (external and necropsy) pathological examination of the parental animals and the pups did not reveal findings that could be attributed to treatment. However, increases in both mean absolute and relative liver and kidney weights were observed in adult animals in both generations – at the intermediate and high doses in males and at the high dose in females (Table 24) – and also in the pups. Taking into consideration the results of histopathological examination and the lack of clinical chemistry analysis in this study, parallel significant increases in absolute and relative organ weights are considered treatment related, as these findings cannot be assigned to lower body weight. In the low-dose male groups, an effect on liver weight was also noted, but it was weaker and not accompanied by histopathological findings. Statistical significance was gained for either absolute or relative liver weight. On balance, this finding was not considered adverse at this dose.

Liver histopathology in the adult animals revealed hepatocellular hypertrophy, in line with the higher liver weights, at the middle and high doses in both males and females of the F_0 generation. The increase was dose related. Single-cell necrosis was also observed in the same groups, but with a clear dose–response relationship only in females. A nearly identical pattern was seen in the F_1 adults, with the exception that single-cell necrosis was a rare event in this generation, affecting only one male and one female in each of the mid- and high-dose groups. Other liver findings were equally distributed among the groups in both generations. However, it is worth noting that an adenoma was seen in one high-dose F_0 female and in one high-dose F_1 dam, but also in one low-dose F_1 male.

Table 24. Organ weight data in parental rats in the two-generation reproduction study

Parameter	0 ppm	150 ppm	750 ppm	1 500 ppm
Mean liver weight				
F_0 males				
Number examined	25	25	25	24
Absolute weight (g)	21.59	23.52*	25.82*	27.15**
Relative weight (%)	3.66	3.84	4.37**	4.63**
F_1 males				
Number examined	24	25	23	24
Absolute weight (g)	22.77	24.00	24.97*	26.47**
Relative weight (%)	3.61	3.82*	4.00**	4.62**
F_0 females				
Number examined	23	20	18	21
Absolute weight (g)	14.95	14.38	15.24	17.64**
Relative weight (%)	4.52	4.36	4.80	5.86**
F_1 females				
Number examined	20	19	19	21
Absolute weight (g)	15.59	15.67	16.90	18.76**
Relative weight (%)	4.62	4.39	4.77	5.41**
Mean kidney weight				
F_0 males				
Number examined	25	25	25	24
Absolute weight (g)	3.82	3.94	4.54**	5.32**
Relative weight (%)	0.65	0.65	0.77**	0.92**
F_1 males				
Number examined	24	25	23	24
Absolute weight (g)	3.76	3.96	4.39**	6.84*
Relative weight (%)	0.60	0.63	0.70**	1.20**
F_0 females				
Number examined	23	20	18	21
Absolute weight (g)	2.49	2.46	2.48	2.56
Relative weight (%)	0.75	0.75	0.78	0.86**
F_1 females				
Number examined	20	19	19	21
Absolute weight (g)	2.58	2.71	2.96**	3.20**
Relative weight (%)	0.77	0.76	0.83**	0.93**

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Dunnett's test)
Source: Eschbach et al. (1994)

Microscopic examination of the kidneys did not reveal nephrotoxicity when the range of findings and the number of affected animals were considered. However, the severity of renal changes tended to be greater at the high dose. In particular, in high-dose F_1 males, there was an extreme variability in absolute kidney weights, as demonstrated by a standard deviation of 4.83 compared with 0.35 in the control group. This was mainly due to three animals with kidney weights of 10.15 (88M), 15.80 (57M) and 25.80 g (55M). A similarly high kidney weight of 13.70 g was noted only in one high-dose F_0 male (97M), whereas 5 g was never exceeded in the controls and only seldom in the other treated groups. Histopathology of these animals revealed that 97M had chronic progressive nephrosis of grade 4. In line with that, both kidneys were markedly enlarged and had a granulated surface. 55M and 57M macroscopically exhibited strongly enlarged kidneys with irregular surface. Microscopic examination revealed chronic progressive nephrosis of grade 4, combined with pelvic dilatation of grade 2, in both animals. In 88M, the enlarged kidneys were accompanied by cortical tubular dilatation and tubular hyperplasia, but also, in other kidney regions, by basophilia and atrophy. In addition, lymphoid cell infiltration and tubular casts were reported, suggesting an inflammatory process. However, all these animals survived until scheduled termination, even though they also suffered from liver changes.

Examination of the reproductive or hormone-producing organs and of gross lesions in the adult rats did not reveal findings that could be attributed to treatment.

There were no effects on fertility or reproductive performance in any generation or mating up to the high dose. Mating behaviour (including time to conception), gestation index and length, parturition, litter size, pup birth weight and sex ratio in pups were not altered. In the second mating of the F_0 generation, there was a statistically significantly higher frequency of stillborn pups at the low and high doses. A nearly identical pattern was observed after the first mating of the F_1 generation (Table 25). Interpretation is difficult, as there was obviously no dose–response relationship. If the increase at the low dose is considered to have occurred by chance, there is little reason for a different view on the high-dose incidences. At the high dose, this finding was not confirmed in the F_{1A} and F_{2B} litters. Furthermore, there was no increase in the number of litters with stillborn pups with dose, and complete litter loss (i.e. litters with all stillborn) was rare and confined to the second mating of the F_0 generation. Overall, an impact of norflurazon on the frequency of stillborn pups is not likely.

Table 25. Number and percentage of stillborn pups, lactation index and terminal pup weight (males and females combined) in the two-generation reproduction study in rats

Parameter	0 ppm	150 ppm	750 ppm	1 500 ppm
F_{1A} litters				
Number of litters	23	20	20	22
Number of litters with stillborn pups	7	8	8	2
Number of pups in total	319	275	291	305
Number of stillborn pups	14	13	14	14
Percentage stillborn	4.4	4.7	4.8	4.6
Lactation index (%)	95	86	94	87
Mean pup weight on day 21 (g)	51.45	50.84	49.14	47.91
F_{1B} litters				
Number of litters	23	20	18	22
Number of litters with stillborn pups (with all stillborn)	5 (0)	7 (1)	7 (0)	8 (1)
Number of pups in total	334	287	267	331

Parameter	0 ppm	150 ppm	750 ppm	1 500 ppm
Number of stillborn pups	12	28**	11	32**
Percentage stillborn	3.6	9.8	4.1	9.7
Lactation index (%)	90	90	93	95
Mean pup weight on day 21 (g)	49.36	50.31	50.34	47.31
F_{2A} litters				
Number of litters	19	19	23	21
Number of litters with stillborn pups	4	7	4	2
Number of pups in total	257	250	305	277
Number of stillborn pups	5	18**	6	17**
Percentage stillborn	1.9	7.2	2.0	6.1
Lactation index (%)	92	92	76	88
Mean pup weight on day 21 (g)	51.30	50.94	46.03	48.74
F_{2B} litters				
Number of litters	20	19	20	21
Number of litters with stillborn pups	3	9	6	5
Number of pups in total	286	257	285	256
Number of stillborn pups	5	26**	10	7
Percentage stillborn	1.7	10.1	3.5	2.7
Lactation index (%)	87	70	64	59
Mean pup weight on day 21 (g)	50.33	49.05	43.81	42.15*

ppm: parts per million; *: $P < 0.05$ (Dunnett's test); **: $P < 0.01$ (chi-squared and Fisher's exact test)
Source: Eschbach et al. (1994)

Following the second mating of the F_1 generation, the lactation index tended to be lower in treated groups, but the difference relative to the control groups did not gain statistical significance. No effects on pup viability or body weight development were observed in any other generation or litter, but an effect of longer-lasting treatment cannot be excluded, even though it seems rather equivocal. It is worth noting that, in the F_{2B} litters, mean pup weight was statistically significantly lower at the high dose on lactation day 14 and at termination on day 21. In the F_{1A} and F_{1B} litters, mean pup weight at the high dose was the lowest, and it was consistently lower than in the control group. A similar trend was obvious at the intermediate dose, even though no statistical significance was achieved (Table 25). To conclude, a weak effect at least of high dose administration on pup body weight appears likely.

Organ weight changes were observed in male (Table 26) and female pups (Table 27) at the intermediate and high doses. In both sexes, the increases in absolute and relative liver weights were most likely treatment related, whereas a slightly higher relative kidney weight likely reflected a lower pup weight. In F_{2B} male pups, absolute kidney weight was reduced. This was the only organ weight decrease observed in this study. Because of its isolated occurrence and because it seems to be in contrast to the general pattern, this finding is considered incidental and not treatment related.

Table 26. Organ weight data in male rat pups in the two-generation reproduction study

Parameter	0 ppm	150 ppm	750 ppm	1 500 ppm
Mean liver weight				
F_{1A} litters				
Number examined	22	19	19	19
Absolute weight (g)	2.21	2.05	2.22	2.37
Relative weight (%)	4.06	3.92	4.36	4.71**
F_{1B} litters				
Number examined	22	17	15	18
Absolute weight (g)	2.16	2.16	2.30	2.34
Relative weight (%)	4.20	4.15	4.58	4.97**
F_{2A} litters				
Number examined	19	19	21	20
Absolute weight (g)	2.26	2.87	2.26	2.52
Relative weight (%)	4.28	4.37	4.71*	4.95**
F_{2B} litters				
Number examined	19	15	14	15
Absolute weight (g)	2.36	2.32	2.31	2.21
Relative weight (%)	4.31	4.48	4.75**	4.87**
Mean kidney weight				
F_{1A} litters				
Number examined	22	19	19	19
Absolute weight (g)	0.59	0.57	0.58	0.58
Relative weight (%)	1.10	1.09	1.15	1.17*
F_{1B} litters				
Number examined	22	17	15	18
Absolute weight (g)	0.58	0.58	0.56	0.55
Relative weight (%)	1.14	1.11	1.13	1.17
F_{2A} litters				
Number examined	19	19	21	20
Absolute weight (g)	0.61	0.60	0.57	0.62
Relative weight (%)	1.15	1.15	1.21	1.24**
F_{2B} litters				
Number examined	19	15	14	15
Absolute weight (g)	0.65	0.61	0.59	0.55*
Relative weight (%)	1.20	1.17	1.22	1.22

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Dunnett's test)
Source: Eschbach et al. (1994)

Table 27. Organ weight data in female rat pups in the two-generation reproduction study

Parameter	0 ppm	150 ppm	750 ppm	1 500 ppm
Mean liver weight				
F_{1A} litters				
Number examined	22	19	18	22
Absolute weight (g)	1.98	2.01	2.26	2.34*
Relative weight (%)	3.93	3.99	4.58**	4.91**
F_{1B} litters				
Number examined	21	18	17	20
Absolute weight (g)	2.02	2.05	2.26	2.37
Relative weight (%)	4.03	4.14	4.56**	5.01**
F_{2A} litters				
Number examined	19	19	21	20
Absolute weight (g)	2.27	2.36	2.31	2.52
Relative weight (%)	4.40	4.56	5.02**	5.17**
F_{2B} litters				
Number examined	20	15	16	17
Absolute weight (g)	2.39	2.30	2.26	2.30
Relative weight (%)	4.51	4.62	4.93**	5.14**
Mean kidney weight				
F_{1A} litters				
Number examined	22	19	18	22
Absolute weight (g)	0.56	0.59	0.58	0.58
Relative weight (%)	1.12	1.17	1.18*	1.21**
F_{1B} litters				
Number examined	21	18	17	20
Absolute weight (g)	0.57	0.57	0.59	0.59
Relative weight (%)	1.14	1.17	1.19	1.26**
F_{2A} litters				
Number examined	19	19	21	20
Absolute weight (g)	0.64	0.63	0.58	0.64
Relative weight (%)	1.24	1.22	1.27	1.33*
F_{2B} litters				
Number examined	20	15	16	17
Absolute weight (g)	0.66	0.62	0.59	0.60
Relative weight (%)	1.25	1.24	1.29	1.35**

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Dunnett's test)
Source: Eschbach et al. (1994)

The NOAEL for parental toxicity was 150 ppm (equal to 10.2 mg/kg bw per day), based on decreases in body weight and body weight gain as well as higher liver and kidney weights and histopathological findings in liver and kidney at 750 ppm (equal to 51.0 mg/kg bw per day). The NOAEL for reproductive toxicity was 1500 ppm (equal to 102.5 mg/kg bw per day), the highest dose tested. The NOAEL for offspring toxicity was 150 ppm (equal to 10.2 mg/kg bw per day), based on impaired body weight development and higher relative liver and kidney weights in pups and on an equivocal impact on viability in the F_{2B} litter at 750 ppm (equal to 51.0 mg/kg bw per day) (Eschbach et al., 1994).

From the parameters investigated, there was no evidence of endocrine disrupting properties. However, in contrast to current data requirements and standards, landmarks of sexual maturation of the pups (i.e. preputial separation or vaginal opening), sperm parameters, estrous cycle and ovarian follicles were not investigated in this rather old study. Thus, no firm conclusion on endocrine disrupting properties may be drawn. However, norflurazon was subject to testing in the Endocrine Disruptor Screening Program of the USEPA. In the Tier I assay battery of this programme, along with other parameters, sexual maturation of male and female pups was investigated in pubertal assays. As they may complement the information obtained from this reproduction study, these studies are reported in section 2.6.

(b) Developmental toxicity

Rats

In a developmental toxicity study, norflurazon (batch no. 0004; purity not provided) was administered by oral gavage to presumably pregnant female Sprague Dawley rats (20 per dose) on gestation days (GDs) 6 through 15. The test substance was applied in 1% carboxymethylcellulose with 0.2% Tween 80 at a dose of 0, 100, 200 or 400 mg/kg bw per day. The doses were selected on the basis of a preliminary range-finding experiment, which had apparently (most likely erroneously) been performed with an 80% wettable powder formulation instead of the technical active ingredient. Dams were euthanized on GD 20. Parameters evaluated in dams were body weight, feed consumption and efficiency, survival, clinical signs, reproductive outcomes and gross pathology. Parameters evaluated in fetuses were fetal weight, sex and incidences of external, visceral and skeletal malformations and variations.

One mid-dose dam died on day 10 of gestation. Necropsy revealed no gross lesions. This isolated premature death was not considered treatment related because of dose considerations. There were no clinical signs (with the possible exception of alopecia in one high-dose rat from GD 10 onwards) or abortions among the pregnant dams. Slight maternal toxicity was observed in all treated groups, as mean body weight gain was reduced during the first 3 days of treatment. In high-dose dams, a small body weight loss (a mean of 4 g) was noted. In all these groups, body weight reduction was accompanied by a dose-related decrease in feed efficiency, whereas feed consumption was lower only at the high dose. Because all these parameters normalized during later stages of the study, and taking into account the degree of observed initial changes, these probably treatment-related effects were considered adverse only at the high dose. There were no remarkable gross findings at necropsy.

Resorption rate was slightly higher in the high-dose group (1.1 per litter compared with 0.8 in the control group), but this was mainly due to a single litter with seven resorptions.

No evidence of developmental toxicity was observed in the fetuses. The only malformations were seen in one mid-dose fetus and in one control group fetus. The first one had thoracic spina bifida with haemorrhagic spinal cord, exencephaly and absent tail. The affected control fetus also had spina bifida, but in the lower thoracic and lumbar regions, as well as internal hydrocephalus. Because of the group allocation of these malformations, they cannot be attributed to treatment.

There was no increase in external, visceral or skeletal variations.

The NOAEL for maternal toxicity was 200 mg/kg bw per day, based on initial effects on body weight, feed consumption and feed efficiency at 400 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 400 mg/kg bw per day, the highest dose tested (Hartman & Hrab, 1972).

Rabbits

In a preliminary range-finding study, small groups of five mated New Zealand white rabbit does were administered norflurazon (lot no. 80561; purity 98.4%) via oral gavage at a dose of 0, 10, 40, 80, 100 or 400 mg/kg bw per day from GDs 7 through 19. The test substance was dissolved in 1% carboxymethylcellulose with 0.2% Tween 80. The dosing volume was 1 mL/kg bw. On the last day of treatment, does were killed and necropsied, and live and dead fetuses, resorptions and corpora lutea were counted.

Maternal toxicity was apparent from 40 mg/kg bw per day onwards. Body weight loss was seen at this dose, and it became more pronounced at higher doses. At 80 and 100 mg/kg bw per day, locomotor activity was decreased, the amount of faeces was reduced and resorptions were increased. At the highest dose (400 mg/kg bw per day), does aborted and eventually died or had to be killed on GD 15 or 16.

Based on these results, doses of 10, 30 and 60 mg/kg bw per day were proposed for the main study (Hrab, 1983a).

In the main developmental toxicity study, norflurazon (lot no. 80561; purity 98.4%) was administered to presumably pregnant New Zealand white rabbits (15 per group) on GDs 7–19. The test substance was dissolved in 1% carboxymethylcellulose with 0.2% Tween 80 and administered by oral gavage at a dose of 0, 10, 30 or 60 mg/kg bw per day. The dosing volume was 1 mL/kg bw. Control group animals received the vehicle only. The animals were killed and necropsied on day 30. Parameters evaluated in does were survival, clinical signs, occurrence of abortions, body weight, body weight gain, feed consumption, gravid uterine weights and gross pathology. Live and dead fetuses, early and late resorptions, and corpora lutea were counted. Parameters evaluated in fetuses were fetal weight, fetal sex ratio and incidences of external, visceral and skeletal malformations and variations.

Two high-dose females aborted on GD 20 and GD 24, respectively. Even though both incidents were noted after treatment had been finished, a delayed effect of the test substance cannot be excluded. It was not known whether the abortions were a sign of maternal toxicity or occurred because of severe fetal anomalies. There were no further abortions in any group, premature deaths or clinical signs that could be attributed to treatment. A remarkable finding was delivery of 12 full-term pups by a low-dose animal on day 12. The only explanation was a pre-existing pregnancy of this doe, calling the study quality into question. The doe and her pups were excluded from analysis.

A significant body weight loss was noted in the high-dose group, which was most pronounced during the first week of treatment. This effect was partly reversed by a compensatory increase in body weight gain in the period following termination of treatment. Low- and mid-dose females gained less weight over the course of the treatment period, but the difference relative to the control group was not statistically significant (Table 28). At the intermediate dose, occasional body weight losses (e.g. by 4 g on average from GD 7 to GD 8 or by 14 g from GD 15 to GD 16) were noted, but were much less pronounced than in the group receiving the high dose. On the same days of treatment, the mean losses in the high-dose group were 44 g and 29 g, respectively. The relevance of the lower body weight gain or of sporadic weight losses in the groups receiving 10 and 30 mg/kg bw per day is questionable, as large differences between all the groups were noted in the first week of gestation (i.e. before treatment commenced) (Table 28). The high-dose body weight loss during treatment, in contrast, was clearly adverse when its extent is taken into consideration.

Table 28. Body weight development during gestation

	Mean maternal body weight change (g)			
Days	**0 mg/kg bw per day**	**10 mg/kg bw per day**	**30 mg/kg bw per day**	**60 mg/kg bw per day**
0–7	11.7	−39.1	−13.9	35.1
7–13	120.3	52.6	41.5	−106.5**
7–19	230.0	157.1	113.7	−129.4**
19–25	42.2	59.8	53.1	193.5*

bw: body weight; *: $P < 0.05$; **: $P < 0.01$ (analysis of variance/Dunnett’s test)
Source: Hrab (1983b)

Feed consumption was not reported. Therefore, it is not clear whether altered body weight gain was (at least partly) due to changes in feed intake.

There were no differences between the control and treated groups with regard to reproductive parameters such as number of corpora lutea, number of implantations, resorption rate, litter size, number of live or dead fetuses and fetal sex ratio. However, mean fetal weight was reduced at the high dose; even in the absence of statistical significance, this difference is considered indicative of a treatment-related effect (Table 29).

Table 29. Mean fetal weight and skeletal anomalies (fetal/litter incidences)

Parameter	**0 mg/kg bw per day**	**10 mg/kg bw per day**	**30 mg/kg bw per day**	**60 mg/kg bw per day**
Number of live fetuses per litter	8.5	7.0	7.0	8.8
Mean fetal weight (g)	46.3	46.6	46.5	40.5
Number of fetuses examined	128	98	105	114
Vertebrae absent or malformed	1/1	1/1	1/1	1/1
Fused ribs	0/0	0/0	1/1	1/1
Incomplete ossification, frontal skull bones	10/4	15/6	18*/8	22*/9
Incomplete ossification, caudal vertebrae (16 present in total)	18/11	11/6	28*/10	33*/12
Rudimentary 13th rib	49/14	47/12	38/13	74*/12
Metacarpal bone, not ossified	9/5	6/4	15/7	25*/8
Forelimb digit V, middle phalanx not ossified	5/3	7/5	12*/6	15*/5
Tibia, proximal epiphysis not ossified	39/11	52*/11	38/11	64*/13

bw: body weight; *: $P < 0.05$; **: $P < 0.01$ (analysis of variance/Dunnett’s test)
Source: Hrab (1983b)

External malformations were confined to two control group fetuses in which acephaly, absence or anomalies of the forelimbs, lordosis, delayed ossification of or malformed vertebrae, and a short tail were described. The only visceral malformation was hydrocephalus in one high-dose fetus (116 fetuses examined). Although it was an isolated finding, historical control data suggest that this type of malformation is rarely seen in this rabbit strain (2/1494 fetuses affected, according to the study report), even though no detailed information on the composition of this historical database is

available. In addition, the incidence of skeletal variations, mainly delayed ossification, was increased at the intermediate and high doses (Table 29). The differences relative to the control group were occasionally statistically significant. However, as the litter (and not the number of affected fetuses) is considered the relevant unit in teratology, there was not much difference between the different doses. The only clearly treatment-related effect was the incomplete ossification of skull bones. It must be emphasized that the total number of fetuses or litters with skeletal or other variations was not affected by treatment. Nonetheless, ossification delay would be well in line with lower mean fetal weight at the high dose. Fused ribs and absent or misaligned vertebrae were considered by the study author as skeletal malformations, but their respective occurrence was rare and not clearly dose related.

The NOAEL for maternal toxicity was 30 mg/kg bw per day, based on abortions and body weight loss at the high dose of 60 mg/kg bw per day. The latter finding was strongest during the first week of treatment. Because there are no data for the individual days, it is considered, by default, to be an acute effect.

The NOAEL for embryo and fetal toxicity was 10 mg/kg bw per day, based on an increase in skeletal variations, mainly ossification delay, at 30 mg/kg bw per day. By its nature, such an effect is not considered an acute one. A teratogenic potential at the high dose (hydrocephalus) cannot be excluded, and its extent could have been masked by the abortions. Even though such a teratogenic effect should be considered an acute one, it would be confined to the high-dose group (Hrab, 1983b).

2.6 Special studies

(a) Neurotoxicity

No studies were submitted.

(b) Immunotoxicity

No studies were submitted.

(c) Mechanistic studies

No mechanistic studies were submitted. However, there was one article of interest retrieved from the open literature. Because of similar effects in plants, the impact of norflurazon on fatty acids in rat liver cells and liver cell microsomes was investigated in vitro. It was shown that the test compound at concentrations of 0.1 and 1.0 mmol/L in the medium, when incubated for 20 or 60 minutes, reduced the amount of desaturated and chain-elongated fatty acids in rat liver cells. In microsomes, norflurazon at 1.0 mmol/L inhibited desaturation of essential fatty acids, but the mechanism behind it could not be elucidated. The possible toxicological relevance of this finding is unknown, as is its potential to occur in vivo (Hagve, Christophersen & Boger, 1985).

(d) Studies on endocrine properties

A number of studies were performed under the Endocrine Disruptor Screening Program of the USEPA. In total, 10 studies that belong to the Tier I assay battery are accessible on the USEPA website. In all these studies, high-purity norflurazon (99.5 ± 0.5% weight/weight; batch no. 609218), which had been provided by Syngenta Ltd, was applied. This test material may be similar to, but is not the same as, that used in the older toxicological studies reported in this monograph. Only two of these 10 studies are reported here, as they may be used to amend data on the sexual maturation of young animals, an end-point that was apparently not investigated in the study by Eschbach et al. (1994).

Young female Crl:CD(SD) rats were administered norflurazon at a dose of 250 or 500 mg/kg bw per day via oral gavage from postnatal days 22 through 42. A concurrent control group received only the vehicle (i.e. 1% carboxymethylcellulose in deionized water). Each group comprised 15 animals. The rats were monitored for clinical signs of toxicity twice a day. In addition, they were observed for vaginal opening. Once this developmental step had been noted, daily vaginal lavage was performed in each female to determine the stage of estrus. Body weight and feed intake were determined daily. At termination on postnatal day 42, the rats were killed and necropsied. Selected organs (liver, kidneys, adrenals, ovaries, uterus, thyroid, pituitary gland) were taken, weighed and preserved. Clinical chemistry parameters, including levels of thyroxine (T_4) and thyroid stimulating hormone (TSH), were measured.

All animals survived to scheduled termination, and there were no clinical signs of toxicity that could be attributed to treatment. However, when compared with the controls, body weight gain was 7.8% or 9.2% lower in the groups receiving 250 or 500 mg/kg bw per day, respectively. These reductions resulted in a slightly lower mean terminal body weight in these treated groups. These findings cannot be attributed to lower feed intake. Mean age of vaginal opening was 35.5 days in the controls, 36.1 days in the low-dose group and 37.6 days in the high-dose group. The significant delay in vaginal opening at 500 mg/kg bw per day might be related to the impaired body weight development, as two females with the strongest delay gained less weight over the course of the study than the average. At termination, these animals had the lowest absolute body weights among all rats on study and exhibited no or reduced corpora lutea in the ovaries. There was no delay in this developmental landmark in the group receiving norflurazon at 250 mg/kg bw per day. Estrous cycle was not affected at any dose.

Clinical chemistry revealed higher mean bilirubin levels and a decrease in mean bile acid levels at both doses. T_4 levels were significantly reduced at 250 and 500 mg/kg bw per day, by 13% and 23%, respectively. Perhaps in line with that, colloid area in the thyroid was reduced in the high-dose group in 7/15 animals. A similar finding was noted in both the control and the low-dose groups in only two animals each. This alteration was microscopically characterized by smaller follicles and a lower amount of colloid. As there was a concomitant and dose-related increase in absolute and relative liver weights at both doses, increased metabolism of thyroid hormones was suspected. However, TSH levels were not affected. Surprisingly, the lowest mean TSH level was noted at the high dose (5.6 ng/mL compared with 6.3 ng/mL in both the controls and the low-dose group) (Coder, 2012a).

Young male Crl:CD(SD) rats were administered norflurazon at a dose of 185 or 375 mg/kg bw per day via oral gavage from postnatal days 23 through 53. A concurrent control group received only the vehicle (i.e. 1% carboxymethylcellulose in deionized water). Each group comprised 15 animals. The rats were monitored for clinical signs of toxicity twice a day. In addition, beginning from postnatal day 30, the males were observed for preputial separation. Body weight and feed intake were determined daily. At termination on postnatal day 53, the rats were killed and necropsied. Selected organs (liver, kidneys, adrenals, thyroid, pituitary gland and reproductive organs) were taken, weighed and preserved. Clinical chemistry parameters, including levels of testosterone, T_4 and TSH, were determined.

All animals survived to scheduled termination, and there were no clinical signs of toxicity that could be attributed to treatment. Similar to what was observed in the study on young females (Coder, 2012a), body weight gains were 7.4% and 8.2% lower in the groups receiving 185 and 375 mg/kg bw per day, respectively. These reductions resulted in slightly lower mean terminal body weights in these groups (5.7% and 6.6%, respectively). These findings cannot be clearly attributed to lower feed intake, even though lower feed consumption was initially noted in the high-dose group as well as sporadically later in the study. Mean age of complete balanopreputial separation was 45.7 days in the controls, 46.1 days in the low-dose group and 47.5 days in the high-dose group. Mean body weight at attainment of this developmental landmark was similar in all groups. Therefore, the delay was attributed by the study author to the lower body weight gain at the high dose.

Hormone levels were not affected in any group, and the only change in clinical chemistry parameters was a decrease in mean bile acid levels by 54% and 58% at 185 and 375 mg/kg bw per day, respectively.

Absolute and relative liver weights were increased at both doses, whereas mean absolute and relative adrenal weights were reduced in the high-dose group. Decreases in absolute weights of some reproductive organs (prostate, seminal vesicle) were attributed to the lower terminal body weight. Gross and histopathological examination did not reveal findings that could be attributed to treatment (Coder, 2012b).

3. Observations in humans

No data were submitted, and no information on poisoning incidents was retrieved from the public literature.

One case report suggests skin sensitization in a farmer by a plant protection product (Predict®) containing norflurazon at a concentration of 78.6%. A patch test was positive for this formulation in a 53-year-old man who suffered from sunburn-like symptoms that caused almost complete closing of his eyes. Later on, he developed urticaria in the same area (Leow & Maibach, 1996).

In an epidemiological study from the San Joaquin valley of California, USA, a possible association between the occurrence of congenital heart defects and residential proximity of the mothers to commercial agricultural pesticide application during a 3-month periconceptional window was investigated. In this case–control study, including 569 heart defect cases and 785 controls born between 1997 and 2006, norflurazon was specifically included as an example of pyradizinone compounds. Based on 74 cases, the risk for coarctation of the aorta (i.e. narrowing of aorta lumen close to the heart) was increased in infants up to 1 year of age and in fetuses when the mothers had lived within 500 m of pesticide application during the periconceptional period. In fact, six mothers from this group of 74 reported norflurazon exposure, compared with 23 women from the control group giving birth to 785 children. The odds ratio was 2.9, with a confidence interval of 1.1–7.5. Confounding factors were taken into account, but no information on the extent of exposure was available. The San Joaquin valley is an area with heavy pesticide use in general, and therefore co-exposure to other agrochemicals is likely. A similar increase in odds ratio for aortal coarctation was noted with exposure to propiconazole, but also to petroleum oil. For seven other heart defect phenotypes, no association with norflurazon exposure could be detected (Carmichael et al., 2014).

Comments

Biochemical aspects

Following oral administration of a single dose of ^{14}C-labelled norflurazon at 2 mg/kg bw to female rats, more than 90% of the administered dose was absorbed, based on a comparison of excretion following oral and intravenous dosing. Absorption after administration of a single high dose of 110 mg/kg bw was similar. Elimination was nearly complete within 96 hours. The major route of excretion was the faeces, with approximately 20% excreted via the urine. Tissue residues were generally low, with the highest relative radioactivity observed in liver and kidneys (Saunders et al., 1985).

Biotransformation was extensive, with only 2% or less of the administered doses excreted as unchanged parent. The main metabolic pathways were *N*-demethylation and glutathione conjugation. Numerous metabolites were found in urine and faeces, nine of which could be identified. Only one of them (metabolite 5, a sulfoxide) accounted for more than 10% of the applied dose (up to 39% in urine, following intravenous administration), whereas the others represented only 1–2% (Saunders et al., 1985).

An older study provided some evidence that excretion and metabolism are similar in male and female rats (Karapally, 1974).

Toxicological data

Where no reliable information was available on the active ingredient, toxicological data on the 80% formulation were evaluated.

In rats, the acute oral LD_{50} of an 80% formulation was 1080 mg/kg bw (Gardner, 1987a), and the acute dermal LD_{50} was greater than 2000 mg/kg bw (Gardner, 1987b). Although these studies were performed under GLP, there is no reliable information on the acute oral or dermal toxicity of the active ingredient itself.

In a GLP- and guideline-compliant study of the active substance in rats, the acute inhalation LC_{50} was greater than 2.4 mg/L (Hoffman, 1996).

In a study of limited reliability, norflurazon active ingredient did not cause skin or eye irritation in rabbits (Bagdon, 1972). In contrast, the 80% formulation was slightly irritating to the eyes and very slightly irritating to the skin of rabbits (Liggett & Smith, 1987, 1988). For the same formulation, there was no evidence of skin sensitization in a Buehler test in guinea-pigs (Kynoch & Parcell, 1987), whereas an older study with the active ingredient was inconclusive (Bagdon, 1973).

Short-term toxicity feeding studies were performed in rats, dogs and, to a lesser extent, mice. In all three species, the liver was a common target organ. Adverse effects on the kidney, thyroid and haematopoietic system (some evidence of anaemia in dogs) were also observed, but did not occur consistently across species and studies.

A poorly reported 28-day study in the mouse was performed as a range-finding experiment. Dietary concentrations were 0, 70, 210, 420 and 2520 ppm (equivalent to 0, 10.5, 31.5, 63 and 378 mg/kg bw per day, respectively). Liver and kidney weights were increased in males from 210 ppm onwards, and a liver weight increase was also observed in females at the highest dose. In males and females at 2520 ppm, gross appearance of the liver suggested some fatty degeneration (Tisdel, 1971).

In a 28-day feeding study in rats, the dietary concentrations were 0, 500, 1000 and 5000 ppm (equal to 0, 52, 105 and 517 mg/kg bw per day for males and 0, 70, 140 and 717 mg/kg bw per day for females, respectively). The NOAEL was 500 ppm (equal to 52 mg/kg bw per day), based on relative liver and kidney weight increases, accompanied by histopathological lesions, at 1000 ppm (equal to 105 mg/kg bw per day) (Fogleman, 1970).

In a 90-day feeding study in rats, the dietary concentrations were 0, 250, 500 and 2500 ppm (equal to 0, 24, 45 and 248 mg/kg bw per day for males and 0, 26, 52 and 275 mg/kg bw per day for females, respectively). Body weight was slightly reduced in both sexes at the high dose. In the same groups, liver and kidney weights were increased, but no concomitant histopathological lesions were noted. In contrast, a higher thyroid weight in high-dose males was accompanied by moderate follicular cell hypertrophy, moderate depletion of colloid and a slight to moderate increase in interstitial vascularity, suggesting some stimulation of thyroid activity. The NOAEL was 500 ppm (equal to 45 mg/kg bw per day) (Fogleman, 1971a).

In a 6-month study, dogs were fed norflurazon at a concentration of 0, 50, 150 or 450 ppm (equal to 0, 1.5, 5.0 and 14.3 mg/kg bw per day for males and 0, 1.6, 4.8 and 17.8 mg/kg bw per day for females, respectively). The NOAEL was 50 ppm (equal to 1.5 mg/kg bw per day), based on liver weight increase, minor histopathological findings in liver and thyroid and increases in cholesterol and alkaline phosphatase at 150 ppm (equal to 4.8 mg/kg bw per day) (Klotzsche & Carpy, 1973).

In a guideline- and GLP-compliant toxicological study with norflurazon, dogs were administered a dietary concentration of 0, 50, 200 or 800 ppm (equal to 0, 1.7, 6.2 and 27 mg/kg bw per day for males and 0, 1.5, 6.3 and 23 mg/kg bw per day for females, respectively) for 1 year. The NOAEL was 50 ppm (equal to 1.5 mg/kg bw per day), based on impairment of nutritional parameters, such as body weight (gain), feed consumption and feed efficiency, in females and alterations in clinical chemistry suggestive of liver toxicity and increased liver weights in both sexes at 200 ppm (equal to 6.2 mg/kg bw per day). Kidney findings of equivocal toxicological significance, including renal tubular pigmentation, were noted in males at 200 ppm and above (Warren et al., 1990).

The overall NOAEL for the two dog studies was 50 ppm (equal to 1.5 mg/kg bw per day), and the overall LOAEL was 150 ppm (equal to 4.8 mg/kg bw per day).

In a combined long-term toxicity and carcinogenicity study in mice with in utero exposure, norflurazon was administered at a dietary concentration of 85, 340 or 1360 ppm (equivalent to 13, 51 and 200 mg/kg bw per day, respectively) for up to 2 years. Two control groups were included. The study suffered from many deficiencies, and there is good reason to exclude one of the control groups and the low-dose group, as the animals were not treated in parallel with the other groups. The parents of the animals under investigation had already been exposed to norflurazon as part of a reproduction study. Except for a slight reduction in body weight in high-dose males, there were no in-life observations that could be attributed to treatment. Liver weight was increased in all male groups and in mid- and high-dose females, showing a clear dose–response relationship. In females, there was no increase either in neoplastic or in non-neoplastic liver findings in any group (Tisdel, 1975a).

Histopathology of the livers in male mice was evaluated independently by three pathologists (Richter, 1975; Tisdel, 1975a; Rust, 1980) and subjected to an additional statistical reanalysis (Andriano, 1981). Based on the evaluations considered most appropriate by the Meeting (Rust, 1980; Andriano, 1981), there was a clear increase only in liver cell adenoma at 1360 ppm (equivalent to 200 mg/kg bw per day), with a NOAEL for carcinogenicity of 340 ppm (equivalent to 51 mg/kg bw per day). No NOAEL could be identified for non-neoplastic effects, as the lower doses were not sufficiently investigated. The LOAEL was 340 ppm (equivalent to 51 mg/kg bw per day), the lowest dose for which reliable data were available, based on non-neoplastic liver lesions in males, increases in liver weight in both sexes and non-neoplastic microscopic lesions in organs other than the liver (i.e. kidneys, spleen, pancreas, ovaries and bone marrow) mainly in females at the highest dose.

The Meeting concluded that norflurazon at a dose of 200 mg/kg bw per day caused an increase in benign liver tumours in male mice that was preceded by non-neoplastic changes at lower doses. No increase in the incidence of carcinomas was observed in this study. No information on the mode of action is available; thus, human relevance cannot be excluded.

In a combined long-term toxicity and carcinogenicity study in rats, norflurazon was administered at a dietary concentration of 125, 375 or 1025 ppm (equivalent to 6.25, 18.9 and 51.3 mg/kg bw per day, respectively) for 2 years. Two control groups of the same size were included and fed on untreated basal diet. Again, there were a number of deficiencies in this study, including the unusual study design, in which the parents had previously been treated with equal or lower concentrations of the test substance. There was no evidence of carcinogenicity in this study. There were no in-life observations attributable to treatment. The NOAEL was 125 ppm (equivalent to 6.25 mg/kg bw per day), based on increased kidney weights and histopathological changes (increased hyaline casts and nephritis) in the kidneys of females at 375 ppm (equivalent to 18.9 mg/kg bw per day). The uterus was identified as an additional target organ, as there was a higher incidence of squamous metaplasia and endometritis, at least in high-dose females. In low- and mid-dose females, the uteri were not subject to histopathological examination (Tisdel, 1975b).

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

Norflurazon was tested for genotoxicity in a limited range of in vitro assays. There was no evidence of genotoxicity in the Ames test (Brusick, 1977; Anonymous, 1980) or the rec assay in bacteria (Anonymous, 1980) or of chromosomal aberrations in mammalian (Chinese hamster ovary) cells (Putman, 1985). The substance gave negative results for unscheduled DNA synthesis in primary rat hepatocytes (Curren, 1985), but was positive in a more recently published comet assay in an aquatic bioindicator organism, but only at cytotoxic concentrations (Horvat et al., 2005).

In spite of the generally poor database, the Meeting concluded, on the weight of evidence, that norflurazon is not genotoxic in vitro and that it is unlikely to be genotoxic in vivo.

In view of the limited evidence of genotoxicity in vitro and taking into account the tumour spectrum of this compound (i.e. adenoma in one sex and one species only at the highest dose), the Meeting concluded that norflurazon is unlikely to pose a carcinogenic risk to humans from the diet.

The reproductive toxicity of norflurazon was investigated in one of the few studies that was performed under GLP and broadly complied with modern test guidelines. In a two-generation study in rats, the test substance was administered at a dietary concentration of 0, 150, 750 or 1500 ppm (equal to 0, 10.2, 51.0 and 102.5 mg/kg bw per day, respectively). No adverse effects on fertility or reproductive performance were observed. Accordingly, the NOAEL for reproductive toxicity was 1500 ppm (equal to 102.5 mg/kg bw per day), the highest dose tested. Parental effects in the adult animals comprised decreases in body weight and body weight gain, but also higher liver and kidney weights and histopathological findings in the liver and kidney, at 750 ppm (equal to 51.0 mg/kg bw per day), with a NOAEL for parental toxicity of 150 ppm (equal to 10.2 mg/kg bw per day). The same NOAEL was identified for offspring toxicity, based on impaired body weight development, higher relative weights of liver and kidney in pups and an equivocal impact on viability (lactation index) in the F_{2B} litters at 750 ppm (equal to 51.0 mg/kg bw per day) (Eschbach et al., 1994).

It was noted that developmental landmarks were not investigated in this study. However, relevant information was available from recent studies from the Endocrine Disruptor Screening Program of the USEPA.

In a pubertal assay in intact young female rats, a delay in vaginal opening was observed at the high dose of 500 mg/kg bw per day, but not at 250 mg/kg bw per day. This effect was attributed to lower body weight gain in this group. Estrous cycle was not affected. An impact on the thyroid was evident, with lower T_4 levels at both doses and histological findings (smaller follicles, lower amount of colloid) in the animals receiving the higher dose (Coder, 2012a).

In a similar study in intact young male rats, balanopreputial separation was slightly delayed at the high dose of 375 mg/kg bw per day. Again, this observation was made in parallel to a decrease in body weight gain. Testosterone, T_4 and TSH levels were not affected (Coder, 2012b).

In a developmental toxicity study in rats, norflurazon was administered by gavage at a dose of 0, 100, 200 or 400 mg/kg bw per day on GDs 6 through 15. The NOAEL for maternal toxicity was 200 mg/kg bw per day, based on initial body weight loss and lower feed consumption and feed efficiency at 400 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 400 mg/kg bw per day, the highest dose tested (Hartman & Hrub, 1972).

In a preliminary range-finding study, pregnant rabbits received norflurazon by oral gavage from GDs 7 through 19 at a dose of 0, 10, 40, 80, 100 or 400 mg/kg bw per day. At 40 mg/kg bw per day, maternal toxicity was apparent as a result of body weight loss, which became more pronounced at higher doses. Resorption rate was increased at 80 mg/kg bw per day and higher. At 400 mg/kg bw per day, does aborted and eventually died or had to be killed on GD 15 or 16 (Hrab, 1983a).

In the main developmental toxicity study, daily doses of 0, 10, 30 or 60 mg/kg bw were administered to pregnant rabbits from GDs 7 through 19. The NOAEL for maternal toxicity was 30 mg/kg bw per day, based on abortions and body weight loss at the high dose of 60 mg/kg bw per day. The latter finding was most marked during the first week of treatment and was considered by the Meeting to be an acute effect. The NOAEL for embryo and fetal toxicity was 10 mg/kg bw per day, based on an increase in skeletal variations, mainly ossification delay, at 30 mg/kg bw per day. A teratogenic potential at the high dose cannot be excluded, as hydrocephalus was observed in one high-dose fetus (of 116 fetuses examined). This is a very rare malformation in New Zealand white rabbits (historical background 0.1%), the strain used in this study, and the extent could have been masked by the abortions (Hrab, 1983b).

The Meeting concluded that norflurazon is not teratogenic in rats, but it could not exclude the possibility that norflurazon is teratogenic in rabbits, based on the occurrence of hydrocephalus in a single rabbit fetus in the high-dose group.

No studies on neurotoxicity or immunotoxicity were submitted. There was no evidence for such effects in the routine toxicological studies, but it must be taken into consideration that the range of parameters in these mostly very old and pre-guideline studies was relatively limited. Therefore, the Meeting could not conclude on these end-points.

Toxicological data on metabolites and/or degradates

Desmethyl norflurazon

The desmethyl metabolite of norflurazon (plant metabolite) was found in the urine of male rats (accounting for 2.9%) following oral administration of 10 mg of norflurazon for 15 days (Karapally, 1974). Desmethyl norflurazon was detected in the urine of female rats receiving a single norflurazon dose of 2 mg/kg bw at a maximum of 7% (lower amounts found in other treatment groups). In faeces, the same metabolite accounted for 2.2–5%, depending on the treatment group (Saunders et al., 1985).

This metabolite and its conjugates are considered to be covered by studies of the parent compound.

6-Methyl sulfoxide norflurazon

The metabolite 6-methyl sulfoxide norflurazon appears in rat urine at greater than 10%.

This metabolite is considered to be covered by the studies of the parent compound.

NOA-452075

The metabolite 1-({5-chloro-6-oxo-1-[3-(trifluoromethyl)phenyl]hydropyridazin-4-yl}-ureido)-4-hydroxybutaneurea (i.e. NOA-452075) occurs in the rat only at trace concentrations.

For chronic toxicity, the threshold of toxicological concern (TTC) approach (Cramer class III) could be applied.

Human data

No data were submitted. A case of skin sensitization due to a commercial product containing norflurazon has been reported in the open literature (Leow & Maibach, 1996).

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

Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) of 0–0.005 mg/kg bw for norflurazon, on the basis of an overall NOAEL of 1.5 mg/kg bw per day in the 6-month and 1-year studies in dogs, using a safety factor of 300. This NOAEL was based on impairment of nutritional parameters, such as body weight (gain), feed consumption and feed efficiency, in females and alterations in clinical chemistry suggestive of liver toxicity and increased liver weights in both sexes. An additional factor of 3 was used because of the poor quality of the database. The upper range of the ADI provides a margin of 40 000 relative to the LOAEL for liver adenomas in male mice.

The Meeting established an acute reference dose (ARfD) of 0.3 mg/kg bw from the NOAEL for maternal toxicity of 30 mg/kg bw per day in a developmental toxicity study in rabbits, based on abortions and reductions in maternal body weight at 60 mg/kg bw per day, using a safety factor of 100.

The ADI and ARfD apply to the metabolites desmethyl norflurazon and its conjugates and 6-methyl sulfoxide norflurazon.

Levels relevant to risk assessment of norflurazon

Species	Study	Effect	NOAEL	LOAEL
Mouse	Two-year study of toxicity and carcinogenicity[a]	Toxicity	–	340 ppm, equivalent to 51 mg/kg bw per day[b]
		Carcinogenicity	340 ppm, equivalent to 51 mg/kg bw per day	1 360 ppm, equivalent to 200 mg/kg bw per day
Rat	Two-year study of toxicity and carcinogenicity[a]	Toxicity	125 ppm, equivalent to 6.25 mg/kg bw per day	375 ppm, equivalent to 18.9 mg/kg bw per day
		Carcinogenicity	1 025 ppm, equivalent to 51.3 mg/kg bw per day[c]	–
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	1 500 ppm, equal to 102.5 mg/kg bw per day[c]	–
		Parental toxicity	150 ppm, equal to 10.2 mg/kg bw per day	750 ppm, equal to 51.0 mg/kg bw per day
		Offspring toxicity	150 ppm, equal to 10.2 mg/kg bw per day	750 ppm, equal to 51.0 mg/kg bw per day
	Developmental toxicity study[d]	Maternal toxicity	200 mg/kg bw per day	400 mg/kg bw per day
		Embryo and fetal toxicity	400 mg/kg bw per day[c]	–
Rabbit	Developmental toxicity study[d]	Maternal toxicity	30 mg/kg bw per day	60 mg/kg bw per day
		Embryo and fetal toxicity	10 mg/kg bw per day	30 mg/kg bw per day
Dog	Six-month and 1-year toxicity studies[a,e]	Toxicity	50 ppm, equal to 1.5 mg/kg bw per day	150 ppm, equal to 4.8 mg/kg bw per day

[a] Dietary administration.
[b] Lowest dose tested for which reliable data were available.
[c] Highest dose tested.
[d] Gavage administration.
[e] Two or more studies combined.

Acceptable daily intake (ADI) (applies to norflurazon, desmethyl norflurazon and its conjugates and 6-methyl sulfoxide norflurazon, expressed as norflurazon)

0–0.005 mg/kg bw

Acute reference dose (ARfD) (applies to norflurazon, desmethyl norflurazon and its conjugates and 6-methyl sulfoxide norflurazon, expressed as norflurazon)

0.3 mg/kg bw

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

Full evaluation of potential for genotoxicity in vivo, neurotoxicity and immunotoxicity; comparative metabolism study in toxicological species and humans; results from epidemiological, occupational health and other observational studies of human exposure

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

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	>80%
Dermal absorption	No data
Distribution	Highest concentrations in liver and kidney
Potential for accumulation	Low
Rate and extent of excretion	Nearly complete via faeces (65–80%) and urine (19–27%) within 96 hours
Metabolism in animals	Extensive by a variety of pathways (e.g. *N*-demethylation and glutathione conjugation), resulting in numerous metabolites, of which only a sulfoxide accounted for more than 10% of the administered dose
Toxicologically significant compounds in animals and plants	Norflurazon
Acute toxicity	
Rat, LD_{50}, oral	1 080 mg/kg bw (80% formulation)
Rat, LD_{50}, dermal	>2 000 mg/kg bw (80% formulation)
Rat, LC_{50}, inhalation	>2.4 mg/L (active substance)
Rabbit, dermal irritation	Very slightly irritating (80% formulation)
Rabbit, ocular irritation	Slightly irritating (80% formulation)
Guinea-pig, dermal sensitization	Not sensitizing (Buehler, 80% formulation)
Short-term studies of toxicity	
Target/critical effect	Liver changes, alterations in body weight, feed consumption and feed efficiency
Lowest relevant oral NOAEL	1.5 mg/kg bw per day (dog)
Lowest relevant dermal NOAEL	No data
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Decrease in body weight; liver weight increase, histological lesions in liver and kidney
Lowest relevant NOAEL	6.25 mg/kg bw per day (rat)
Carcinogenicity	Carcinogenic in male mice, but not in female mice or rats[a]
Genotoxicity	
	Negative in vitro[a]

Reproductive toxicity	
Target/critical effect	No effects on reproduction
Lowest relevant parental NOAEL	10.2 mg/kg bw per day
Lowest relevant offspring NOAEL	10.2 mg/kg bw per day
Lowest relevant reproductive NOAEL	102.5 mg/kg bw per day, highest dose tested
Developmental toxicity	
Target/critical effect	Abortions and body weight losses in does; skeletal variations and developmental delay in rabbit fetuses
Lowest relevant maternal NOAEL	30 mg/kg bw per day (rabbit)
Lowest relevant embryo/fetal NOAEL	10 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	No data
Studies on toxicologically relevant metabolites	
	No studies available on toxicologically relevant metabolites
Human data	
	Evidence of skin sensitization (case report)

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

Summary

	Value	**Study**	**Safety factor**
ADI	0–0.005 mg/kg bw[a]	Short-term toxicity studies in dogs	300
ARfD	0.3 mg/kg bw[a]	Developmental toxicity study in rabbits	100

[a] Applies to norflurazon, desmethyl norflurazon and its conjugates and 6-methyl sulfoxide norflurazon, expressed as norflurazon.

References

Andriano K (1981). Supplemental analyses and comments – hepatic proliferative change data for the mouse carcinogenicity study of herbicide 9789 (norflurazon). Unpublished report no. T-204. Sandoz Inc. Submitted to WHO by Tessenderlo Kerley Inc., Phoenix, AZ, USA.

Anonymous (1980). Norflurazon. Report on mutagenicity test on NP-52 in bacteria. Unpublished report no. 13/81 (English translation). Biosafety Research Center, Japan. Submitted to WHO by Tessenderlo Kerley Inc., Phoenix, AZ, USA.

Bagdon RE (1972). Acute oral rat toxicity, rabbit eye irritation and rabbit dermal irritation studies of herbicide 9789, 48-511 and a mixture of 95% 9789:%% 48-511. Unpublished report no. T-1-10/24/72. Sandoz-Wander, Inc. (USA). Submitted to WHO by Tessenderlo Kerley Inc., Phoenix, AZ, USA.

Bagdon RE (1973). Guinea pig sensitization test of herbicide 9789. Unpublished report no. T-1-5/16/73. Sandoz-Wander, Inc. (USA). Submitted to WHO by Tessenderlo Kerley Inc., Phoenix, AZ, USA.

Brusick D (1977). Mutagenicity evaluation by the Ames test of norflurazon. Unpublished report no. T-1-6/6/77. Sandoz Project T-0993, Sandoz-Wander, Inc. (USA). Submitted to WHO by Tessenderlo Kerley Inc., Phoenix, AZ, USA.

Bukva NF (1970). Dermal LD_{50} test with SAN 9789 in rabbits. Unpublished report no. 9789-525. Affiliated Medical Enterprises, Inc. Submitted to WHO by Tessenderlo Kerley Inc., Phoenix, AZ, USA.

Carmichael SL, Yang W, Roberts E, Kegley SE, Padula AM, English PB et al. (2014). Residential agricultural pesticide exposures and risk of selected congenital heart defects among offspring in the San Joaquin valley of California. Environ Res. 135:133–8.

Charache S, Grisolia S, Fiedler AJ, Hellegers AE (1970). Effect of 2,3-diphosphoglycerate on oxygen affinity of blood in sickle cell anemia. J Clin Invest. 49:806–12.

Coder PS (2012a). Norflurazon – pubertal development and thyroid function in intact juvenile/peripubertal female rats. Unpublished report no. WIL-639116. WIL Research Laboratories, Ashland, OH, USA, on behalf of Syngenta, Ltd. Submitted to WHO by Tessenderlo Kerley Inc., Phoenix, AZ, USA.

Coder PS (2012b). Norflurazon – pubertal development and thyroid function in intact juvenile/peripubertal male rats. Unpublished report no. WIL-639117. WIL Research Laboratories, Ashland, OH, USA, on behalf of Syngenta, Ltd. Submitted to WHO by Tessenderlo Kerley Inc., Phoenix, AZ, USA.

Curren R (1985). Norflurazon technical. Unscheduled DNA synthesis in rat hepatocytes. Unpublished report (number not provided). Microbiological Associates, Bethesda, MD, USA. MBA Study No. T4030.380. Submitted to WHO by Tessenderlo Kerley Inc., Phoenix, AZ, USA.

Eschbach B, Aerni R, Hertl P, Müller F (1994). Norflurazon: two generation reproduction study in rats. Unpublished report no. 94/123 I. Study no. 440R (BS4809), Sandoz Agro Ltd, Development Region Basle, Department of Toxicology. Submitted to WHO by Tessenderlo Kerley Inc., Phoenix, AZ, USA.

Fogleman RW (1970). Evaluation of the subacute oral toxicity of SAN 9789 to male and female rats. Unpublished report (number not provided). Affiliated Medical Enterprises, Inc. Submitted to WHO by Tessenderlo Kerley Inc., Phoenix, AZ, USA.

Fogleman RW (1971a). Evaluation of the subchronic (90-day) toxicity effects of SAN-9789 in rats. Unpublished report (number not provided). Affiliated Medical Enterprises, Inc. Submitted to WHO by Tessenderlo Kerley Inc., Phoenix, AZ, USA.

Fogleman RW (1971b). 90-day toxicity of SAN-9789 in beagle dogs. Unpublished report (number not provided). Affiliated Medical Enterprises, Inc. Submitted to WHO by Tessenderlo Kerley Inc., Phoenix, AZ, USA.

Gardner JR (1987a). Acute oral toxicity to rats of Norflurazon 80 DF. Unpublished report no. 87872D/SNC 1/AC. Huntingdon Research Centre, Ltd. Submitted to WHO by Tessenderlo Kerley Inc., Phoenix, AZ, USA.

Gardner JR (1987b). Acute dermal toxicity to rats of Norflurazon 80 DF. Unpublished report no. 87873D/SNC 2/AC. Huntingdon Research Centre, Ltd. Submitted to WHO by Tessenderlo Kerley Inc., Phoenix, AZ, USA.

Hagve TA, Christophersen BO, Boger P (1985). Norflurazon – an inhibitor of essential fatty acid desaturation in isolated liver cells. Lipids. 20(10):719–22.

Harris D (1972). 9-month feeding study in rats F_0 generation. Unpublished report (number not provided). WARF Institute, Inc., Madison, WI, USA. Submitted to WHO by Tessenderlo Kerley Inc., Phoenix, AZ, USA.

Hartman H, Hrab R (1972). Compound 9789. Investigation of teratogenic potential in the rat. Unpublished report no. T-1-8/7/72. Sandoz Research Department. Submitted to WHO by Tessenderlo Kerley Inc., Phoenix, AZ, USA.

Hoffman GM (1996). An acute (4-hour) inhalation toxicity study of norflurazon a.i. in the rat via nose-only exposure. Unpublished report (number not provided). Study no. 96-5280, Huntingdon Life Sciences. Submitted to WHO by Tessenderlo Kerley Inc., Phoenix, AZ, USA.

Horvat T, Kalafatić M, Kopjar N, Kovačević G (2005). Toxicity testing of herbicide norflurazon on an aquatic bioindicator species – the planarian *Polycelis felina* (Daly.) Aquat Toxicol. 73:342–52.

Hrab R (1983a). Dose range–finding study of norflurazon in pregnant rabbits. Unpublished report no. T-1-4/19/83. Project no. T-1793, Sandoz Inc. Submitted to WHO by Tessenderlo Kerley Inc., Phoenix, AZ, USA.

Hrab R (1983b). Investigation of teratogenic potential of norflurazon in the rabbit – segment II. Unpublished report no. T-1-8/31/83. Project no. T-1794, Sandoz Inc. Submitted to WHO by Tessenderlo Kerley Inc., Phoenix, AZ, USA.

Juel R, Milam JO (1979). 2,3-Diphosphoglycerate: its role in health and disease. CRC Crit Rev Clin Lab Sci. 10(2):113–46.

Karapally JC (1974). Metabolism of (^{3}H, ^{14}C)-norflurazon in rat. Unpublished report no. JK 74007. Sandoz Inc. Submitted to WHO by Tessenderlo Kerley Inc., Phoenix, AZ, USA.

Klotzsche C, Carpy S (1973). SAN 9789 (Zorial) six month feeding study in dogs. Unpublished report no. FB73008. Sandoz Ltd, Basle, Switzerland. Submitted to WHO by Tessenderlo Kerley Inc., Phoenix, AZ, USA.

Kynoch SR, Parcell BI (1987). Delayed contact hypersensitivity in the guinea-pig with Norflurazon 80 DF. Unpublished report no. 871420D/SNC 5/SS. Huntingdon Research Centre, Ltd. Submitted to WHO by Tessenderlo Kerley Inc., Phoenix, AZ, USA.

Lamberth C (2017). Pyridazine chemistry in crop protection. J Heterocyclic Chem. 54:2974.

Leow Y-H, Maibach HL (1996). Allergic contact dermatitis from norflurazon (Predict®). Contact Dermatitis. 35:369.

Liggett MP, Smith PA (1987). Irritant effects on the rabbit eye of Norflurazon 80 DF. Unpublished report no. 87843D/SNC 4/SE. Huntingdon Research Centre, Ltd. Submitted to WHO by Tessenderlo Kerley Inc., Phoenix, AZ, USA.

Liggett MP, Smith PA (1988). Irritant effects on rabbit skin of Norflurazon 80 DF. Unpublished report no. 87873D/SNC 4/SE. Huntingdon Research Centre, Ltd. Submitted to WHO by Tessenderlo Kerley Inc., Phoenix, AZ, USA.

Moritz A, Schwendenwein I, Kraft W (2014). Hämatologie. In: Moritz A, editor. Klinische Labordiagnostik in der Tiermedizin, 7th edition. Stuttgart, Germany: Schattauer.

Putman D (1985). Norflurazon technical. Chromosome aberrations in CHO cells. Unpublished report (number not provided). MBA study no. T4030-337, Microbiological Associates, Bethesda, MD, USA. Submitted to WHO by Tessenderlo Kerley Inc., Phoenix, AZ, USA.

Quistad GB, Saunders AL, Skinner WS, Reuter CC, Collier KD (1989). Metabolism of norflurazon by rats. J Agric Food Chem. 37:1412–6.

Rapoport SM (1983). Medizinische Biochemie [Medical Biochemistry], 8th edition. Berlin, German Democratic Republic: Volk und Gesundheit; 334–6, 555.

Richter W (1975). Letter report to Sandoz Pharmaceutical Research Laboratories on histopathological evaluation of a series of liver tissue slides. Unpublished report (number not provided). A.J. Carlson Animal Research Facility, University of Chicago, Chicago, IL, USA. Submitted to WHO by Tessenderlo Kerley Inc., Phoenix, AZ, USA [included as part of Tisdel, 1975a].

Rust JH (1980). Assessment of the data derived from a histologic examination of liver tissues and certain lung tissues taken from male mice participating in a two-year oral carcinogenicity study (Sandoz project T-204) with a substance identified by 9789. Submitted to WHO by Tessenderlo Kerley Inc., Phoenix, AZ, USA [included as part of Tisdel, 1975a].

Saunders AL, Skinner WS, Reuter C, Collier KD, Quistad GB (1985). Metabolism of norflurazon by female rats. Unpublished report no. 3760-22-01-85. Biochemistry Department, Zoecon Corp., Palo Alto, CA, USA. Submitted to WHO by Tessenderlo Kerley Inc., Phoenix, AZ, USA.

Tisdel M (1971). Range finding toxicity study – mice. Compound 9789. Final report. Unpublished report (number not provided). WARF Institute, Inc., Madison, WI, USA. Submitted to WHO by Tessenderlo Kerley Inc., Phoenix, AZ, USA.

Tisdel M[2] (1973). SAH 9789: long term feeding study in rats. One year report. Unpublished report no. T-1-7/25/73. WARF Institute, Inc., Madison, WI, USA. Submitted to WHO by Tessenderlo Kerley Inc., Phoenix, AZ, USA.

Tisdel M[2] (1975a). Two year feeding study of 9789 in mice. Unpublished report no. T-1-8/5/75. WARF Institute, Inc., Madison, WI, USA. Submitted to WHO by Tessenderlo Kerley Inc., Phoenix, AZ, USA.

Tisdel M[2] (1975b). 9789: two year feeding study in rats. Unpublished report no. T-1-4/29/75. WARF Institute, Inc., Madison, WI, USA. Submitted to WHO by Tessenderlo Kerley Inc., Phoenix, AZ, USA.

Tisdel M (1975c). Compound 9789. Reproduction studies in rats and mice. Final report. Volume 1 – Reproduction study on rats. Unpublished report no. T-2-4/29/75. WARF Institute, Inc., Madison, WI, USA. Submitted to WHO by Tessenderlo Kerley Inc., Phoenix, AZ, USA.

Tisdel M (1975d). Compound 9789. Reproduction studies in rats and mice. Final report. Volume 2 – Reproduction study on mice. Unpublished report no. T-2-4/29/75. WARF Institute, Inc., Madison, WI, USA. Submitted to WHO by Tessenderlo Kerley Inc., Phoenix, AZ, USA.

USEPA (2001). Norflurazon. HED risk assessment for tolerance reassessment eligibility decision (TRED). Memorandum from W.J. Hazel, dated 12/20/01. United States Environmental Protection Agency Washington, DC (https://www3.epa.gov/pesticides/chem_search/hhbp/R047666.pdf; accessed 5 June 2018).

Warren SFP, Müller F, Hopley JP, Karapally J, Hertl P (1990). SAN 9789 H (norflurazon). A one year feeding study in beagle dogs to evaluate chronic toxicity. Unpublished report no. AGRO DOK CBK I.7399/90. Project 422-D, Sandoz Agro Ltd, Central Development Basel Toxicology, Basel, Switzerland. Submitted to WHO by Tessenderlo Kerley Inc., Phoenix, AZ, USA.

---

[2] In the sponsor’s study directory, these studies were quoted as “Harris”. However, Dr Harris approved the studies in his or her responsibility as “Chief pathologist/toxicologist” as indicated in the reports. The study director was apparently Meryl Tisdel, who signed the reports. It is more common to mention the name of the study director when citing a study.

PYDIFLUMETOFEN

First draft prepared by
Dr Chris Schyvens,[1] Dr Midori Yoshida[2] and Dr Carl Cerniglia[3]

[1] Scientific Assessment and Chemical Review Program, Australian Pesticide and Veterinary Medicines Authority, Canberra, Australia
[2] Food Safety Commission, Tokyo, Japan
[3] Division of Microbiology, National Center for Toxicological Research, Food and Drug Administration, Jefferson, Arkansas, United States of America (USA)

Explanation....476
Evaluation for acceptable intake....476
1. Biochemical aspects....476
1.1 Absorption, distribution and elimination....477
(a) Oral route....477
(b) Dermal route....488
1.2 Biotransformation....488
(a) Mice....488
(b) Rats....489
2. Toxicological studies....495
2.1 Acute toxicity....495
(a) Lethal doses....495
(b) Dermal irritation....495
(c) Ocular irritation....496
(d) Dermal sensitization....496
2.2 Short-term studies of toxicity....496
(a) Oral administration....496
(b) Dermal application....506
(c) Exposure by inhalation....507
2.3 Long-term studies of toxicity and carcinogenicity....507
2.4 Genotoxicity....512
(a) In vitro studies....512
(b) In vivo studies....514
2.5 Reproductive and developmental toxicity....514
(a) Multigeneration studies....514
(b) Developmental toxicity....516
2.6 Special studies....520
(a) Neurotoxicity....520
(b) Immunotoxicity....522
(c) Toxicity of metabolites....523
(d) Mechanistic studies....529
(e) Microbiological effects....537
3. Observations in humans....538
Comments....538
Toxicological evaluation....541
References....544
Appendix 1: List of animal metabolites....550
Appendix 2: Mode of action of pydiflumetofen-induced liver tumours: analysis of their human relevance using the IPCS/ILSI framework....570
Appendix 3: Blood concentrations of pydiflumetofen in toxicity studies....579

Explanation

Pydiflumetofen (Fig. 1) is the International Organization for Standardization–approved common name for the 50:50 enantiomer ratio of (*S*)-3-difluoromethyl-1-methyl-1*H*-pyrazole-4-carboxylic acid methoxy-[1-methyl-2-(2,4,6-trichlorophenyl)-ethyl]-amide (SYN546968) and (*R*)-3-difluoromethyl-1-methyl-1*H*-pyrazole-4-carboxylic acid methoxy-[1-methyl-2-(2,4,6-trichlorophenyl)-ethyl]-amide (SYN546969) (International Union of Pure and Applied Chemistry), with the Chemical Abstracts Service number 1228284-64-7.

Fig. 1. Chemical structures of pydiflumetofen enantiomers

***S*-enantiomer** ***R*-enantiomer**

Source: European Commission (2018)

Both pydiflumetofen enantiomers are biologically active. Pydiflumetofen is a member of the *N*-methoxy-(phenyl-ethyl)-pyrazole-carboxamide group of fungicides. Its mode of action (MOA) is respiration inhibition at complex II (succinate dehydrogenase) in mitochondria of phytopathogenic fungi.

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

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

Evaluation for acceptable intake

1. Biochemical aspects

The positions of the radiolabels used in the absorption, distribution, metabolism and excretion studies with pydiflumetofen are shown in Fig. 2.

Fig. 2. Positions of radiolabels in the two pydiflumetofen enantiomers

Positions of the radiolabels: *: [pyrazole-5-^{14}C]pydiflumetofen (*left*); *: [phenyl-U-^{14}C]pydiflumetofen (*right*)
Source: MacDonald & Jewkes (2015)

1.1 Absorption, distribution and excretion

(a) Oral route

Mice

In a pharmacokinetics study, CD-1 mice (eight of each sex per dose) were administered pydiflumetofen (lot/batch no. 2637-BA/110; purity 99.5%) by gavage in 0.5% weight per volume (w/v) carboxymethylcellulose containing 0.5% volume per volume (v/v) Tween 80 in distilled water for either a single day or daily for 7 days at 10, 30, 100, 200, 300, 500, 750 or 1000 mg/kg body weight (bw). An additional group of mice (12 of each sex) was administered pydiflumetofen intravenously in dimethyl sulfoxide (DMSO):10% w/v aqueous hydroxypropyl-β-cyclodextrin (5:95 v/v) at 1 mg/kg bw as a single dose. Blood was taken from four animals of each oral dose group at 1, 2, 4, 8, 12 and 24 hours after dosing on day 1 and day 7. Animals were terminated at the time of the third blood sample (12 or 24 hours). Animals retained for analysis on day 7 did not give blood on day 1. For the intravenously administered animals, blood was taken at 5 minutes and at 0.5, 1, 4, 6, 12 and 24 hours. The limit of quantification (LOQ) was 5 ng/mL blood.

The results of the pharmacokinetics study following intravenous or oral administration of pydiflumetofen are summarized in Table 1.

Intravenous administration of pydiflumetofen demonstrated rapid clearance and a high volume of distribution.

The total systemic exposure (area under the concentration–time curve [AUC]) to orally administered pydiflumetofen increased in an approximately dose-proportional manner in both sexes after a single dose (day 1), but sub-dose-proportionally after 7 days of repeated dosing, whereas the maximum concentration (C_{max}) increased sub-dose-proportionally at both times. The absolute oral bioavailability was low after 1 and 7 days of dosing (<10%) and lower after 7 days than after 1 day (<3%); it was also lower at higher doses.

After oral administration, the systemic exposure achieved on day 7 for each dose was markedly less than that on day 1, reflecting enzyme induction and increased first-pass clearance. Although it appeared that systemic exposure to pydiflumetofen was greater in females than in males at doses of 200–1000 mg/kg bw, no consistent sex-related trends were observed. The absolute oral bioavailability of pydiflumetofen was considered low and ranged from 3.6% to 10% in males and from 3.1% to 7.9% in females, across the respective doses on day 1 (Punler & Harris, 2014a).

The excretion of [phenyl-U-^{14}C]pydiflumetofen (lot/batch no. RDR-XIX-18; purity 98.9%) and [pyrazole-5-^{14}C]pydiflumetofen (lot/batch no. 5318IYC001-1; purity 98.5%) was investigated following the administration of a single oral dose of 10 or 300 mg/kg bw to groups of four male and four female CD-1 mice. Excretion samples were obtained over a 7-day period. After this period, the mice were humanely killed, and residual radioactivity was measured in the gastrointestinal tract and remaining carcass. The nature and identity of metabolites present in selected excreta and cage wash samples were also investigated (see section 1.2 below).

After the administration of [phenyl-U-^{14}C]pydiflumetofen or [pyrazole-5-^{14}C]pydiflumetofen at 10 mg/kg bw, a mean of 97–103% of the administered dose was eliminated in urine and faeces (including cage wash) over 7 days. The majority of administered radioactivity (87–97%) was excreted in the first 24 hours. The routes and rates of excretion were similar for both radiolabels and for both sexes. The majority of the dose was excreted in the faeces (63–79%), whereas urinary excretion accounted for 15–30% of the dose.

After the administration of [phenyl-U-^{14}C]pydiflumetofen or [pyrazole-5-^{14}C]pydiflumetofen at 300 mg/kg bw, a mean of 95–110% of the administered dose was eliminated in urine and faeces (including cage wash) over 7 days. The majority of administered radioactivity (88–103%) was excreted in the first 24 hours. The routes and rates of excretion were similar for both radiolabels and for both

Table 1. Pharmacokinetics (whole blood) of pydiflumetofen in mice following a single intravenous (1 mg/kg bw) or a single or multiple (10–1000 mg/kg bw) oral administration of radiolabelled pydiflumetofen

	Nominal dose (mg/kg bw)	C_{max} (ng/mL)	T_{max} (h)	AUC_{0-t} (ng·h/mL)	$AUC_{0-\infty}$ (ng·h/mL)	$t_{½}$ (h)	F[a] (%)	Rsq	MRT (h)	CL (mL/h·kg)	V_d (mL/kg)
Intravenous											
Male	1	236	0.08	155	156	0.63	–	0.985	0.83	7 100	6 490
Female	1	214	0.10	154	167	1.40	–	0.842	1.39	6 750	13 500
Oral											
Male											
Day 1	10	47.9	1.00	88.0	104	1.25	6.60	0.990	–	–	–
	30	138	1.00	250	257	1.51	6.25	0.996	–	–	–
	100	601	1.00	1 560	1 590	1.22	9.51	1.00	–	–	–
	200	694	1.00	2 850	2 860	1.39	10.0	1.00	–	–	–
	300	598	1.00	3 490	3 630	2.28	7.54	0.998	–	–	–
	500	591	1.00	3 340	3 470	2.30	4.38	0.966	–	–	–
	750	798	0.667	5 710	6 040	2.55	5.79	0.918	–	–	–
	1 000	845	0.500	5 350	5 370	2.78	3.56	0.993	–	–	–
Day 7	10	14.7	1.00	20.7	NC	NC	NC	NC	–	–	–
	30	41.8	1.00	66.4	67.5	0.56	1.74	0.867	–	–	–
	100	80.8	0.500	345	358	2.33	2.18	0.968	–	–	–
	200	35.5	4.00	265	289	2.98	1.03	0.951	–	–	–
	300	54.7	2.00	370	384	2.19	0.818	0.971	–	–	–
	500	46.2	2.00	345	406	4.15	0.518	0.894	–	–	–
	750	47.9	8.00	487	NC	NC	NC	NC	–	–	–
	1 000	88.3	4.00	725	3 390[b]	25.9[b]	2.35[b]	0.187[b]	–	–	–

	Nominal dose (mg/kg bw)	C_{max} (ng/mL)	T_{max} (h)	AUC_{0-t} (ng·h/mL)	$AUC_{0-\infty}$ (ng·h/mL)	$t_{½}$ (h)	F^{a} (%)	Rsq	MRT (h)	CL (mL/h·kg)	V_d (mL/kg)
Female											
Day 1	10	44.4	1.00	65.3	83.4[b]	1.44[b]	4.81[b]	0.686[b]	–	–	–
	30	113	1.00	136	138	0.916	3.27	0.970	–	–	–
	100	442	2.00	1 530	1 540	1.54	7.62	0.928	–	–	–
	200	577	2.00	2 100	2 100	1.41	7.87	0.878	–	–	–
	300	475	2.00	2 820	2 880	1.99	5.63	0.925	–	–	–
	500	447	1.00	2 450	2 570	2.47	3.07	0.950	–	–	–
	750	681	0.704	4 810	5 830	4.84	5.09	0.866	–	–	–
	1 000	809	0.500	4 150	4 390[b]	6.52[b]	2.79[b]	0.723[b]	–	–	–
Day 7	10	11.6	1.00	31.5	37.6[b]	2.43[b]	2.28[b]	0.680[b]	–	–	–
	30	28.1	1.00	56.0	57.4	0.604	1.34	0.970	–	–	–
	100	85.7	1.00	315	334[b]	2.31[b]	2.13[b]	0.775[b]	–	–	–
	200	50.2	1.00	390	415	2.85	1.37	0.800	–	–	–
	300	96.8	8.00	752	NC	NC	NC	NC	–	–	–
	500	68.8	4.00	601	789[b]	5.35[b]	0.946[b]	0.910[b]	–	–	–
	750	178	12.0	1 070	NC	NC	NC	NC	–	–	–
	1 000	108	12.0	1 140	NC	NC	NC	NC	–	–	–

AUC: area under the concentration–time curve; bw: body weight; C_{max}: maximum concentration; *F*: bioavailability; CL: clearance; MRT: mean residence time; NC: not calculable, a terminal phase unable to be characterized; Rsq: square of the correlation coefficient for the terminal phase regression line; *t*: time; $t_{½}$: half-life; T_{max}: time to reach maximum concentration; V_d: volume of distribution

[a] Bioavailability on day 7 calculated using exposure estimates after single intravenous administration.

[b] Coefficient of determination was less than 0.800 and/or the extrapolation of the AUC to infinity represents more than 20% of the total.

Source: Punler & Harris (2014a)

sexes, with 76–94% of the dose excreted in the faeces and 7.2–15% in the urine. Nearly 50% of the 300 mg/kg bw dose was considered unabsorbed.

At both doses, excretion was essentially complete in all animals by 168 hours post-dosing, with 0.3% or less remaining in the carcass and gastrointestinal tract (Tomlinson et al., 2015).

Rats

In a preliminary pharmacokinetics study, Han Wistar rats were given a single dose of pydiflumetofen radiolabelled (^{14}C) in either the pyrazole position (lot/batch no. 5222MF-O00101, purity 97.8%; or lot/batch no. RDR-XIV-49, purity 97.2%) or the phenyl position (lot/batch no. DAD-XIII-38-5; purity 98.7%) intravenously at 1 mg/kg bw (one of each sex per radiolabel) or by gavage at 5 or 1000 mg/kg bw (six of each sex per radiolabel). The dosing vehicles were 5% w/v DMSO in 40% w/v aqueous hydroxypropyl-β-cyclodextrin (1 mg/kg bw); 5% v/v DMSO and 0.5% v/v Tween 80 in 0.5% w/v aqueous carboxymethylcellulose (5 mg/kg bw); and 20% v/v DMSO and 0.5% v/v Tween 80 in 0.5% w/v aqueous carboxymethylcellulose (1000 mg/kg bw). Blood was taken from 0.08 to 48 hours after dosing for intravenous administration and at the same time points and 72 hours after dosing for oral treatment. The following samples were collected from all animals to identify the biotransformation pathway (see section 1.2 below): urine and faeces (from predosing to 168 hours), cage wash (from 8 hours to termination of animals), expired air (predosing, 0–24 hours and 24–48 hours) and carcass (168 hours).

The pharmacokinetics of [^{14}C]pyrazole- or [^{14}C]phenyl-labelled pydiflumetofen in rats (whole blood) is summarized in Table 2. Mean recoveries of radioactivity were greater than 90% in all groups except for 1000 mg/kg bw females (recovery >85%). Blood and plasma radiolabel profiles were qualitatively similar for both radiolabels and both sexes, but significant differences were seen between the profiles for the 5 and 1000 mg/kg bw oral doses. Bioavailability was high at 5 mg/kg bw (63–87%), but much lower at 1000 mg/kg bw (<36%), resulting in an extended time to C_{max} (T_{max}) and a sub-dose-proportional AUC_{0-t}. At 5 mg/kg bw, absorption was rapid, with C_{max} reached in under an hour. Elimination of radiolabel was moderately slow, with the half-life between 20 and 51 hours, indicating that moderate accumulation of metabolites in plasma is likely to occur in repeated-dose studies.

Table 2. Pharmacokinetics (whole blood) of [^{14}C]pyrazole- or [^{14}C]phenyl-labelled pydiflumetofen following administration of a single intravenous (1 mg/kg bw) or a single oral (5 or 1000 mg/kg bw) dose to rats

	Males			**Females**		
	Intravenous	**Oral**		**Intravenous**	**Oral**	
Parameter	**1 mg/kg bw**	**5 mg/kg bw**	**1 000 mg/kg bw**	**1 mg/kg bw**	**5 mg/kg bw**	**1 000 mg/kg bw**
Pyrazole radiolabel						
C_{max} (ng equiv/g)	352	418	20 600	296	472	20 200
AUC_{0-t} (ng equiv·h/g)	2 150	6 800	724 000	2 020	7 800	724 000
T_{max} (h)	0.08	0.5	30	0.08	1	24
$t_{½}$ (h)	28	46	22	33	43	20
MRT (h)	35	–	–	38	–	–
F (%)	–	63.3	33.7	–	77.2	35.8
V_{ss} (g/kg)	12 100	–	–	13 600	–	–
Recovery (% of dose)						
Urine	18.67	20.83	21.93	17.23	26.01	27.16

	Males			Females		
	Intravenous	Oral		Intravenous	Oral	
Parameter	**1 mg/kg bw**	**5 mg/kg bw**	**1 000 mg/kg bw**	**1 mg/kg bw**	**5 mg/kg bw**	**1 000 mg/kg bw**
Faeces	74.29	73.54	66.64	78.24	70.38	54.90
Cage wash	1.11	2.14	1.90	1.84	1.89	3.58
Carcass	0.32	0.22	0.13	0.22	0.15	0.12
Total	94.39	96.73	90.60	97.53	98.43	85.76
Phenyl radiolabel						
C_{max} (ng equiv/g)	298	562	11 200	358	715	10 100
T_{max} (h)	0.08	1	24	0.08	1	24
$AUC_{0–t}$ (ng equiv·h/g)	2 140	8 520	449 000	2 930	12 800	353 000
$t_{½}$ (h)	51	46	22	34	43	20
MRT (h)	59	–	–	41	–	–
F (%)	–	79.6	21	–	87.4	12.0
V_{ss} (g/kg)	16 200	–	–	9 650	–	–
Recovery (% of dose)						
Urine	23.7	23.49	13.19	20.55	19.02	15.07
Faeces	64.65	74.3	93.17	72.75	81.24	67.7
Cage wash	2.04	2.62	1.44	0.97	0.70	2.33
Carcass	0.31	0.32	0.11	0.24	0.19	0.14
Total	90.81	100.79	107.95	94.55	101.19	85.28

AUC: area under the concentration–time curve; bw: body weight; C_{max}: maximum concentration; equiv: equivalents; F: bioavailability; MRT: mean residence time; t: time; $t_{½}$: half-life; T_{max}: time to reach maximum concentration; V_{ss}: volume of distribution at steady state
Source: Webbley & Williams (2015)

The predominant route of excretion in both sexes following oral administration was faecal, which accounted for between 55% and 93% of the administered dose, with urine accounting for less than 30%. At the low dose, the faecal content was due primarily to excretion in the bile, whereas at the high dose, unabsorbed pydiflumetofen contributed substantially to the faecal content. The observation of a peak in blood levels in females at 6–8 hours after intravenous administration was characteristic of enterohepatic recirculation; this peak was also seen at around 10 hours following administration of a 5 mg/kg bw oral dose in both sexes. Excretion was largely complete within the first 72 hours. Residual radiolabel in the carcass accounted for less than 0.5% of the administered dose, and negligible amounts were recovered in expired air (Webbley & Williams, 2015).

In a study of the absorption and excretion of pydiflumetofen, Han Wistar rats were administered pydiflumetofen radiolabelled (^{14}C) in either the pyrazole position (lot/batch no. 5271GAR001-4, purity 99.2%; or lot/batch no. 5283RJP001-2, purity 98.8%) or the phenyl position (lot/batch no. RDR-X-94; purity 99.1%) as a single intravenous dose of 1 mg/kg bw (four rats of each sex per radiolabel) or a single oral dose (24 rats of each sex per dose for each radiolabel) of 5 mg/kg bw for both sexes, 100 mg/kg bw for females only or 300 mg/kg bw for males only. Blood samples were taken for analysis of pharmacokinetics over 4 days for the oral doses and 2 days for the intravenous dose. The dosing vehicles

were DMSO:10% aqueous hydroxypropyl-β-cyclodextrin (5:95 v/v) for intravenous dosing and 0.5% w/v aqueous carboxymethylcellulose containing 0.5% Tween 80 for oral dosing. After oral dosing, blood samples were taken from subgroups of four rats of each sex at 0.25 and 1 hour; 0.5 and 2 hours; 4 and 12 hours; 8 and 24 hours; 30 and 72 hours; or 48 and 96 hours. Each rat gave two non-sequential samples, with the second sample taken at termination; for example, four rats of each sex were sampled at 0.25 hour and then at termination at 1 hour, four rats of each sex were sampled at 0.5 and 2 hours, and so on. The intravenously dosed animals (four rats of each sex) were each sampled at 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 30 and 48 hours.

After intravenous administration of 1 mg/kg bw, the pharmacokinetics parameters were similar for both radiolabels and both sexes (Table 3). The long half-life in females for the phenyl label was attributable to a low coefficient of determination (i.e. broad scatter of data), preventing reliable estimation of the value. Overall, the half-life was of the order of 20–40 hours, and the volume of distribution (V_d) was large, at around 13–20 L/kg bw.

Following oral administration, there were no marked sex- or radiolabel-based differences in pharmacokinetics parameters (Table 4). The bioavailability was approximately 50% at 5 mg/kg bw, but closer to 30% at 100/300 mg/kg bw, and T_{max} was delayed at the higher doses. These observations, together with a sub-dose-proportional AUC at the higher doses, indicate a saturation of absorption at the higher doses (Hutton & O'Hagan, 2015).

Table 3. Pharmacokinetics (whole blood) of pydiflumetofen following a single intravenous (1 mg/kg bw) administration of radiolabelled pydiflumetofen to rats[a]

	Phenyl label		Pyrazole label	
Parameter	**Males**	**Females**	**Males**	**Females**
C_0 (µg equiv/g)	0.463	0.366	0.439	0.341
$t_{½}$ (h)	39.4[b]	182[b]	25.3	20.7
$AUC_{0–t}$ (µg equiv·h/g)	1.89	1.95	1.54	1.62
$AUC_{0–t}/D$	2.07	2.28	1.66	1.87
$AUC_{0–\infty}$ (µg equiv·h/g)	2.6	5.96	1.93	1.91
$AUC_{0–\infty}/D$	2.84[b]	6.90[b]	2.10	2.24
AUC_{extrap} (%)	26.8[b]	50.7[b]	18.9	15.8
$MRT_{0–\infty}$ (h)	37.3[b]	235[b]	38.7	32.7
CL (mL/h·kg)	355[b]	216[b]	475	447
V_d (mL/kg bw)	19 950[b]	27 300[b]	17 300	13 400

AUC: area under the concentration–time curve; AUC_{extrap}: percentage of AUC to infinity estimated by extrapolation of the apparent terminal phase; bw: body weight; C_0: initial concentration; CL: clearance; *D*: dose administered; equiv: equivalents; MRT: mean residence time; *t*: time; $t_{½}$: half-life; V_d: volume of distribution

[a] Pharmacokinetics parameters were derived using WinNonlin, version 5.2.1, Pharsight Corp., Mountain View, CA, USA.

[b] Coefficient of determination was less than 0.800 and/or the extrapolation of the AUC to infinity represents more than 20% of the total area – i.e. values are quantitatively unreliable.

Source: Hutton & O'Hagan (2015)

In a study on absorption, excretion and tissue distribution, Han Wistar rats (four of each sex per dose for each radiolabel) were administered a single dose of pydiflumetofen radiolabelled (^{14}C) in either the pyrazole position (lot/batch no. 5271GAR001-4, purity 99.2%; or lot/batch no. 5283RJP001-2, purity 98.8%) or the phenyl position (lot/batch no. RDR-XV-94; purity 99.1%) by gavage. The doses were 5 mg/kg bw for both sexes and 300 mg/kg bw for males only or 100 mg/kg bw for females only. The dosing vehicle was 0.5% w/v aqueous carboxymethylcellulose containing 0.5% Tween 80. The dosing regimen was repeated with equivalent groups of bile duct–cannulated rats to examine biliary

Table 4. Pharmacokinetics (whole blood) of pydiflumetofen following a single oral (5, 100 or 300 mg/kg bw) administration of radiolabelled pydiflumetofen to rats

	Phenyl label				Pyrazole label			
	5 mg/kg bw		300 mg/kg bw	100 mg/kg bw	5 mg/kg bw		300 mg/kg bw	100 mg/kg bw
Parameter	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
C_{max} (µg equiv/g)	0.63	0.72	8.1	3.8	0.27	0.45	4.7	2.1
T_{max}[a] (h)	2	1	8	8	2	0.5	8	8
$t_{½}$ (h)	116[b]	82.1[b]	163[b]	160[b]	75.3[b]	68.5[b]	196[b]	NC
$AUC_{0–t}$ (µg equiv·h/g)	7.19	8.16	218	89.7	5.6	5.86	167	54.0
$AUC_{0–t}/D$	1.48	1.66	0.778	0.936	1.1	1.15	0.605	0.632
$AUC_{0–\infty}$ (µg equiv·h/g)	12.2[b]	11.7	488[b]	165[b]	8.05	7.84[b]	358[b]	NC
$AUC_{0–\infty}/D$	2.51[b]	2.38[b]	1.74[b]	1.72[b]	1.58[b]	1.53[b]	1.3[b]	NC
F (%)[c]	55	55	27.9	33.7	51.8	48.3	27.0	26.4

AUC: area under the concentration–time curve; bw: body weight: C_{max}: maximum concentration; D: dose administered; equiv: equivalents; F: bioavailability; NC: not calculable, a terminal phase unable to be characterized; t: time; $t_{½}$: half-life; T_{max}: time to reach maximum concentration

[a] Median reported for T_{max}.

[b] Coefficient of determination was less than 0.800 and/or the extrapolation of AUC to infinity represents more than 20% of the total area – i.e. values are quantitatively unreliable.

[c] F (%) was calculated using $AUC_{0–48\ h}$.

Source: Hutton & O'Hagan (2015)

excretion. Excretion samples were collected from non-cannulated animals for 7 days and from bile duct–cannulated animals over 3 days. At the end of this period, radiolabel was determined in blood, excreta, selected tissues, carcass and cage wash.

Following oral administration of phenyl- or pyrazole-labelled pydiflumetofen at 5 mg/kg bw to intact rats, 97–99% of the administered dose was eliminated in urine and faeces (including cage wash), with the majority (91–96%) recovered in the first 48 hours. The major route of elimination was in bile (67–76%), with urinary elimination accounting for 18–26%. Excretion was similar in both sexes and for both radiolabel positions.

In intact male rats given 300 mg/kg bw, 101–103% of the dose was recovered in excreta, with 98–100% recovered in the first 48 hours. Radiolabel recovery from the faeces accounted for 91–92% of the dose, and urine, 6.7–7.8%. For intact females given 100 mg/kg bw, 101–102% of the administered dose was eliminated in urine and faeces, 99% in the first 48 hours, with 84–85% in the faeces and 14–15% in the urine.

Absorption was limited by dose, from approximately 85–90% of the 5 mg/kg bw dose to 19–24% at 300 mg/kg bw in males and 50–55% at 100 mg/kg bw in females. The majority of the absorbed dose was excreted in faeces via bile elimination.

For each of these groups, excretion was essentially complete by 168 hours, with 0.1% or less remaining in the carcass and gastrointestinal tract. Radiolabel was undetectable in the majority of tissues. Low levels of radiolabel were found in the liver and kidney, consistent with the biliary and urinary elimination of absorbed pydiflumetofen (Table 5). Moreover, radioactive residues were not above the limit of detection (LOD) in the majority of tissues.

Table 5. Tissue levels[a] of radiolabelled pydiflumetofen in rats after a single oral dose

	Tissue levels (µg equivalents/g)							
	Males				Females			
	Phenyl label		Pyrazole label		Phenyl label		Pyrazole label	
Tissue	**5 mg/kg bw**	**300 mg/kg bw**	**5 mg/kg bw**	**300 mg/kg bw**	**5 mg/kg bw**	**100 mg/kg bw**	**5 mg/kg bw**	**100 mg/kg bw**
Lung	0.01	0.3	0.013	0.2	0.018	0.1	0.017	0.1
Liver	0.09	2.7	0.134	2.4	0.048	0.5	0.06	0.4
Kidney	0.035	0.9	0.041	0.6	0.024	0.2	0.02	0.1

bw: body weight; LOD: limit of detection for radioactivity

[a] All other tissues were at or close to the LOD: for 5 mg/kg bw: 0.03 µg equivalents/g; for 100 and 300 mg/kg bw: 0.2 and 0.07 µg equivalents/g for males and females, respectively.

Source: Hutton (2015a)

In bile duct–cannulated rats given 5 mg/kg bw, 97–99% of the administered dose was eliminated in urine, bile and faeces (including cage wash) over 72 hours. The routes and rates of excretion were similar for both radiolabels and both sexes, with the exception that urinary excretion for males was approximately twice that for females. The majority of the dose was excreted in the bile (67–81%), but radioactivity measured in the faeces accounted for 10–15% of the dose, and urinary excretion accounted for 6.3–13% of the dose.

In bile duct–cannulated males given 300 mg/kg bw, 98–100% of the administered dose was eliminated in urine, bile and faeces (including cage wash) over 72 hours. The routes and rates of excretion were similar for both radiolabels, with 76–79% of the dose excreted in the faeces, 15–18% in the bile and 2.5–4.4% in the urine. In bile duct–cannulated females given 100 mg/kg bw, 97–99% of the administered dose was eliminated in urine, bile and faeces (including cage wash) over 72 hours. The routes and rates of excretion were similar for both radiolabels, with 43–49% of the dose excreted in the faeces, 33–41% in the bile and 7–15% in the urine. Excretion was essentially complete in all bile duct–cannulated animals by 72 hours post-dosing, with 0.3% or less remaining in the carcass and gastrointestinal tract (including contents).

Absorption was approximately 85–90% at 5 mg/kg bw. The majority of the absorbed dose was excreted in faeces through bile elimination. Seven days after the administration, radioactive residues were not detectable in the majority of tissues. The highest mean concentrations were found in the liver and kidney, consistent with the biliary and urinary elimination of absorbed ^{14}C-labelled pydiflumetofen (Hutton, 2015a).

In a study on tissue depletion, Han Wistar rats (20 of each sex per dose) were administered a single oral (gavage) dose of pydiflumetofen radiolabelled (^{14}C) in either the pyrazole position (lot/batch no. 5283RJP001-2; purity 98.8%) or the phenyl position (lot/batch no. RDR-XV-94; purity 99.1%) at 5 mg/kg bw for both sexes and 300 mg/kg bw for males only or 100 mg/kg bw for females only. The dosing vehicle was 0.5% w/v aqueous carboxymethylcellulose containing 0.5% Tween 80. The tissue distribution and depletion of radiolabelled pydiflumetofen were ascertained at various time points following dosing. Groups of rats (four of each sex at each time point) were killed at sequential predetermined time points based on a previous pharmacokinetics study (Hutton & O'Hagan, 2015). The first termination occurred at around the T_{max}. For the 5 mg/kg bw dose groups, these were 1 hour (females only), 2 hours (males only), 12, 24, 72 and 120 hours for the phenyl label and 0.5, 12, 24, 48 and 96 hours for the pyrazole label. For the 100 and 300 mg/kg bw dose groups for both labels, these were 8, 24, 48, 72 and 96 hours. Residual radioactivity was measured in selected tissues/organs (adrenals, brain, heart, kidneys, liver, lungs, ovaries, pancreas, spleen, testes, thymus, thyroid, uterus

and the remaining carcasses). Blood was taken at termination. Where appropriate, terminal-phase half-lives of depletion were calculated for individual tissues.

In all cases, the radiolabel was widely distributed, with peak levels in tissues obtained at the first sampling point (selected to correspond to C_{max}). At all doses, peak tissue levels were highest in the liver, kidneys and adrenals. At the time of the last sampling (96/120 hours), all tissue concentrations, with the exception of those in the liver and kidney, were below the blood concentration or were below the LOD of radioactivity (0.003 µg equivalents/g for the 5 mg/kg bw dose group; 0.2 and 0.07 µg equivalents/g for males and females, respectively, for the 100 and 300 mg/kg bw dose groups). By 96/120 hours following dosing, residual levels of radiolabel were less than or equal to 3% in both sexes, for both radiolabels and all doses. Throughout the study, the majority of tissue concentrations were generally slightly higher in females than in males at 5 mg/kg bw.

The depletion profile of radiolabel in tissues reflected that of the blood/plasma, with no evidence of a plateau in any tissue. The half-lives of the radiolabel in tissues were similar to or less than the half-life in blood/plasma (Hutton, 2015b).

Han Wistar rats (four of each sex per dose) were administered pydiflumetofen (purity 99.5%) orally (by gavage) in 0.5% Tween 80 with 0.5% w/v carboxymethylcellulose at a dose of 3, 10, 30, 100, 300, 500 or 1000 (males only) mg/kg bw per day for 7 days or as a single intravenous dose of 1 mg/kg bw. Blood was taken on day 1 (intravenous and oral) and day 7 (oral only) at 0.08 (intravenous only), 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12 and 24 hours after dosing. The LOQ was 5.0 ng/mL of blood.

The results are summarized in Tables 6 and 7. The bioavailability of pydiflumetofen after a single oral dose was only 2–6% in males and 4.8–37% in females. In males, C_{max} and AUC were less than dose proportional above 300 mg/kg bw after a single dose; after repeated dosing for 7 days, the achieved C_{max} was approximately the same for doses of 100–1000 mg/kg bw per day, and AUC was unchanged between 300 and 1000 mg/kg bw per day. In females, the C_{max} and AUC were essentially unchanged between 100 and 500 mg/kg bw after single and repeated administration. In both sexes, the AUC and C_{max} after 7 days of dosing were substantially lower than after a single dose.

The T_{max} tended to increase with increasing dose in both sexes. There was negligible accumulation of pydiflumetofen between day 1 (single administration) and day 7 (repeated administration) at the 3 and 10 mg/kg bw per day doses in both sexes, with systemic exposure appreciably reduced at doses greater than 10 mg/kg bw per day with repeated oral administration. The volume of distribution (V_d) largely reflected extensive distribution beyond the central circulation. Systemic exposure was consistently greater in females than in males throughout the study (Punler & Harris, 2014b).

Rabbits

In a toxicokinetics study, pregnant New Zealand white rabbits (four per dose) were administered pydiflumetofen (batch/lot no. SMU2EP12007; purity 98.5%) as a suspension in 1% w/v aqueous carboxymethylcellulose by gavage as a single dose (on gestation day [GD] 6) or repeated daily doses (GDs 6–27) of 100, 300, 750 or 1000 mg/kg bw. Animals were monitored for clinical signs and mortality daily, and feed intake and body weight were measured daily. At termination, a gross pathology examination was conducted, but no examination of the uterine contents was performed. Blood for analysis of toxicokinetics was taken immediately before dosing, at 2, 4, 6, 8, 12 and 24 hours after dosing and on days 6, 13 and 27. The LOQ was 5 ng/mL.

There were no treatment-related deaths or effects on clinical signs, gross pathology, feed intake or (in general) body weight. One female at 1000 mg/kg bw per day lost body weight between days 24 and 28 and had a very small overall body weight gain of only 0.5 kg. This same animal showed laboured breathing on day 25 of gestation, reduced faecal output on days 25, 27 and 28 and a reduced feed intake.

Table 6. Pharmacokinetics (whole blood) of pydiflumetofen after a single or repeated (7 days) oral administration to rats

	Single administration (mg/kg bw)							Repeated administration (mg/kg bw per day)						
	3	**10**	**30**	**100**	**300**	**500**	**1 000**	**3**	**10**	**30**	**100**	**300**	**500**	**1 000**
Males														
C_{max} (ng/mL)	7.86	12.4	38.9	242	602	380	612	8.5	14.9	17.2	34.3	63.3	41.9	64.5
T_{max} (h)	2.0	2.0	2.0	4.0	6.0	6.0	7.0	2.3	2.0	2.0	6.0	10.0	7.0	9.0
$t_{½}$ (h)	NC	NC	2.76	3.17	3.53	3.76	4.08	NC	NC	NC	3.57	6.34	3.37	5.85
$AUC_{0–t}$ (ng·h/mL)	11.22	41.65	227.4	1 613	6 096	3 644	7 546	13.5	57.8	109	291	666	448	832
$AUC_{0–∞}$ (ng·h/mL)	NC	NC	232.6	1 496	6 358	3 737	7 855	NC	NC	NC	391	2 725	429	1 096
R_{ac}	–	–	–	–	–	–	–	NC	NC	NC	0.2	0.2	0.1	0.1
F (%)	NC	2.8[a]	3.0	6.0	6.3	2.3	2.6	NC	2	1	1	0.9	0.3	0.3
Females														
C_{max} (ng/mL)	76	178	527	674	639	640	–	76.5	146	272	259	252	286	–
T_{max} (h)	1.0	2.0	3.0	5.0	7.0	8.0	–	1.0	2.0	3.0	4.0	9.0	10.0	–
$t_{½}$ (h)	2.74	2.96	3.0	3.15	5.69	7.02	–	2.19	2.99	4.43	2.89	3.46	3.24	–
$AUC_{0–t}$ (ng·h/mL)	273	774	4 410	8 124	9 827	10 170	–	244	612	1 674	1 946	2 471	3 035	–
$AUC_{0–∞}$ (ng·h/mL)	296	820	4 489	8 266	10 740	11 060	–	264	768	1 868	2 045	2 544	3 438	–
R_{ac}	–	–	–	–	–	–	–	0.9	1.1	0.4	0.3	0.3	0.3	–
F (%)[b]	23.0	21.0	36.8	20.8	7.6	4.8	–	24	18	16	6	2	2	–

AUC: area under the concentration–time curve; bw: body weight; C_{max}: maximum concentration; *F*: bioavailability; NC: not calculable; R_{ac}: accumulation ratio compared with single dose; *t*: time; $t_{½}$: half-life; T_{max}: time to reach maximum concentration

[a] Value unreliable. Coefficient of determination was less than 0.800 and/or the extrapolation of AUC to infinity represents more than 20% of the total area.

[b] Approximated from dose-normalized AUC day 7 / single-dose intravenous AUC (see Table 7).

Source: Punler & Harris (2014b)

Table 7. Pharmacokinetics (whole blood) of pydiflumetofen after a single intravenous administration (1 mg/kg bw) to rats

Parameter	Males	Females
C_0 (ng/mL)	727	411
$t_{½}$ (h)	1.26	1.75
$AUC_{0–t}$ (ng·h/mL)	253	344
$AUC_{0–\infty}$ (ng·h/mL)	266	361
CL (mL/h·kg)	3 414	2 644
V_d (mL/kg)	6 134	6 632
MRT (h)	1.1	2.0

AUC: area under the concentration–time curve; bw: body weight; C_0: initial concentration; CL: clearance; MRT: mean residence time; *t*: time; $t_{½}$: half-life; V_d: volume of distribution
Source: Punler & Harris (2014b)

The toxicokinetics parameters on day 6 and day 27 are shown in Table 8. Blood levels increased sub-proportionally to dose, with the AUC and C_{max} at 750 and 1000 mg/kg bw essentially similar at all time points. The T_{max} was extended at 750 and 1000 mg/kg bw. These observations reflect extended and incomplete absorption at the higher doses. Where calculable, the elimination half-life was approximately 6 hours.

Table 8. Toxicokinetics (whole blood) of pydiflumetofen following repeated oral administration to pregnant rabbits

	Day 6				Day 27			
Parameter	**100 mg/kg bw**	**300 mg/kg bw**	**750 mg/kg bw**	**1 000 mg/kg bw**	**100 mg/kg bw**	**300 mg/kg bw**	**750 mg/kg bw**	**1 000 mg/kg bw**
C_{max} (ng/mL)	26.4	44.1	71.2	79.3	87.5	118	102	116
T_{max} (h)	2–8	2–24	4–8	4–12	4–12	4–8	8	4–12
$t_{½}$ (h)	NC	NC	NC	NC	5.4	6.4	NC	NC
$AUC_{0–t\ last}$ (ng·h/mL)	254	722	1 010	1 140	1 110	1 560	1 850	2 050
$AUC_{0–24\ h}$ (ng·h/mL)	344	722	1 010	1 140	1 110	1 560	1 850	2 050
AUC_{∞} (ng·h/mL)	NC	NC	NC	NC	758	2 300	–	–
Dose-proportionality ratio versus lowest dose	–	3.0	7.5	10.0	–	3.0	7.5	10.0
Dose-proportionality ratio (C_{max})	–	1.7	2.7	3.0	–	1.3	1.2	1.2
Dose-proportionality ratio ($AUC_{0–24\ h}$)	–	2.1	2.9	3.3	–	1.4	1.7	1.8

–: not relevant; AUC: area under the concentration–time curve; bw: body weight; C_{max}: maximum concentration; NC: not calculable; *t*: time; $t_{½}$: half-life; T_{max}: time to reach maximum concentration
Source: Penn (2015a)

Overall, the toxicokinetics of pydiflumetofen in pregnant rabbits indicated a subproportional increase in systemic exposure with dose, with no apparent increase in systemic exposure between 750 and 1000 mg/kg bw per day (Penn, 2015a).

(b) Dermal route

No studies were submitted.

1.2 Biotransformation

(a) Mice

The biotransformation study in mice was conducted as part of the absorption and excretion study described in section 1.1 above (Tomlinson et al., 2015).

Up to 86% of the administered radioactivity was structurally identified following administration of a single oral dose of pydiflumetofen (SYN545974) to mice. The major metabolites present in urine and faeces were qualitatively and quantitatively similar between males and females and across doses. Some quantitative differences were observed in faeces from male and female mice, but these were not considered biologically relevant. Unchanged pydiflumetofen was not detected in urine. Although unchanged pydiflumetofen accounted for less than 4.4% of the dose in the faeces at 10 mg/kg bw, the unchanged parent was the major component recovered at up to 48.8% of the administered dose at 300 mg/kg bw.

Metabolites detected in urine were numerous and generally comprised a low percentage of the administered dose. The major components detected in urine were those formed following the cleavage of the parent compound and included the phenyl-derived metabolites 2,4,6-trichlorophenol (2,4,6-TCP) sulfate, 3-hydroxy-2,4,6-TCP sulfate and 2,4,6-TCP glucuronide. The major pyrazole-derived metabolite was identified as SYN548263. Other components identified included demethylated and/or hydroxylated pydiflumetofen and their corresponding glucuronide and sulfate conjugates.

Metabolites detected in faeces were mostly identified as demethylated and/or hydroxylated and/or dechlorinated pydiflumetofen. Only one label-specific metabolite was observed, and this was the pyrazole-derived metabolite SYN548263, at up to 4.7% of the dose. Desmethyl-hydroxy pydiflumetofen isomers and didesmethyl hydroxy pydiflumetofen isomers accounted for a large percentage of the administered dose in faeces, at up to 14.0% and 13.5% of the dose, respectively. SYN547897 was detected in nearly all samples and accounted for 2.9–11.4% of the dose. Only two other metabolites accounted for more than 5% of the dose: SYN547890 (6.0%) and dihydroxy SYN545974 (5.8%).

These results indicate that pydiflumetofen was extensively metabolized in the mouse via demethylation, hydroxylation and dechlorination, and subsequently by glucuronidation and sulfation. The molecule also cleaved at the benzylic carbon to yield the phenyl-derived metabolite 2,4,6-TCP and the pyrazole-derived metabolite SYN548263. These cleavage products were further metabolized via direct glucuronidation and sulfation and also following hydroxylation and sulfation to 3-hydroxy-2,4,6-TCP sulfate.

The biotransformation proceeded by:

- hydroxylation to give hydroxylated and dihydroxylated metabolite isomers;
- demethylation to SYN547890;
- demethoxylation to SYN545547, accompanied by hydroxylation;
- hydroxylation and demethylation to give desmethyl hydroxy metabolites and didesmethyl hydroxy metabolites;
- cleavage of pydiflumetofen to give the pyrazole-derived metabolite SYN548263 and the phenyl-derived metabolite 2,4,6-TCP;
- dechlorination and hydroxylation to SYN547894 and other dechlorinated hydroxy and dechlorinated dihydroxy metabolites; and
- glucuronic acid conjugation and sulfate conjugation.

A proposed metabolic pathway for pydiflumetofen in mice is shown in Fig. 3.

Fig. 3. The proposed metabolic pathway for pydiflumetofen in mice following oral administration

Source: Tomlinson et al. (2015)

(b) Rats

In the preliminary pharmacokinetics study described above in section 1.1, metabolism involved both phase I and phase II pathways, including oxidation, glucuronidation and sulfation, plus cleavage at the amide bond to form pyrazole-related metabolites and cleavage of the parent molecule to form 2,4,6-TCP-related metabolites (Webbley & Williams, 2015).

The nature and identity of pydiflumetofen metabolites in faeces, urine, bile, plasma and cage wash were ascertained from intact and bile duct–cannulated Han Wistar rats following administration

of a single oral (gavage) dose of pydiflumetofen radiolabelled in either the pyrazole or phenyl position at 5 mg/kg bw for both sexes, 100 mg/kg bw for females or 300 mg/kg bw for males. Samples were derived from two pharmacokinetics studies (Hutton, 2015a; Hutton & O'Hagan, 2015) whose materials and methods were described above in section 1.1.

Analyses of the radiolabelled moieties in excreta identified up to 84.8% of the administered dose. Pydiflumetofen was extensively metabolized, and all metabolites composing greater than 4.8% of the dose were identified. There were 35 unknown metabolites observed, each accounting for less than 4.8% of the dose. In general, the major metabolites present were qualitatively and quantitatively similar, irrespective of dose and sex.

At 5 mg/kg bw, unchanged parent compound accounted for less than 3.9% of the dose in excreta. At higher doses (100 or 300 mg/kg bw), unchanged pydiflumetofen was the major component recovered, at up to 48.2% of the administered dose, almost all of which was in the faeces (reflecting the dose-limited bioavailability at higher doses). Based on the high bioavailability of radiolabel and the low proportion of circulating radiolabel identified as parent pydiflumetofen, a high level of first-pass metabolism was considered likely.

Only two metabolites (2,4,6-TCP sulfate and SYN548263) were present at more than 10% of the administered dose. Numerous other cleavage products, and others that retained both the phenyl and pyrazole ring moieties, were identified. Cleavage of pydiflumetofen occurred following hydroxylation of the carbon adjacent to the trichlorophenyl ring to give SYN547948, which was then cleaved to yield 2,4,6-TCP and SYN548263. Subsequently, 2,4,6-TCP was sulfated, and this moiety accounted for the largest proportion of the administered dose, at up to 14.9%.

The metabolites identified in the urine and faeces of rats are shown in Tables 9 and 10.

Table 9. Metabolite profile in urine and faeces after administration of a single oral dose of 5 mg/kg bw [phenyl-U-^{14}C]pydiflumetofen or [pyrazole-U-^{14}C]pydiflumetofen to rats

	% of administered dose					
	Males			Females		
Compounds	**Urine[a]**	**Faeces[b]**	**Total excreta**	**Urine[a]**	**Faeces[b]**	**Total excreta**
[phenyl-U-^{14}C]Pydiflumetofen						
Pydiflumetofen	ND	2.2	2.2	ND	3.9	3.9
TCPM glucuronide	1.7	ND	1.7	ND	ND	ND
Dihydroxy pydiflumetofen	ND	4.4	4.4	0.2	2.0	2.2
Dechlorinated dihydroxy thiomethyl pydiflumetofen	ND	ND	ND	ND	1.9	1.9
Desmethyl hydroxy pydiflumetofen[c]	ND	8.1	8.1	ND	5.0	5.0
Desmethyl hydroxy pydiflumetofen sulfate	0.1	ND	0.1	ND	ND	ND
2,4,6-TCP sulfate	14.9	ND	14.9	7.8	ND	7.8
SYN547948	ND	2.7	2.7	ND	ND	ND
Hydroxy pydiflumetofen[d]	ND	ND	ND	ND	4.6	4.6
SYN547891 glucuronide	ND	ND	ND	0.4	ND	0.4
Hydroxy SYN545547	ND	ND	ND	ND	2.1	2.1
2,4,6-TCP	4.0	ND	4.0	6.6	ND	6.6
SYN547894	ND	2.1	2.1	ND	3.4	3.4

Compounds	% of administered dose					
	Males			Females		
	Urine[a]	Faeces[b]	Total excreta	Urine[a]	Faeces[b]	Total excreta
SYN547897	ND	5.2	5.2	0.1	8.2	8.3
Hydroxy pydiflumetofen sulfate	ND	ND	ND	0.2	ND	0.2
Dechlorinated hydroxy thiomethyl pydiflumetofen	ND	1.9	1.9	ND	6.3	6.3
SYN547890	ND	2.3	2.3	ND	5.9	5.9
SYN545547	ND	1.3	1.3	ND	0.9	0.9
Unidentified components	0.8[e]	27.4[f]	28.2	3.2[g]	17.0[h]	20.2
Post-extraction solids	NA	13.1	13.1	NA	11.0	11.0
Total identified	20.7	30.2	50.9	15.3	44.0	59.3
Total unidentified	0.8	27.4	28.2	3.2	17.0	20.2
Total accounted for	21.5	70.7	92.2	18.5	72.0	90.5
Losses/gains	0.0	2.6	2.6	-0.1	3.8	3.7
Total	21.5	73.3	94.8	18.4	75.8	94.2
LOQ	0.3	1.8	NA	0.3	1.8	NA
[pyrazole-U-^{14}C]Pydiflumetofen						
Pydiflumetofen	ND	2.6	2.6	ND	3.1	3.1
SYN548264	2.2	ND	2.2	0.7	ND	0.7
Desmethyl SYN548265 glucuronide	0.4	ND	0.4	0.5	ND	0.5
Desmethyl SYN548263	2.0	ND	2.0	1.3	ND	1.3
SYN548265 glucuronide	1.4	ND	1.4	1.4	ND	1.4
SYN548263	8.9	5.1	14.0	4.3	ND	4.3
SYN548265	0.2	ND	0.2	0.1	ND	0.1
Dihydroxy pydiflumetofen	ND	6.0	6.0	ND	1.4	1.4
Dechlorinated dihydroxy thiomethyl pydiflumetofen	ND	ND	ND	ND	1.0	1.0
Desmethyl hydroxy pydiflumetofen[i]	ND	2.1	2.1	ND	2.7	2.7
Desmethyl hydroxy pydiflumetofen sulfate	0.5	ND	0.5	ND	ND	ND
Dechlorinated dihydroxy pydiflumetofen	ND	ND	ND	ND	1.9	1.9
SYN547948	ND	ND	ND	ND	2.5	2.5
Hydroxy SYN547891	ND	2.6	2.6	ND	ND	ND
SYN547891 glucuronide	0.1	ND	0.1	ND	ND	ND
Hydroxy pydiflumetofen	ND	1.6	1.6	ND	0.6	0.6
SYN547894	ND	1.4	1.4	ND	3.9	3.9
SYN547897	ND	3.0	3.0	ND	4.8	4.8
Dechlorinated hydroxy thiomethyl pydiflumetofen	ND	1.4	1.4	ND	5.9	5.9

Compounds	% of administered dose					
	Males			Females		
	Urine[a]	Faeces[b]	Total excreta	Urine[a]	Faeces[b]	Total excreta
SYN547890	ND	2.5	2.5	ND	4.4	4.4
SYN545547	ND	0.7	0.7	ND	ND	ND
Unidentified components	10.3[j]	21.7[k]	32.0	10.0[l]	24.8[m]	34.8
Post-extraction solids	NA	11.8	11.8	NA	10.2	10.2
Total identified	15.7	29.0	44.7	8.3	32.2	40.5
Total unidentified	10.3	21.7	32.0	10.0	24.8	34.8
Total accounted for	26.0	62.5	88.5	18.3	67.2	85.5
Losses/gains	0.1	4.6	4.7	0.0	2.7	2.7
Total	26.1	67.1	93.2	18.3	69.9	88.2
LOQ	0.2	1.4	NA	0.3	1.7	NA

bw: body weight; LOQ: limit of quantification; NA: not applicable; ND: not detected; TCP: trichlorophenol; TCPM: trichlorophenol methanol

[a] 0–72 hours.
[b] 0–96 hours.
[c] Aggregate of two retention times for desmethyl hydroxy pydiflumetofen.
[d] Aggregate of two retention times for hydroxy pydiflumetofen.
[e] A total of six unidentified components, with no individual component accounting for greater than 0.3% of the dose.
[f] A total of 19 unidentified components, one of which accounted for 3.2% of the dose and another for 2.7% of the dose, with no other individual component accounting for greater than 2.0% of the dose.
[g] A total of 27 unidentified components, with no individual component accounting for greater than 0.4% of the dose.
[h] A total of 14 unidentified components, one of which accounted for 2.7% of the dose and another for 2.5% of the dose, with no other individual component accounting for greater than 1.9% of the dose.
[i] Aggregate of two retention times for desmethyl hydroxy pydiflumetofen.
[j] A total of 24 unidentified components, one of which accounted for 4.4% of the dose and another for 1.1% of the dose, with no other individual component accounting for greater than 0.5% of the dose.
[k] A total of 23 unidentified components, with no individual component accounting for greater than 1.8% of the dose.
[l] A total of 35 unidentified components, one of which accounted for 2.8%, with no other individual component accounting for greater than 0.6% of the dose.
[m] A total of 31 unidentified components, one of which accounted for 2.3% of the dose and another two for 1.8% of the dose, with no other individual component accounting for greater than 1.3% of the dose.

Source: MacDonald & Jewkes (2015)

Table 10. Metabolite profile in plasma after administration of a single oral dose of [phenyl-U-^{14}C]pydiflumetofen to rats

Compound	% of AUC[a]			
	Males		Females	
	5 mg/kg bw	300 mg/kg bw	5 mg/kg bw	100 mg/kg bw
Pydiflumetofen	1.9	1.3	2.8	5.0
Dihydroxy pydiflumetofen glucuronide	ND	0.9	1.4	1.0
HTCP sulfate	6.1	4.8	9.3	9.2
TCPM glucuronide	3.4	3.4	0.9	2.4
Dechlorinated dihydroxy pydiflumetofen glucuronide[b]	2.2	1.9	1.2	0.9

Compound	% of AUC[a]			
	Males		Females	
	5 mg/kg bw	300 mg/kg bw	5 mg/kg bw	100 mg/kg bw
Hydroxy pydiflumetofen glucuronide	1.0	3.1	1.6	1.0
2,4,6-TCP sulfate	41.1	44.1	41.0	32.2
SYN547948	ND	ND	2.5	3.6
Desmethyl hydroxy pydiflumetofen sulfate	ND	1.8	ND	ND
SYN547891 glucuronide	2.7	1.8	3.6	1.8
2,4,6-TCP	4.3	2.4	5.2	5.3
SYN547897	0.8	1.5	4.3	2.5
Unidentified components	11.3[c]	4.7[d]	10.9[e]	8.3[f]
Additional extracts	NA	<LOQ	NA	<LOQ
Post-extraction solids	18.8	18.3	13.1	12.5
Total identified	63.5	67.0	73.8	64.9
Total unidentified	11.3	4.7	10.9	8.3
Total accounted for	93.6	90.0	97.8	85.7
Losses/gains	6.4	10.0	2.2	14.3
Total	100.0	100.0	100.0	100.0
LOQ	0.8	1.2	0.9	1.2

AUC: area under the concentration–time curve; bw: body weight; HTCP: hydroxylated 2,4,6-trichlorophenol; LOQ: limit of quantification; NA: not applicable; ND: not detected; TCP: trichlorophenol

[a] 0–96 hours.

[b] Values aggregated for dechlorinated dihydroxy pydiflumetofen glucuronide from different retention times.

[c] A total of eight unidentified components, with no individual component accounting for greater than 2.5% of the AUC.

[d] A total of five unidentified components, with no individual component accounting for greater than 1.7% of the AUC.

[e] A total of 11 unidentified components, with no individual component accounting for greater than 2.0% of the AUC.

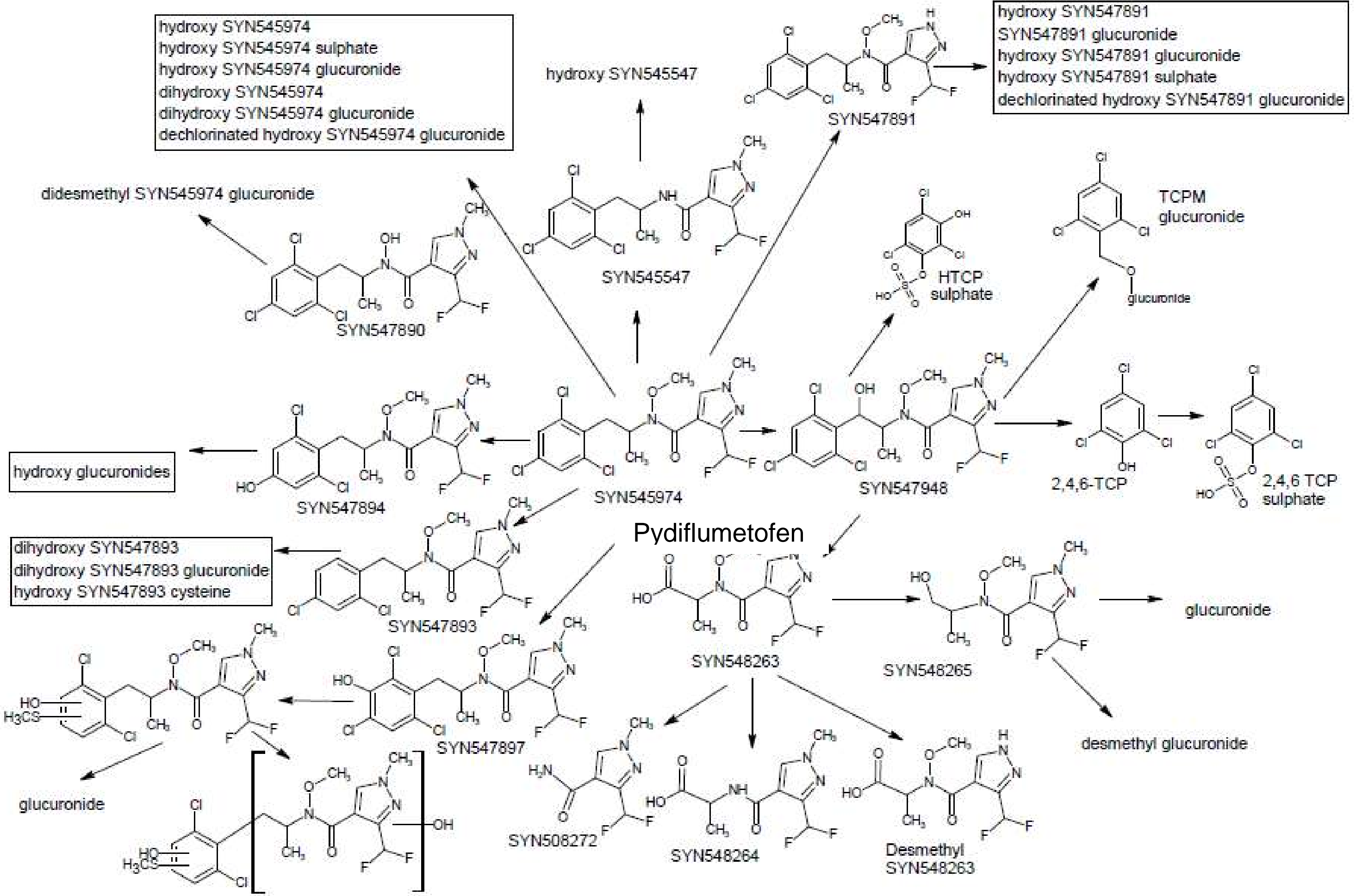
[f] A total of 10 unidentified components, with no individual component accounting for greater than 1.9% of the AUC.

Source: MacDonald & Jewkes (2015)

The proposed metabolic pathway of pydiflumetofen in rats is shown in Fig. 4.

Overall, the biotransformation proceeded by hydroxylation to SYN547897, SYN547948 and other hydroxylated and dihydroxylated isomers, demethylation to SYN547890 and *N*-desmethyl SYN547890, demethoxylation to SYN545547, followed by subsequent hydroxylation to hydroxy SYN545547, hydroxylation and demethylation to hydroxy SYN547891 and other desmethyl hydroxy metabolites, cleavage of pydiflumetofen to give the pyrazole-derived metabolites SYN548265, SYN548263, SYN548264, desmethyl SYN548263 and SYN508272 and the phenyl-derived metabolite 2,4,6-TCP, dechlorination to SYN547893, dechlorination and hydroxylation to SYN547894 and other dechlorinated hydroxyl and dechlorinated dihydroxy metabolites, glutathione conjugation followed by metabolism of the conjugate to give dechlorinated hydroxy thiomethyl SYN545974 and dechlorinated dihydroxy thiomethyl SYN545974, and glucuronic acid conjugation and some sulfate conjugation (MacDonald & Jewkes, 2015).

Fig. 4. Biotransformation pathway for pydiflumetofen in rats



Source: MacDonald & Jewkes (2015)

2. Toxicological studies

2.1 Acute toxicity

The acute toxicity of pydiflumetofen is summarized in Table 11.

Table 11. Summary of acute toxicity of pydiflumetofen

Study (species)	Purity (%); lot/batch no.	Result	Reference
Oral (rats)	98.5; SMU2EP12007	LD_{50} > 5 000 mg/kg bw	Petus-Árpásy (2012)
Dermal (rats)	98.5; SMU2EP12007	LD_{50} > 5 000 mg/kg bw	Petus-Árpásy (2013)
Inhalation (rats)	98.5; SMU2EP12007	LC_{50} > 5.11 mg/L	Nagy (2013)
Skin irritation (rabbits)	98.5; SMU2EP12007	Not irritating	Török-Bathó (2012a)
Eye irritation (rabbits)	98.5; SMU2EP12007	Mildly irritating	Török-Bathó (2012b)
Skin sensitization (LLNA) (mice)	98.5; SMU2EP12007	Not sensitizing	Hargitai (2013)

bw: body weight; LC_{50}: median lethal concentration; LD_{50}: median lethal dose; LLNA: local lymph node assay

(a) Lethal doses

The oral median lethal dose (LD_{50}) for pydiflumetofen (purity 98.5%) using carboxymethylcellulose (0.5% v/v) as the vehicle in female CRL:(WI) Wistar rats was greater than 5000 mg/kg bw (limit dose). No deaths occurred at 5000 mg/kg bw, and no significant clinical observations or macroscopic findings were noted (Petus-Árpásy, 2012).

The dermal LD_{50} for pydiflumetofen (purity 98.5%) using tap water as the vehicle in male and female CRL:(WI) Wistar rats was studied by application of pydiflumetofen to a shaved area representing 10% of the body surface. The shaved area was then covered with a gauze pad, and the entire trunk was wrapped in a semi-occlusive plastic wrap for 24 hours. No deaths occurred at 5000 mg/kg bw (limit dose). Clinical signs were limited to decreased activity in all animals on day 1 after treatment. There were no treatment-related effects on body weight or body weight gain (Petus-Árpásy, 2013).

The inhalation median lethal concentration (LC_{50}) for pydiflumetofen (purity 98.5%) was greater than 5.11 mg/L (mass median aerodynamic diameter 3.54 µm ± 2.32 geometric standard deviation) in air for male and female CRL:(WI) Wistar rats after a 4-hour nose-only exposure. One death occurred in a group of five female rats after exposure on day 0. Clinical signs included wet fur, puffed coat and fur staining on the day of treatment and for several days after treatment. In addition, animals showed signs of laboured, gasping and noisy respiration, sneezing, decreased activity, prostration and ataxia on the day of exposure. Signs of noisy respiration or weakened condition were noted in some animals in the first 2 days after exposure. There were no significant effects on body weight during the 14-day observation period (Nagy, 2013).

(b) Dermal irritation

The skin irritating potential of pydiflumetofen (purity 98.5%) was investigated in three male New Zealand white rabbits. Pydiflumetofen was applied as an undiluted powder to intact clipped skin on the back and flanks of each rabbit. Test substance (0.5 mg) was applied to the skin under a patch of gauze (2.5 cm × 2.5 cm) that was held in place with adhesive bandage, and the entire trunk was wrapped in plastic wrap and elastic stocking for 4 hours. Treated areas were washed with water and scored at 24,

48 and 72 hours after removal of the patch. Under the conditions of the test, there was no evidence of skin irritation by pydiflumetofen (Török-Bathó, 2012a).

(c) Ocular irritation

The eye irritating potential of pydiflumetofen (purity 98.5%) was investigated in three male New Zealand white rabbits. Pydiflumetofen (0.1 g) was applied as an undiluted powder to one eye of each rabbit, while the other eye served as the control. Eyes were rinsed with saline and scored at 1, 24, 48 and 72 hours post-treatment. All animals demonstrated a slight initial pain reaction on instillation of pydiflumetofen. There was a slight discharge in one rabbit and conjunctival redness in all rabbits at 1 hour. At 24 and 48 hours, conjunctival redness was noted in only one rabbit. Fluorescein staining was negative in all animals at all time points. There were no additional clinical signs or effects on body weight. As all clinical signs had reversed by 72 hours, the study was subsequently terminated. Under the conditions of the test, pydiflumetofen was considered mildly irritating to the eyes of rabbits (Török-Bathó, 2012b).

(d) Dermal sensitization

Pydiflumetofen (purity 98.5%) was tested for dermal sensitization potential using the local lymph node assay in female CBA/JRj mice. Pydiflumetofen (50%, 25% or 25% w/v) was applied to the dorsal surface of the animals' ears (25 µL/ear) for 3 consecutive days using an acetone:olive oil (4:1 v/v) vehicle. Five hours prior to termination on day 6, tritiated methyl thymidine was injected via the tail vein. Cell proliferation in the local lymph nodes was measured by incorporation of the tritiated methyl thymidine. There were no deaths, signs of systemic toxicity or effects on body weight during the study. In addition, there were no signs of irritation at the site of application. Precipitate of the test substance was noted on the ears of animals treated with either 50% or 25%. The appearance of the lymph nodes was normal in all pydiflumetofen-treated animals compared with positive controls (25% w/v α-hexylcinnamaldehyde). There were no significant increases in the stimulation index for pydiflumetofen in this study. Under the conditions of the test, pydiflumetofen was not considered to cause skin sensitization (Hargitai, 2013).

2.2 Short-term studies of toxicity

(a) Oral administration

Mice

In a 28-day toxicity study, CD-1 mice (six of each sex per group) were given pydiflumetofen (lot/batch no. LOT2491-DC/110; purity 98.6%) in the diet at a concentration of 0, 500, 1500, 4000 or 7000 parts per million (ppm) (equal to 0, 76, 213, 612 and 1115 mg/kg bw per day for males and 0, 96, 266, 701 and 1312 mg/kg bw per day for females, respectively). Satellite groups (six of each sex) that received 0 or 7000 ppm (equal to 925 and 1132 mg/kg bw per day for males and females, respectively, euthanized on day 4; and 1053 and 1595 mg/kg bw per day for males and females, respectively, euthanized on day 8) were euthanized on days 4 and 8 for the analysis of plasma levels of pydiflumetofen and cell proliferation activity in the liver. The results of the latter analysis (Haines, 2012) are described in section 2.6(d) below.

There were no treatment-related effects on mortality, clinical signs, feed consumption, haematology, gross pathology or histopathology. Body weight gains were lower in all treated male groups over the first 11 days, resulting in lower terminal body weights. Feed consumption values, however, were highly variable and were not a clear indication as to the cause of the lower body weight gains. The relationship between dose and terminal body weight was weak, however, except at the highest dose, and the relationship with treatment at 500 and 1500 ppm is questionable, with the terminal body weight at 1500 ppm comparable to control values. In the absence of any other toxicologically relevant findings at the lower doses and the absence of similar effects in females, the apparent reduction

in body weight gains at doses less than 4000 ppm was considered to be of uncertain relationship to treatment. Triglycerides were higher (2.54 mmol/L vs 1.18 mmol/L in controls) and phosphates were lower (1.56 mmol/L vs 2.31 mmol/L in controls) in males at 7000 ppm. Although there were only three animals per group used for clinical chemistry, all values for triglycerides at 7000 ppm were higher than the highest control value. Absolute liver weights were increased in both sexes at 7000 ppm on days 4 and 8 and in all treated groups at termination. Toxicokinetic analysis was unreliable and unusable, with blood levels of pydiflumetofen in some control animals similar to or higher than those in animals dosed at 500 or 1500 ppm.

The no-observed-adverse-effect level (NOAEL) was 4000 ppm (equal to 612 mg/kg bw per day), based on reduced body weight gain, an increase in absolute liver weights and an increase in serum triglyceride levels at 7000 ppm (equal to 1115 mg/kg bw per day) (Strepka, 2012a).

In a 91-day toxicity study, 10 Crl:CD-1(ICR) mice of each sex per group were administered pydiflumetofen (lot/batch no. 2637-BA/110; purity 99.5%) in the diet at a concentration of 0, 100, 500, 4000 or 7000 ppm (equal to 0, 17.5, 81.6, 630 and 1158 mg/kg bw per day for males and 0, 20.4, 106, 846 and 1483 mg/kg bw per day for females, respectively). In addition to test guideline parameters, a detailed functional observational battery (FOB) study (including motor activity) was undertaken pretrial and at week 12. Whole blood concentrations of pydiflumetofen were analysed from samples taken at various time points on days 2, 16, 30 and 91. Histopathological examinations were conducted only for mice in the 0 and 7000 ppm groups, except for the liver, which was also examined in the 100, 500 and 4000 ppm groups.

There were no effects of treatment on mortality, clinical signs, feed consumption and feed utilization, water intake, haematology or gross pathology. Dose-related and consistent changes in body weight gain were not observed in either sex. In the blood, levels of cholesterol in males at 4000 ppm and in both sexes at 7000 ppm and levels of triglyceride in both sexes at 7000 ppm were statistically significantly increased. Slight (approximately 10%) increases in globulin levels in males of all treated groups were not considered treatment related due to a lack of dose–response relationship. A slight (approximately 15%) decrease in glucose levels in females in the higher dose groups was not considered toxicologically relevant, as the decreases were within the historical control range (11.09 ± 0.62 mmol/L, mean ± standard deviation) for the testing laboratory (Shearer & Robertson, 2016). Absolute and relative (to body weight) liver weights were increased at 500 ppm and above in males and at 4000 ppm and above in females. These treatment-related changes in the liver correlated with an increased incidence of hepatocyte hypertrophy (minimal) in males and females. Liver hypertrophy in males at 500 ppm was considered adaptive, as there were no findings indicating liver damage in this group.

The key results are summarized in Table 12.

Table 12. Summary of findings from the 91-day toxicity study of pydiflumetofen in mice

	Males					Females				
Parameter	**0 ppm**	**100 ppm**	**500 ppm**	**4 000 ppm**	**7 000 ppm**	**0 ppm**	**100 ppm**	**500 ppm**	**4 000 ppm**	**7 000 ppm**
Blood biochemistry										
Cholesterol	3.9	4.1	4.8	4.9*	5.9**	2.8	2.6	2.7	3.6	3.8**
Triglycerides	1.50	1.26	1.46	1.70	2.79**	1.13	1.14	0.90	1.72	1.77*
Globulin	19	21*	22**	21*	22**	17	17	17	18	18
Glucose	12.3	12.33	12.29	11.88	11.30	12.86	12.30	11.93	10.89*	10.88*
Liver weight										
Absolute (g)	1.94	1.96	2.29*	2.76**	3.24**	1.63	1.64	1.66	2.60**	2.51**

Parameter	Males					Females				
	0 ppm	100 ppm	500 ppm	4 000 ppm	7 000 ppm	0 ppm	100 ppm	500 ppm	4 000 ppm	7 000 ppm
Covariate (g)	1.90	1.99	2.18*	2.82**	3.32**	1.58	1.61	1.71	2.56**	2.59**
Relative (%)[a]	4.7	4.9	5.3	7.0	8.3	5.0	5.1	5.4	8.0	8.4
Histopathology										
No. of mice examined	10	10	10	10	10	10	10	10	10	10
Hepatocellular hypertrophy	0	0	2	4	5*	0	0	0	6*	7**

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$
[a] Relative to body weight. No statistical analyses performed on relative liver weights.
Source: Shearer (2015)

Concentrations of pydiflumetofen in blood were generally below the LOQ (5 ng/mL) in control animals, but values were above the LOQ in 18/160 samples taken from control animals. In general, females had substantially higher levels in blood than males at each dose (see Appendix 3).

The NOAEL was 500 ppm (equal to 81.6 mg/kg bw per day), based on increased cholesterol concentrations associated with liver hypertrophy at 4000 ppm (equal to 630 mg/kg bw per day) (Shearer, 2015).

Rats

In a 28-day toxicity study, six Han Wistar rats of each sex per group were administered pydiflumetofen (lot/batch no. 2491-DC/110; purity 98.6%) in the diet at a concentration of 0, 500, 4000, 8000 or 16 000 ppm (equal to 0, 43, 343, 677 and 1322 mg/kg bw per day for males and 0, 40, 322, 619 and 1174 mg/kg bw per day for females, respectively). An additional six rats of each sex for the control and high-dose groups provided satellite groups for termination on days 3 and 7. Satellite groups were subjected to necropsy, gross pathology and organ weight measurement, but not histology. Toxicokinetics was investigated using blood samples taken from the main group on days 4 and 28 of the study, and cell proliferation studies were conducted on satellite animals but not reported. At necropsy, tissues from animals in the control and high-dose groups were examined histologically, and the liver was examined in all groups except females at 500 ppm. Organ weights were also analysed by analysis of covariance (ANCOVA) using terminal kill body weight as the covariate, but group mean organ to body weight ratios (relative organ weights) were reported without any statistical analysis.

There were no treatment-related effects on mortality, clinical signs, water consumption (visual estimation only) or gross pathology. Body weight was lower in both sexes at 16 000 ppm. For females, the lower body weight was due to an initial body weight loss on the first day, which took until day 7 to return to pretreatment levels. The reduced body weight gain was associated with a marked reduction in feed consumption in both sexes over the first 1 or 2 days (in males, approximately 50% reduction at 8000 and 16 000 ppm; in females, approximately 40–75% reduction without dose dependency at 4000 ppm and above on day 1, and 50% reduction at 16 000 ppm on day 2). The lower feed consumption in this early phase was suggested by the study author to be related to lower palatability and may have caused the lower body weight gain, rather than systemic toxicity per se. However, the slight (approximately 10%), but consistent, reduction in body weight gain throughout the study period in males at 16 000 ppm was considered adverse.

Lower white blood cell counts (5.95×10^9/L, 8.32×10^9/L, 7.06×10^9/L and 7.54×10^9/L vs 9.2×10^9/L in controls) and lymphocyte counts (4.62×10^9/L, 6.94×10^9/L, 5.52×10^9/L and 6.24×10^9/L vs 7.65×10^9/L in controls) in all treated groups of males were not considered treatment related owing to a lack of dose dependency and reproducibility in 90-day and long-term studies in rats. In

females, a substantially lower serum glutamate dehydrogenase level in all treated groups (4.0, 3.9, 3.0 and 3.0 IU/L vs 9.6 IU/L in controls) was not considered toxicologically relevant owing to the decrease, not increase, in magnitude and lack of change in related parameters. A lower glucose level was observed in all treated female groups, which was statistically significant but without a dose–response relationship across a 32-fold dose range, and which was within the historical control range for the testing laboratory. Slightly, but statistically significantly, lower alanine aminotransferase (ALT) activity in females at 8000 and 16 000 ppm (29 and 28 IU/mL vs 40 IU/mL in controls) was unlikely to be adverse in isolation. Higher urinary volumes in females at 4000 ppm and higher were observed, with a correspondingly slightly lower specific gravity. There was no real dose–response relationship for specific gravity, and the increase in volume was not statistically significant at any dose, owing to high variability in individual values.

Covariate liver weights (liver weight adjusted for covariance to terminal body weight) were increased in males at 4000 ppm and higher and in females at 500 ppm and higher (10.89, 11.47, 13.31, 14.25 and 14.64 g for males; 6.50, 7.36, 8.34, 8.68 and 9.160 g for females; at 0, 500, 4000, 8000 and 16 000 ppm, respectively). In the satellite group (0 and 16 000 ppm) for cell proliferation activity, covariate liver weights were increased statistically significantly on both examined days (approximately 20% and 30% increases on days 4 and 8, respectively).

Treatment-related findings were confined to minimal centrilobular hypertrophy in males at 4000 ppm and above and in females at 8000 ppm and above (0, 0, 4, 5 and 6 in males at 0, 500, 4000, 8000 and 16 000 ppm, respectively; and 0, 0, 3 and 5 in females at 0, 4000, 8000 and 16 000 ppm, respectively). Minimal inflammatory cell foci were scattered in all dose groups including controls in both sexes. Their incidence increased in males at 8000 and 16 000 ppm (incidence: 1, 1, 1, 3 and 3 at 0, 500, 4000, 8000 and 16 000 ppm, respectively). However, this finding was not related to hepatocellular hypertrophy except at 16 000 ppm, and inflammatory changes in the liver were not noted in either the 91-day or chronic toxicity study in rats. Therefore, this finding was not considered treatment related. As no findings indicated liver damage in the treated groups, the liver hypertrophy observed at 500 ppm and above was considered adaptive.

The toxicokinetics data appear to be unreliable. Pydiflumetofen was detected in both control and treated groups, values were highly variable across individuals in each group and there was not a relationship between levels in blood and dose. The study report does not discuss the source of the errors/inconsistencies. Regardless of the source of the errors in this study, the toxicokinetics from this study are unusable.

The NOAEL was 8000 ppm (equal to 619 mg/kg bw per day), based on reduction of body weight gain at 16 000 ppm (equal to 1174 mg/kg bw per day) (Strepka, 2012b).

In a 91-day toxicity study, Crl:WI(Han) rats (10 of each sex per dose) were administered pydiflumetofen (lot/batch no. 2637-BA/110; purity 99.5%) in the diet at a concentration of 0, 250, 1500, 8000 or 16 000 ppm (equal to 0, 18.6, 111, 587 and 1187 mg/kg bw per day for males and 0, 21.6, 127, 727 and 1325 mg/kg bw per day for females, respectively). In addition to test guideline parameters, a detailed FOB, including motor activity and body temperature, was conducted pretest and at week 12. Blood samples (50 µL) were collected on days 2, 9, 28 and 91 from all animals to determine concentrations of pydiflumetofen in whole blood. Histopathological examination was conducted on only the control and high-dose animals, except for the liver and thyroid glands, which were also examined at 250 and 1500 ppm. The left lateral lobes of the liver were placed in separate RNA-free tubes for analysis of microsomal protein and uridine diphosphate (UDP) glucuronosyltransferase (UGT) activity, which was conducted as a separate study from the present study and is described in section 2.6(d).

The results are summarized in Table 13. There were no treatment-related effects on mortality, clinical signs, ophthalmoscopy, FOB, body temperature, pathology, haematology, coagulation rate or urine analysis. Body weights in males at 16 000 ppm were statistically significantly and consistently

Table 13. Summary of findings from the 91-day toxicity study of pydiflumetofen in rats

Parameter	Males 0 ppm	Males 250 ppm	Males 1 500 ppm	Males 8 000 ppm	Males 16 000 ppm	Females 0 ppm	Females 250 ppm	Females 1 500 ppm	Females 8 000 ppm	Females 16 000 ppm
Body weight (g)										
Day 0	246	242	248	244	242	161	160	160	163	172
Day 1	249	247	249	234	232*	165	163	161	156	161
Day 4	263	261	265	250	246*	170	171	169	166	172
Day 7	279	278	279	264	259*	181	182	177	176	181
Day 14	304	300	301	286	280*	194	193	189	187	192
Day 28	344	337	336	313	302**	207	206	203	199	207
Day 56	393	380	386	359	347*	229	227	223	216	228
Day 91	434	418	419	382*	368**	237	238	230	223	229
Feed consumption (g/day)										
Day 0	22.4	22.4	23.4	20.4	22.6	15.7	13.7	15.6	19.1	16.7
Day 1	23.3	22.9	18.5*	11.0**	11.3**	16.2	14.2	15.8	9.9*	7.2**
Day 2	20.8	19.9	20.5	13.1**	12.6**	14.6	14.8	11.9	15.3	9.0
Day 3	22.9	23.0	23.4	22.7	22.1	17.7	15.5	14.5	10.5*	11.1*
Day 91	23.6	23.9	23.0	21.3	22.0	17.7	16.9	16.9	18.1	17.3
Blood biochemistry										
ALP (U/L)	93	89	65**	64**	57**	59	45	35**	34**	36**
Chol (mmol/L)	2.0	2.1	2.3	1.9	1.9	1.7	1.7	2.0	2.3**	2.3**
Liver weight (g)										
Absolute	14.51	14.39	17.57*	17.80**	18.30**	7.63	8.19	8.85**	10.38**	10.71**
Covariate	13.07	13.79	16.85**	18.81**	19.90**	7.52	8.04	8.87**	10.58**	10.75**
Histopathology										
No. of rats examined	10	10	10	10	10	10	10	10	10	10
Hepatocellular hypertrophy minimal	0	0	5*	7**	10**	0	0	0	6*	9**
Thyroid, follicular cell hypertrophy	0	0	4	6*	7**	0	0	0	4*	8**

ALP: alkaline phosphatase; Chol: cholesterol; ppm: parts per million; U: units; *: $P < 0.05$; **: $P < 0.01$
Source: Shearer & Robertson (2015)

lower than control values throughout the study period. Body weights in males at 8000 ppm were slightly (within approximately 10%), but consistently, lower after 1 month of treatment. Body weight gain was reduced in both sexes at 8000 and 16 000 ppm, associated with reduced feed consumption over the first 2–3 days of treatment. Body weight gain was also lower in males at 1500 ppm, but did not reach statistical significance. Examination of the individual animal data indicates that four of the 10 animals at 1500 ppm had body weight gains lower than or equal to the lowest body weight gain in control

animals, and there was a clear shift in values downwards. The lower body weight gain was associated with reduced feed utilization and consistent with the overall dose–response relationship. Transient reduced body weight gains on the first 2 days in males at 1500 ppm and higher and in females at 8000 ppm and higher were related to lower feed consumption, suggesting lower palatability in this period. No statistically significant changes in body weight were found in males at 1500 ppm or in females in all treated groups. Therefore, the transient changes in body weight gains in males at 1500 ppm were not considered treatment related.

Compared with controls, alkaline phosphatase (ALP) activity was reduced in both sexes at 1500, 8000 and 16 000 ppm, and cholesterol was higher in females at 8000 and 16 000 ppm. A decrease in the magnitude of ALP is generally accepted as having no toxicological relevance. In addition, there were no corresponding findings in the hepatobiliary system, gastrointestinal tract or bone marrow. Therefore, the lower ALP levels in both sexes at 1500 ppm and above were not considered adverse. In urine analysis, increases in volume accompanied by lower pH were observed in both sexes at 8000 ppm and above. As no other change indicating renal toxicity was observed in these dose groups, these changes were not considered to be toxicologically relevant.

Treatment-related changes in liver and thyroid weights and histopathological observations were found. A dose-related increase in liver weight was observed in both sexes at 1500 ppm and above. Incidences of minimal hepatocyte hypertrophy and minimal to mild follicular cell hypertrophy in thyroid were increased in males at 1500 ppm and above and in females at 8000 ppm and above. The liver findings were associated with a corresponding increase in liver weight, except in females at 1500 ppm. The increase in liver weight in females at 1500 ppm was considered adaptive, as there were no findings related to hepatotoxicity or thyroid effects.

With the exception of some isolated observations of low levels of pydiflumetofen in blood from control animals, these values were generally below the LOQ (5.0 ng/mL). A saturation of absorption appears to occur above 1500 ppm in both sexes, with no or only slight increases in levels in blood at higher doses. In general, females had substantially higher levels in blood than males at each dose (see Appendix 3).

The NOAEL was 250 ppm (equal to 18.6 mg/kg bw per day), based on follicular cell hypertrophy in the thyroid at 1500 ppm (equal to 111 mg/kg bw per day) (Shearer & Robertson, 2015).

Dogs

In a 28-day toxicity study, a single beagle dog of each sex per dose was administered pydiflumetofen (lot/batch no. 2637AA/110; purity 99.3%) by oral capsule at a dose of 0, 100, 300 or 1000 mg/kg bw per day for 28 days. Animals were monitored daily for mortality, clinical signs and feed consumption. Ophthalmoscopy, clinical chemistry, haematology and urine analysis were conducted. Concentrations of pydiflumetofen in blood were determined on days 1 and 28 (LOQ < 5.0 ng/mL). All animals were necropsied after 28 days of treatment, selected organs were weighed, and the liver and any gross lesions were examined for histopathology.

There were no effects of treatment on mortality, clinical signs, body weight gain, feed consumption, ophthalmoscopy, gross pathology, haematology, coagulation, urine analysis parameters or liver histopathology. A clear trend for increased ALP was observed in both sexes at all doses. Relative liver weight showed a clear increase at 300 mg/kg bw per day and above, although no liver histopathology was observed. Pydiflumetofen concentrations in blood increased with increasing dose, although peak levels were slightly less than dose proportional (Strepka & Shearer, 2015).

In a 13-week toxicity study, beagle dogs (four of each sex per dose) were administered pydiflumetofen (lot/batch no. SMU2EP12007; purity 98.5%) in capsules at a dose of 0, 30, 300 or 1000 mg/kg bw per day. In addition to test guideline parameters, a weekly FOB was conducted. Toxicokinetics was investigated on days 1 and 28 and during week 13, with blood samples collected

predosing and 0.5, 1, 1.5, 2, 4, 8, 12 and 24 hours after dosing in all animals, except in control animals, from which only predosing samples were taken.

There were no treatment-related effects on mortality, clinical signs, ophthalmoscopy, organ weights, gross pathology or histopathology (except liver), haematology or urine analysis. At 1000 mg/kg bw per day, two of the four males and all females lost body weight during the initial weeks of dosing, with some taking several weeks to regain this lost body weight and others, most notably females, continuing to show erratic body weight change. Body weight gain was lower for the female group at this dose compared with controls, and their final body weights were notably lower than control values. There was no significant difference in overall body weight gain or final body weight for the male group. There was a corresponding effect on feed consumption in males and females at 1000 mg/kg bw per day. At 300 mg/kg bw per day, one male and two females showed slight body weight losses during the first 2 weeks of the treatment period, but thereafter gained body weight normally. In the absence of a consistent and overall effect on body weight and body weight gain, this was considered not adverse.

Plasma ALP and triglycerides were increased at 300 mg/kg bw per day and above in both sexes, associated with increased liver weights. However, ALT, aspartate aminotransferase (AST) and gamma-glutamyl transferase (GGT) were unaffected. At 300 mg/kg bw per day, there were no indications of liver damage in the histopathology that might correlate with the increased ALP. In addition, no changes were found in bone marrow or adrenals, organs with the potential to affect plasma ALP in dogs. The slight, but continuous, increase in triglycerides in males at 1000 mg/kg bw per day was treatment related. Bilirubin was decreased in males at 300 mg/kg bw per day and higher and in females at 1000 mg/kg bw per day in week 13; however, individual values overlapped with those in the controls, and the relationship was inconsistent across the time points. In week 8, for example, the bilirubin levels were lower in males at 300 and 1000 mg/kg bw per day than in controls. Consequently, the apparent effect on bilirubin was concluded to be incidental.

The only treatment-related histopathological finding was minimal hepatocyte hypertrophy observed at 1000 mg/kg bw per day.

No adverse observations were made in the animals treated with 30 mg/kg bw per day.

Pydiflumetofen levels in blood (LOQ: 5.00 ng/mL) showed a supraproportional increase relative to the dose increments for both the AUC and C_{max}, but without a prolongation of half-life. In males, for example, the C_{max} on day 1 was 24.6, 832 and 3510 ng/mL when animals were given pydiflumetofen at a dose of 30, 300 or 1000 mg/kg bw per day, respectively; and on day 91, the C_{max} was 38.3, 638 and 2070 ng/mL, respectively. Similarly, in males, the $AUC_{0–24 h}$ on day 1 was 85.3, 6710 and 31 400 ng·h/mL when animals were given pydiflumetofen at a dose of 30, 300 or 1000 mg/kg bw per day, respectively; and on day 91, the $AUC_{0–24 h}$ was 191, 6270 and 17 400 ng·h/mL, respectively.

Animals that received 300 or 1000 mg/kg bw per day were continuously exposed over the 24-hour period after dosing, but at 30 mg/kg bw per day, exposure was limited to up to 8 hours after dosing. Exposure tended to be higher in males, most notably at 300 mg/kg bw per day. There was no evidence of accumulation in plasma. Absorption was relatively slow, with a T_{max} of the order of 4–12 hours at 300 and 1000 mg/kg bw per day, and the half-life was varied, between 2.6 and 4.9 hours in males and between 2.4 and 4.5 hours in females, with some (equivocal) indication of a shorter half-life after 90 days of treatment.

The results observed in the 13-week toxicity study in dogs are summarized in Table 14.

Although ALP was increased at 300 mg/kg bw per day, there was no alteration of liver histology at 300 mg/kg bw per day and only minimal hepatocyte hypertrophy at 1000 mg/kg bw per day, with no other compelling evidence of pathology in liver. On this basis, although the increase in ALP is clearly treatment related at 300 and 1000 mg/kg bw per day, it is concluded to be non-adverse.

The NOAEL was 300 mg/kg bw per day, based on reduced body weight gain and increased triglycerides at 1000 mg/kg bw per day (Blunt, 2015a).

Table 14. Summary of findings from the 13-week toxicity study of pydiflumetofen in dogs

Parameters	Males				Females			
	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	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
Body weight (kg)								
Day −1	11.45	11.73	11.55	11.78	10.48	10.08	10.53	10.85
Day 7	11.50	11.93	11.55	11.68	10.55	10.20	10.45	10.48
Day 35	11.53	12.05	11.63	11.73	10.95	10.58	10.50	10.35
Day 63	11.78	12.43	11.85	12.03	11.43	11.18	11.10	10.60
Day 91	11.68	12.58	12.03	11.90	11.63	11.43	11.10	10.83
Weight gain (kg)								
Days −1 to 7	0.05	0.20	0.00	−0.10	0.08	0.13	−0.08	−0.38*
Days −1 to 14	0.15	0.20	−0.05	−0.10	0.18	0.23	−0.15**	−0.38*
Days −1 to 21	0.20	0.43	0.00	0.00	0.28	0.38	−0.13	−0.33*
Days −1 to 28	0.18	0.28	0.00	−0.05	0.40	0.45	−0.03	−0.45*
Days −1 to 35	0.08	0.33	0.08	−0.05	0.48	0.50	−0.03	−0.50*
Days −1 to 49	0.18	0.80	0.63	0.85	0.38	0.18	0.13	−0.28*
Days −1 to 56	0.23	0.83	0.38	0.85	0.05	0.30	−0.20	−0.33*
Days −1 to 63	0.33	0.95	0.70	1.08	0.30	0.45	0.25	−0.13*
Days −1 to 77	0.33	1.05	0.73	1.13	0.35	0.60	0.25	−0.55*
Days −1 to 91	0.23	1.15	0.85	1.35	0.48	0.58	0.13	−0.03**
ALP (U/L)								
−1 week								
Mean	73.5	81.5	89.3	71.5	100.5	83.8	94.5	88.5
SD	19.8	10.4	16.2	23.9	32.3	12.8	35.9	23.0
4 weeks								
Mean	71.5	85.8	251.8	349.0**	94.0	91.0	214.3	328.3*
SD	25.6	5.2	86.6	167.8	25.9	23.1	79.8	149.3
8 weeks								
Mean	74.0	86.0	286.8	379.0*	86.0	87.3	247.5	374.3*
SD	28.1	8.7	134.7	198.1	31.4	20.0	89.0	195.7
13 weeks								
Mean	68.5	70.0	252.3**	384.3**	77.8	76.8	236.5	327.8**
SD	24.0	6.3	132.9	203.8	26.7	17.0	99.7	193.9
Triglycerides (mg/dL)								
−1 week								
Mean	28.5	34.8	36.5	33.5	38.8	42.0	38.8	32.3

Parameters	Males				Females			
	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	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
SD	3.1	8.6	9.5	8.8	7.1	1.4	5.0	2.9
4 weeks								
Mean	28.3	35.8	68.3*	96.8*	50.3	54.8	51.8	79.3
SD	1.7	5.7	20.0	23.5	12.3	14.9	7.7	28.3
8 weeks								
Mean	26.3	31.0	41.3	67.3*	44.5	58.0	54.3	71.0
SD	2.1	4.8	9.9	29.7	6.1	13.3	9.8	29.8
13 weeks								
Mean	30.3	29.0	51.0	74.0**	44.8	52.8	47.5	61.5
SD	6.2	5.2	10.9	37.4	9.4	21.9	8.1	3.1
Liver weight, covariate (g)								
Mean	375.7	382.7	492.9*	529.9**	356.6	355.9	414.3	522.0**
Histopathology								
Hypertrophy, hepatocyte, minimal	0/4[a]	0/4	0/4	4/4	0/4[a]	0/4	0/4	4/4

ALP: alkaline phosphatase; bw: body weight; SD: standard deviation; U: units; *: $P < 0.05$; **: $P < 0.01$
[a] The number of animals with the finding/the number of dogs examined.
Source: Blunt (2015a)

In a 1-year toxicity study, beagle dogs (four of each sex per dose) were administered pydiflumetofen (lot/batch no. SMU2EFP12007; purity 98.5%) in capsules at a dose of 0, 30, 100 or 300 mg/kg bw per day. In addition to test guideline parameters, a weekly FOB was conducted.

Although group mean body weights and body weight gains for the treated groups were generally lower than those in the concurrent controls, there was no dose–response relationship, and the greatest difference was in the 30 mg/kg bw per day group. Body weight gains in this group were substantially below control values, particularly over the first 3 weeks. Body weights were highly variable between individuals within groups, with the body weights of the control males at the end of the study ranging from 11.4 kg to 16.0 kg, for example. The body weight of the single heavy animal in the male controls (16 kg) was 3 kg higher than that of the next heaviest dog (13 kg) and significantly influenced the apparent between-group differences. Consequently, intermittent achievement of statistical significance between groups was concluded to reflect the large variation and small group sizes and was not an adverse effect of treatment.

Plasma ALP was increased in both sexes at 300 mg/kg bw per day at all time points throughout the study (e.g. males: 146.5 U/L vs 38.3 U/L in controls; females: 125.5 U/L vs 40.3 U/L in controls, at week 52). The ALP increases were dose related, but the levels fell with increased age. The increased ALP levels at 300 mg/kg bw per day, although potentially reflecting liver pathology, were not associated with increases in other liver enzymes, such as AST, ALT or GGT.

No statistically significant change was observed in organ weights except for an increased trend in liver weight at 300 mg/kg bw per day. The mean liver weights were increased at 300 mg/kg bw per day due primarily to substantially higher liver weights in one male and two females at this dose. Increased liver weights at 300 mg/kg bw per day were not associated with histological alterations.

Increases in absolute (with or without covariate) and relative thyroid weights were observed in both sexes at 300 mg/kg bw per day, with statistical significance in males only, and only the covariate thyroid weight was statistically significantly increased in males at 100 mg/kg bw per day. There were no corresponding histopathological findings in the thyroid in either sex in any treated dose groups. In the absence of any histological alterations, the toxicological relevance of this observation is doubtful.

The results are summarized in Table 15.

Table 15. Summary of findings from the 1-year toxicity study of pydiflumetofen in dogs

	Males				Females			
Parameters	**0 mg/kg bw per day**	**30 mg/kg bw per day**	**100 mg/kg bw per day**	**300 mg/kg bw per day**	**0 mg/kg bw per day**	**30 mg/kg bw per day**	**100 mg/kg bw per day**	**300 mg/kg bw per day**
ALP (U/L)								
Week −1								
Mean	103.8	114.0	105.0	100.0	81.8	101.5	78.8	98.3
SD	17.0	40.9	15.4	12.8	4.6	40.8	23.6	18.9
Week 13								
Mean	56.8	98.8	104.8	200.5**	52.8	71.8	58.5	144.3*
SD	8.8	23.5	51.8	77.2	16.7	11.5	16.0	69.0
Week 26								
Mean	45.3	83.5	109.0	173.5*	43.8	63.8	55.5	133.5*
SD	11.3	19.9	67.8	64.5	18.2	15.4	22.9	70.7
Week 52								
Mean	38.3	66.5	92.3	146.5 **	40.3	65.0	61.3	125.5*
SD	11.2	20.7	54.6	64.5	27.2	9.6	26.6	75.2
Body weight (kg)								
Mean	13.3	10.9	11.8	12.2	10.4	8.6	10.0	9.4
SD	2.0	0.5	2.3	1.1	1.2	0.8	1.2	0.7
Liver weight								
Absolute (g)								
Mean	376.8	369.9	371.7	468.3	313.5	324.2	339.6	376.7
SD	70.4	37.8	54.0	98.7	30.0	92.8	47.0	48.6
Covariate (g)								
Mean	346.1	398.3	377.2	465.0*	297.8	345.2	330.7	380.4
Relative (%)[a]								
Mean	2.848	3.380	3.174	3.843	3.061	3.732	3.421	4.001
SD	0.433	0.202	0.280	0.689	0.456	0.763	0.559	0.471
Thyroid weight								
Absolute (g)								

Parameters	Males				Females			
	0 mg/kg bw per day	30 mg/kg bw per day	100 mg/kg bw per day	300 mg/kg bw per day	0 mg/kg bw per day	30 mg/kg bw per day	100 mg/kg bw per day	300 mg/kg bw per day
Mean	0.988	1.063	1.215	1.385*	0.795	0.660	1.038	1.138
SD	0.158	0.154	0.231	0.174	0.061	0.139	0.471	0.349
Covariate (g)								
Mean	0.897	1.146	1.231*	1.375**	0.863	0.570	1.076	1.122
Relative (%)[a]								
Mean	0.007 5	0.009 7	0.010 3	0.011 4	0.007 8	0.007 7	0.010 9	0.012 0
SD	0.000 9	0.001 5	0.001 2	0.000 8	0.001 2	0.001 5	0.006 6	0.003 4

ALP: alkaline phosphatase; bw: body weight; SD: standard deviation; U: units; *: $P < 0.05$; **: $P < 0.01$
[a] Relative to body weight.
Source: Blunt (2015b)

Consequently, the increased liver weights at 300 mg/kg bw per day are concluded on balance to be adaptive and non-adverse. Increased thyroid weights in the absence of histological alterations are likely to reflect increased turnover of thyroid hormones secondary to induction of metabolism enzymes, reflected in the increased liver weight.

The NOAEL was 300 mg/kg bw per day, the highest dose tested (Blunt, 2015b).

(b) Dermal application

Rats

In a dermal toxicity study, Han Wistar rats (10 of each sex per dose) were clipped across the back; 24 hours later, pydiflumetofen (lot/batch no. SMU2EP12007; purity 98.5%) was applied at a dose of 0, 100, 300 or 1000 mg/kg bw per day (5 days per week for 28 days) under a surgical gauze pad (5 cm × 5 cm) moistened with 0.5 mL of purified water and held in place with a semi-occlusive dressing and restrainer bandage for 6 hours. After 6 hours, the dressing was removed, and the skin was washed with lukewarm water and dried with disposable paper towels. The fur of the animals was clipped as needed, but at least once weekly. In addition to test guideline parameters, a weekly FOB was conducted. Histopathological analysis was conducted in control and high-dose rats.

There were no effects of treatment on mortality, clinical signs, dermal signs, FOB, feed consumption, body weight, urine analysis, organ weights, gross pathology or histopathology.

A minor increase in the incidence of alveolitis was observed in high-dose males (5/10 vs 3/10 in controls), but not females. This was attributed by the study report's author to be due to accidental inhalation of dust and/or test item, which is not unusual in a dermal study. This conclusion would appear to be reasonable, and the report notes that similar lesions, although lower in incidence, were recorded in control animals.

A statistically significantly lower grip strength of the hind legs in females at 1000 mg/kg bw per day was seen in week 4. The finding is considered incidental, as no effect was observed in the forelegs, and the finding was limited to females only. Some differences in locomotor activity were noted at one interval (20–30 minutes in males at 100 mg/kg bw per day and 30–40 minutes in females at 300 or 1000 mg/kg bw per day) in week 4, but there was no dose dependency, the pattern was opposite in males and females, and total locomotor activity over the whole 1-hour interval was not affected.

Potential pydiflumetofen-attributable effects on clinical chemistry parameters were limited to small changes in values, including higher calcium (2.84 mmol/L vs 2.74 mmol/L in controls),

phospholipid (2.06 mmol/L vs 1.65 mmol/L in controls) and total cholesterol (1.00 mmol/L vs 1.56 mmol/L in controls) levels in females at 1000 mg/kg bw per day, and higher globulin (23.36 g/L vs 21.91 g/L in controls) and total protein (69.01 g/L vs 66.76 g/L in controls) levels in males at 1000 mg/kg bw per day. However, as the values were in general within historical control ranges and/or were not associated with any other pathology, these findings were considered incidental to treatment. Similarly, increased relative monocyte counts in females in low-dose (0.019), mid-dose (0.021) and high-dose (0.019) animals compared with controls (0.013) and absolute monocyte counts in high-dose (0.11×10^9/L) females compared with controls (0.06×10^9/L) were within historical control (95% tolerance) ranges (relative monocytes: 0.011–0.024; absolute monocytes: $0.05–0.14 \times 10^9$/L) and therefore were not considered to be toxicologically relevant.

The NOAEL was 1000 mg/kg bw per day, the highest dose tested (Sieber, 2013).

(c) Exposure by inhalation

No studies were submitted.

2.3 Long-term studies of toxicity and carcinogenicity

Mice

A carcinogenicity study was conducted in which 50 male and 50 female CD-1 (ICR) mice per group were administered pydiflumetofen (lot/batch no. SMU2EP12007; purity 98.5%) in the diet at a concentration of 0, 75, 375 or 2250 ppm (equal to 0, 9.2, 45.4 and 287.9 mg/kg bw per day for males and 0, 9.7, 48.4 and 306.2 mg/kg bw per day for females, respectively) for a period of at least 80 weeks.

Animals were monitored for mortality and clinical signs regularly. Body weights and feed consumption were measured and recorded throughout the study. At week 80, prior to terminal kill, blood samples of all surviving animals were withdrawn for haematological examination. Blood smears were obtained from all surviving animals in weeks 52/53 and week 80. However, analysis of blood cell morphology was not performed, because no treatment-related changes were observed in total and differential white blood cell counts or haematological parameters at termination.

The summary of results is shown in Table 16. Mortality in males and females at 375 and 2250 ppm was lower than that of their respective controls, but this was not statistically significant. There were no treatment-related effects on clinical observations or haematological parameters in males or females in any treated groups. Slight (approximately within 10%), but consistent, depression of body weight and reductions of body weight gain were observed in both sexes at 2250 ppm, although only statistically significantly for females at 2250 ppm for the overall treatment period. The reductions were statistically significant in males throughout the study, and the depressions were in males during 5–11 months and in females from 8 months up to termination. These slight reductions of body weight gain were considered treatment related. Feed intakes were comparable in both sexes throughout the study, but feed efficiency was slightly lowered in males at 2250 ppm. Treatment-related increases in absolute liver weight in males and covariate liver weight in both sexes were observed at 2250 ppm. Macroscopically, an increased incidence of liver mass was observed in males at 2250 ppm. Most of the increased mass corresponded to liver tumours in this group, as described below.

In the microscopic examination, a statistically significantly increased incidence of centrilobular hepatocellular hypertrophy was observed in males at 375 ppm and above. The intensity of hypertrophy was enhanced in a dose-dependent manner. Various types of foci of hepatocellular alteration were observed in mice, including controls. Although the focus is known to be a common change in aged mice, the incidence of eosinophilic foci of cellular alteration was increased in males at 2250 ppm, and the increase was considered to be treatment related. The incidences in males at 375 ppm and lower and in females of all treated groups showed no statistically significant differences from those in the control group.

Table 16. Summary of findings from the carcinogenicity study in mice treated with pydiflumetofen

Parameters	Males				Females			
	0 ppm	**75 ppm**	**375 ppm**	**2 250 ppm**	**0 ppm**	**75 ppm**	**375 ppm**	**2 250 ppm**
Mortality[a]	13/50	12/50	10/50	5/50	24/50	18/50	10/50	15/50
(%)	(26)	(24)	(20)	(10)	(48)	(36)	(20)	(30)
Body weight (g)								
Day 1	34.5	34.4	34.4	33.6	25.0	25.0	24.7	24.7
Day 8	36.0	35.5	35.5	34.4**	26.6	26.8	26.2	26.0
Day 15	37.0	36.7	36.6	35.9	27.3	28.1	27.3	27.0
Day 85	43.1	43.6	43.8	41.2	33.2	34.1	32.9	32.1
Day 169	47.3	47.8	47.0	44.3*	36.3	37.4	37.5	35.3
Day 239	50.4	51.2	50.1	46.7*	40.5	41.2	41.4	37.5*
Day 323	52.0	52.8	51.9	47.9*	42.1	44.2	43.5	39.8
Day 407	52.6	54.5	52.8	49.1	45.1	46.6	44.0	40.9*
Day 561	53.6	54.1	52.8	50.2	46.7	46.8	44.0	41.3*
Body weight gain (g)								
Days 1–92	9.1	10.0	9.5	8.1	7.8	9.4*	9.0	7.5
Days 92–183	3.5	3.4	3.2	2.4*	4.0	2.7*	3.6	2.6*
Days 183–281	4.0	4.0	3.5	3.6	4.9	5.4	5.2	4.3
Days 281–379	1.0	2.0	1.5	1.2	1.8	4.5**	1.8	1.5
Days 379–463	0.9	0.2	0.9	1.6	0.3	−0.3	−0.5	−1.1
Days 463–561	1.6	0.4	0.2*	−0.3**	0.4	−0.2	−0.4	−0.2
Days 1–561	19.6	19.6	18.8	16.8	21.4	22.1	19.5	16.2**
Liver weights								
Absolute (g)	2.943	2.974	3.189	4.034**	2.356	2.275	2.337	2.562
Covariate (g)	2.803	2.877	3.144	4.269**	2.272	2.227	2.344	2.660**
Relative (% of body weight)[b]	5.412	5.541	5.925	7.953	5.019	4.924	5.347	6.331
Microscopic findings								
No. of mice examined	50	50	49[c]	50	48[c]	50	50	48[c]
Hepatocellular hypertrophy, centrilobular	0	0	6*	18**	0	0	0	0
Focus of cellular alteration								
Basophilic	0	2	1	0	0	0	0	0
Clear cell	3	2	2	0	0	0	0	0
Eosinophilic	1	4	6	10*	1	0	1	2
Hepatocellular adenoma, including multiple	4	6	9*	22**	0	0	0	1
Hepatocellular adenoma[d]	4	6	2	8	0	0	0	1

Parameters	Males				Females			
	0 ppm	75 ppm	375 ppm	2 250 ppm	0 ppm	75 ppm	375 ppm	2 250 ppm
Hepatocellular adenoma, multiple[e]	0	0	7**	14**	0	0	0	0
Hepatocellular carcinoma[f]	2	3	4	10*	0	0	0	0
Hepatocellular carcinoma, multiple[g]	0	0	0	2	0	0	0	0

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

[a] No. of animals dead or killed when moribund/no. of animals examined.

[b] No statistical analysis was conducted for relative weights of organs.

[c] A reduced number of livers was examined, as postmortem changes precluded examination.

[d] Historical control data on adenoma in males were 45/250 (18.0%), minimum to maximum range 10–28%.

[e] Minimum to maximum range of historical control data on multiple adenoma in males was 5–14 for 250 mice.

[f] Historical control data on carcinoma in males were 7/250 (2.8%), minimum to maximum range 0–6%.

[g] Minimum to maximum range of historical control data on multiple carcinoma in males was 3–5 for 250 mice.

Source: Robertson (2015)

As for neoplastic changes, the incidences of hepatocellular adenomas and carcinomas were statistically significantly (Fisher's exact test) increased in males at 2250 ppm. Multiple hepatocellular adenomas and carcinomas were also statistically significantly increased in this group. Peto analysis showed a significant dose-related trend in the incidence of hepatocellular adenomas and carcinomas when all treated groups were analysed. However, the trend analysis was not statistically significant when the incidence at 2250 ppm was excluded from the analysis. The increased incidences of altered foci, adenomas and carcinomas in the liver in males at 2250 ppm were considered to be treatment related. No statistically significant differences in liver tumours or foci of hepatocellular alteration, which are accepted as a precancerous lesion of hepatocellular adenoma in rodents, were observed in males at 375 ppm, but the incidence of multiple adenomas was statistically significantly increased at this dose, although the combined incidence of single and multiple adenomas was the average (18%) of historical control data. Increased multiplicity of tumours is accepted as one of the promoting effects for carcinogenesis. Therefore, the marginal increase in hepatocellular adenomas and multiple adenomas at 375 ppm was considered to be treatment related. There were no differences in these lesions in females. Other tumour incidences were not affected by the treatment in either sex.

The NOAEL for toxicity was 375 ppm (equal to 45.4 mg/kg bw per day), based on a reduction of body weight gain in males at 2250 ppm (equal to 287.9 mg/kg bw per day). The NOAEL for carcinogenicity was 75 ppm (equal to 9.2 mg/kg bw per day), based on an increased incidence of liver tumours in male mice at 375 ppm (equal to 45.4 mg/kg bw per day) (Robertson, 2015).

Rats

In a combined chronic toxicity and carcinogenicity study, groups of 52 male and 52 female Han Wistar Crl: WI (Han) rats were treated with pydiflumetofen (lot/batch no. SMU2EP12007; purity 98.5%) in the diet at a concentration of 0, 200, 1000 or 6000 ppm (equal to 0, 9.9, 51.0 and 319 mg/kg bw per day, respectively) for males and 0, 150, 450 or 1500 ppm (equal to 0, 10.2, 31.0 and 102 mg/kg bw per day, respectively) for females for 104 weeks for the carcinogenicity study. For the chronic toxicity study, a further four groups of 12 males and 12 females were included and dosed for 52 weeks at the same dietary concentrations as for the carcinogenicity study.

Animals were monitored regularly for viability and for clinical signs. Body weights and feed consumption were measured and recorded at predetermined intervals from pretrial up until the completion of treatment. Prior to the terminal kill, blood samples were collected from all surviving animals for haematological analysis. Blood smears and clinical pathology samples were obtained from animals in the chronic toxicity study group at weeks 14, 27 and 52. Blood films were made from all

survivors in the carcinogenicity study group; however, blood cell morphology was not performed, because no treatment-related effects on haematological parameters were found at termination. All animals were necropsied and subjected to histopathological examination. Qualitative or quantitative functional observations and motor activity were examined as part of the neurological examination in rats of the chronic toxicity group.

The summary of findings from the combined chronic toxicity and carcinogenicity study in rats is shown in Table 17.

Table 17. Summary of findings in the chronic toxicity and carcinogenicity study of pydiflumetofen in rats

	Males				**Females**			
Parameter	**0 ppm**	**200 ppm**	**1 000 ppm**	**6 000 ppm**	**0 ppm**	**150 ppm**	**450 ppm**	**1 500 ppm**
Mortality								
Toxicity study	1/12	0/12	0/12	0/12	1/12	0/12	1/12	2/12
Carcinogenicity study	14/52	23/52	7/52	10/52	23/52	13/52	10/52	16/52
Combined (%)	23	36	11	16	38	20	17	28
Body weight gain (g) (combined[a])								
Days 1–92	266	268	245**	213**	117.4	116.5	111.5	106.5**
Days 92–190	73	75	63**	62**	24.1	21.8	21.0	18.5**
Days 190–274	44	41	41	43	13.6	16.2	12.6	11.7
Days 274–358	41	44	36	30**	22.7	20.8	15.9*	11.0**
Days 358–442	36	35	30	28**	26.1	19.9	22.2	18.5
Days 442–526	28	20	23	19	20.6	26.2	17.2	17.9
Days 526–610	23	11	16	11	23.5	27.2	23.7	14.2
Days 610–722	32	10*	23	22	25.4	28.2	22.8	34.1
Days 1–722	550	513	476**	429**	267.5	273.8	241.2	230.8*
Body weight (g) (combined[a])								
Day 1	153	152	154	148*	129	128	125*	128
Day 29	295	299	282**	256**	194	195	188*	186**
Day 64	382	381	362**	324**	230	231	223*	220**
Day 120	443	448	420*	383**	252	254	243*	240**
Day 232	513	517	484**	448**	277	275	263**	259**
Day 358	573	578	538**	496**	307	303	285**	276**
Day 484	633	628	576**	535**	345	339	314**	305**
Day 610	671	645	600**	554**	372	375	341*	324**
Day 722	704	664	628**	576**	395	402	365	359*
Feed utilization (g body weight gain/100 g feed consumed) (combined)								
Weeks 1–4	22.3	22.9	20.7**	18.8**	14.2	14.6	14.4	13.7
Weeks 5–8	11.6	11.0	11.6	10.5**	6.1	6.0	6.4	6.3
Weeks 1–13	13.0	13.2	12.4*	11.6**	7.3	7.2	7.2	6.9

Parameter	Males 0 ppm	Males 200 ppm	Males 1 000 ppm	Males 6 000 ppm	Females 0 ppm	Females 150 ppm	Females 450 ppm	Females 1 500 ppm
Blood biochemistry								
GGT (µmol/L)								
Week 14	2.00	2.00	2.00	7.08**	2.18	2.25	2.09	2.17
Week 27	2.00	2.00	2.00	8.08**	2.00	2.00	2.00	2.09
Week 52	2	2	2	11**	2	2	2	2
Liver weight (g, absolute, covariance analysis)								
Chronic toxicity study	15.90	15.28	18.40	21.56**	9.011	9.141	9.785	10.38*
Carcinogenicity study	17.93	19.53	20.01*	22.17**	10.78	11.02	11.59	11.87*
Histopathology								
Liver								
Hepatocellular hypertrophy								
Chronic toxicity study	0/11[b]	0/12	5/12*	11/12**	0/11	0/12	0/11	4/10
Carcinogenicity study	0/52	0/51	3/52	39/52**	0/52	0/52	0/51	3/52
Eosinophilic inclusions	0/52	0/52	0/52	19/52	0/52	0/52	0/52	0/52
Thyroid								
Hyperplasia								
Total	4/52	3/50	3/50	7/52	1/51	3/52	0/51	3/51
Follicular cell, focal	2/52	1/50	1/50	4/52	0/51	1/52	0/51	0/51
Follicular cell, cystic focal	2/52	2/50	2/50	3/52	1/51	2/52	0/51	3/51
Follicular cell adenoma	8/52	3/50	4/50	5/52	1/51	0/52	0/51	3/51
Lymph node								
Erythrophagocytosis, mesenteric								
Chronic toxicity study	1/12	0/12	1/12	7/12*	1/12	1/12	0/12	0/12
Carcinogenicity study	7/35	11/30	16/30	8/37	8/41	7/41	7/34	5/39

GGT: gamma-glutamyl transferase; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$

[a] Twelve and 52 animals were allocated for the chronic toxicity study and the carcinogenicity study, respectively.

[b] Indicating an incidence of finding (X) per the number of animals examined (Y) as X/Y.

Source: Robertson (2016)

No treatment-related changes in clinical observations or increased mortality were observed during the treatment period. Lower body weights and reduced body weight gains were consistently noted throughout the study in both sexes at the two highest doses. These changes were dose dependent, and body weight gains were reduced by more than 10% and 20% at 1000 and 6000 ppm in males, respectively, compared with control animals. In females, the reductions in body weight gain were less than 10% for the 450 ppm treatment group and greater than 10% for the 1500 ppm group, compared with control animals. Slight, but consistent, decreases in feed consumption were observed in males (within a 10% decrease) and females (within a 5% decrease) at the highest doses (males: 6000 ppm;

females: 1500 ppm). Treatment-related lower feed consumption was observed in males at 1000 ppm and above in a dose-dependent manner.

There were no consistent treatment-related findings associated with ophthalmoscopy, neurotoxicity, urine analysis or haematology in either sex. In blood biochemistry, increases in GGT in males at weeks 14, 27 and 52, decreases in glucose and triglycerides in males at week 52, and decreases in glucose in females at week 14 were found at the high doses. The individual GGT values in males at 6000 ppm were more than 3 µmol/L, the upper LOQ at all time points, whereas the GGT values in males of the other treated groups and the control group were less than 3 µmol/L. Most of the GGT values were 2 µmol/L, the lower LOQ in the GGT assay. No other hepatotoxicity was observed at 6000 ppm in either the chronic toxicity or the carcinogenicity study. However, the consistent increase in GGT was considered to be an adverse effect on the liver. The decreases in glucose or triglycerides in males or females at the high doses were considered to reflect the consistently lower body weights at these doses.

The pathological analysis identified increased liver weights and an increased incidence of hepatocellular hypertrophy in males at 1000 ppm and above and in females at 1500 ppm (the highest dose tested in females) in both the chronic toxicity and carcinogenicity studies. The incidence and severity of the hypertrophy increased with dose and in males at 6000 ppm were associated with cytoplasmic eosinophilic inclusions. Although liver hypertrophy was consistently observed with treatment, precancerous lesions or tumours in the liver were not increased by the treatment. The toxicological relevance of eosinophilic inclusions in hepatocytes was not determined, but the change was considered to be treatment related. In the thyroid, there was a slight numerical increase in the incidence of follicular cell adenomas in females at 1500 ppm, which did not reach statistical significance on pairwise comparison or show a dose-related trend (Peto $P = 0.16$). The incidence of preneoplastic or related findings, focal follicular cell hyperplasia or follicular cell hypertrophy, was not increased in this group. In addition, the incidence at 1500 ppm was within the historical control range (mean 2.2%, range 0–10% in 1276 rats). Therefore, the increase in follicular cell adenoma in females was not considered to be treatment related. The increased incidence of erythrophagocytosis in mesenteric lymph nodes in males at 6000 ppm in the chronic toxicity group was considered incidental, as there was no increase in this lesion in the carcinogenicity study. Although various histopathological findings were found in this study, there were no treatment-related changes other than those described above.

The liver hypertrophy found in males at 1000 ppm was considered to be adaptive but not adverse because there was no indication of hepatotoxicity.

The NOAEL for toxicity was 200 ppm (equal to 9.9 mg/kg bw per day), based on lower body weight and reduced body weight gain in males at 1000 ppm (equal to 51.0 mg/kg bw per day). No carcinogenicity was observed in rats in this study (Robertson, 2016).

2.4 Genotoxicity

The genotoxicity of pydiflumetofen and its individual isomers was examined in an adequate range of in vitro and in vivo studies (Table 18). Select studies are described briefly below.

(a) In vitro studies

Chromosomal aberration test using human lymphocytes

Three independent experiments were performed. In experiment I, the exposure was 4 hours with and without metabolic activation (S9 mix). In experiment IIA, the exposures were 4 hours with S9 mix and 22 hours without S9 mix. In experiment IIB, the exposure period was 22 hours without S9 mix. The chromosomes were prepared 22 hours after the start of treatment with the test substance.

Precipitation of the test material was noted at the end of treatment at and above 28.1 µg/mL without S9 mix, at and above 49 µg/mL with S9 mix (experiment I), at and above 16.1 µg/mL with and without S9 mix (experiment IIA) and at and above 20.0 µg/mL without S9 mix (experiment IIB).

Table 18. Results of genotoxicity assays on pydiflumetofen

Test system	Test object	Concentration/ dose	Purity	Results		Reference
				Without S9	With S9	
In vitro						
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537; *Escherichia coli* WP2*uvr*A/pKM101, WP2pKM101	5 000 µg/plate	98.5	Negative[a]	Negative[b]	Sokolowski (2012)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537; *E. coli* WP2*uvr*A/pKM101, WP2pKM101	5 000 µg/plate	96.7	Negative[a]	Negative[a]	Sokolowski (2014a)
Chromosomal aberration, mammalian	Human lymphocytes	150.8 µg/mL −S9 4 332 µg/mL +S9	98.5	Positive[c]	Negative[d]	Bohnenberger (2013a)
Point mutation (*tk* locus) assay	Mouse lymphoma L5178Y cells	60 µg/mL −S9 110 µg/mL +S9	98.5	Negative	Negative	Wollny (2013a)
In vivo						
Micronucleus induction	Mouse bone marrow	2 000 mg/kg bw (ip)	98.5	Negative	NA	Roth (2012)
Micronucleus induction	Mouse bone marrow	2000 mg/kg bw (ip)	96.7	Negative	NA	Dony (2014a)

bw: body weight; ip: intraperitoneal; NA: not applicable; S9: 9000 × *g* supernatant fraction from rat liver homogenate; *tk*: thymidine kinase

[a] Precipitation observed at and above 1000 µg/plate.

[b] Precipitation observed at and above 333 µg/plate.

[c] Precipitation observed at and above 20.0 µg/mL (22-hour exposure) and at and above 28.1 µg/mL (4-hour exposure).

[d] Precipitation observed at and above 16.1 µg/mL (4-hour exposure).

No clastogenicity was observed in the presence of S9 mix at concentrations up to 4332 µg/mL. In the absence of S9 mix, clastogenicity was not observed after a 4-hour exposure, but was observed after a 22-hour exposure. In experiment IIA in the absence of S9 mix, one statistically significant increase (6.5% aberrant cells, excluding gaps) above the range of the historical solvent control data (0.0–3.0% aberrant cells, excluding gaps) was observed after treatment with 5.3 µg/mL, but without a dose–response relationship. In the confirmatory experiment IIB, statistically significant increases occurred after treatment with 20.0 and 40.0 mg/mL (7.5% and 9.5% aberrant cells, excluding gaps), clearly exceeding the laboratory historical solvent control range of 0.0–3.0% and showing a dose–response relationship. Concordant with the test facility and applicant analysis, pydiflumetofen was found to induce structural chromosomal aberrations in human lymphocytes in vitro in the absence of metabolic activation.

(b) In vivo studies

Two mouse bone marrow micronucleus tests were conducted, one of which was on the same batch of material (lot/batch no. SMU2EP12007) that gave a positive response in the in vitro chromosomal aberration test. The second study was conducted on a batch of material spiked with potential impurities (lot/batch no. SMU4FL762) to support the technical specification. Both in vivo micronucleus tests gave negative results. The in vivo mouse bone marrow micronucleus tests did not include an assessment of bone marrow exposure to pydiflumetofen; however, clinical signs observed in both in vivo studies were indicative of systemic absorption of the test material. In addition, a high volume of distribution was observed in pharmacokinetics of a single intravenous treatment with pydiflumetofen in mice (Punler & Harris, 2014a). Therefore, bone marrow will have been exposed to pydiflumetofen in the in vivo mouse micronucleus studies.

Overall, the positive in vitro clastogenicity finding for pydiflumetofen did not correspond to genotoxicity in vivo.

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

A two-generation reproductive toxicity study was conducted in which Han Wistar rats (24 of each sex per dose) were administered pydiflumetofen (lot/batch no. SMU2EP12007; purity 98.5%) in the diet at a concentration of 0, 150, 750 or 4500 ppm for males and 0, 150, 450 or 1500 ppm for females (in the F_0 generation: pre-pairing, equal to 0, 9.1, 46.1 and 277 mg/kg bw per day for males and 0, 11.9, 36.1 and 116 mg/kg bw per day for females, respectively; during gestation, equal to 0, 12.6, 33.7 and 115 mg/kg bw per day for females, respectively; during lactation, equal to 0, 30.0, 87.0 and 291 mg/kg bw per day for females, respectively; in the F_1 generation: pre-pairing, equal to 0, 11.9, 59.1 and 364 mg/kg bw per day for males and 0, 14.1, 42.4 and 141 mg/kg bw per day for females, respectively; during gestation, equal to 0, 15.1, 39.5 and 141 mg/kg bw per day for females, respectively; and during lactation, equal to 0, 26.3, 77.8 and 267 mg/kg bw per day for females, respectively). Doses were selected based on the results of toxicokinetics studies, to be within the linear range for the dose–blood level relationship. Animals were treated for at least 10 weeks prior to pairing for the parental (F_0) generation from birth until pairing for mating for the F_1 generation. In addition to the test guideline parameters, the number of follicles in the ovaries was counted in the control and high-dose females, and sperm motility and concentration were assessed for all males killed at terminal necropsy.

In the F_0 generation, there were no treatment-related effects on mortality, clinical signs, gross pathology, reproductive performance, mating behaviour, conception, sperm parameters, pup survival or pup development. In the male parents at 4500 ppm, body weight gains were reduced during the first 5 weeks. Females at 1500 ppm had slightly longer estrous cycles (3.93 ± 0.10 days at 0 ppm vs 4.05 ± 0.24 days at 1500 ppm); however, the finding was not repeated in the F_1 generation and was within the historical control range (in four studies during 2009–2014, ranges of estrous cycles were 4, 3.8–4.5, 4.0–6.0 and 4.0–6.3 days per 23–25 females), so the observations were considered likely to be incidental.

Females at 1500 ppm had a slightly and statistically significantly shorter gestation length than controls (22.04 days compared with 22.29 days in controls; $P < 0.05$). However, this was incidental owing to a single animal delivering at 21.0 days, with all others delivering between 22.0 and 22.5 days, which is within the range of the concurrent control animals (days 21.5–23 of gestation). All pregnant females gave birth to live litters. Absolute and relative (to body weight) liver weights were increased in males at the intermediate and high doses, and the incidence of hepatocellular hypertrophy was increased in both sexes at the high dose. A statistically significant increase in thyroid weight and a corresponding increased incidence of minimal thyroid follicular cell hypertrophy were observed in males at 4500 ppm. The liver hypertrophy without findings related to liver damage or thyroid effects was considered adaptive.

In the F_1 generation, there were no effects of treatment on mortality, clinical signs, estrous cycle, fertility, mating performance, gestation length, postnatal survival, live births, sex ratio, sperm parameters or ovarian follicle counts. At weaning, the mean body weight of males at 4500 ppm was lower than the control value; body weight gains remained below control values until necropsy, and feed consumption was commensurately lower. At weaning, females at 1500 ppm also had lower body weights than controls, but body weight gains and feed consumption were comparable to those of controls thereafter. Although all animals delivered litters between days 21.0 and 23.5 of gestation, at 1500 ppm, mean gestation length was shorter; this was considered to be an artefact of comparison with a concurrent control range that was unusually narrow (22.0–23.0 days). Historical control data provided showed a gestation length range of 22–24 days. Absolute and relative liver weights were increased in males at 750 ppm and above and were associated with hepatocellular hypertrophy at 4500 ppm. Relative liver weights were elevated in females at 450 and 1500 ppm, but there were no histological correlates. Absolute thyroid weights were higher in all treated male groups; however, thyroid weights were within the normal range (0.012–0.032 g), with the exception of one male at 750 ppm. The observation was therefore concluded to be potentially incidental, but is consistent with the observations in the F_0 generation. There were no treatment-related effects on mortality, clinical signs, anogenital distance, body weights or gross pathology in litters from the F_1 generation.

There were no treatment-related changes in F_2 generation pups.

The results of the multigeneration study of pydiflumetofen in rats are summarized in Table 19.

Table 19. Summary of findings from the multigeneration reproductive toxicity study of pydiflumetofen in rats

Parameters / generation	Males				Females			
	0 ppm	**150 ppm**	**750 ppm**	**4 500 ppm**	**0 ppm**	**150 ppm**	**450 ppm**	**1 500 ppm**
Body weight gain (g)								
F_0 generation								
0–17 weeks	214.0	223.2	215.0	192.7*	–	–	–	–
0–10 weeks (pre-pairing)	–	–	–	–	96.2	87.4*	91.8	91.3
0–20 weeks (gestation)	–	–	–	–	107.3	112.1	106.5	103.2
1–21 weeks (lactation)	–	–	–	–	31.1	30.6	32.2	36.3
F_1 generation								
0–17 weeks	364.3	367.2	354.5	328.6**	–	–	–	–
0–10 weeks (pre-pairing)	–	–	–	–	157.7	159.8	161.8	157.4
0–20 weeks (gestation)	–	–	–	–	101.4	99.7	105.5	99.0
1–21 weeks (lactation)	–	–	–	–	33.3	30.7	33.9	36.6
Body weight (g)[a]								
F_1 generation before weaning								
Lactation day 1	–[a]	–[a]	–[a]	–[a]	6.69	6.64	6.72	6.45
Lactation day 4	–[a]	–[a]	–[a]	–[a]	10.17	9.98	10.30	9.27
Lactation day 7	–[a]	–[a]	–[a]	–[a]	16.83	15.99	16.50	14.89**
Lactation day 14	–[a]	–[a]	–[a]	–[a]	33.97	32.75	33.11	29.91**
Lactation day 21	–[a]	–[a]	–[a]	–[a]	51.63	50.63	50.28	46.29**

Parameters / generation	Males				Females			
	0 ppm	150 ppm	750 ppm	4 500 ppm	0 ppm	150 ppm	450 ppm	1 500 ppm
F_2 generation before weaning								
Lactation day 1	– [a]	– [a]	– [a]	– [a]	6.23	6.45	6.60	6.35
Lactation day 4	– [a]	– [a]	– [a]	– [a]	8.16	7.81	8.76	8.05
Lactation day 7	– [a]	– [a]	– [a]	– [a]	12.60	12.32	13.47	12.95
Lactation day 14	– [a]	– [a]	– [a]	– [a]	30.32	29.58	30.93	29.33
Lactation day 21	– [a]	– [a]	– [a]	– [a]	48.94	47.27	49.60	47.32
Hepatocyte hypertrophy (minimal to slight)[b]								
F_0 generation	0/24	0/24	0/24	19/24	0/24	0/24	0/24	8/24
F_1 generation	0/24	0/24	0/24	18/24	0/24	0/24	0/24	0/24
Follicular hypertrophy in thyroid (minimal)[b]								
F_0 generation	1/24	1/24	2/24	7/24	0/24	0/24	0/24	0/24
F_1 generation	2/24	0/24	1/24	7/24	0/24	0/24	0/24	0/24

F_0: parental generation; F_1: first filial generation; F_2; second filial generation; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$

[a] Body weights given under the “Females” heading are mean values for males and females combined.

[b] The number of animals with the finding/the number of rats examined.

Source: Hackford (2015)

The NOAEL for parental toxicity was 750 ppm (equal to 46.1 mg/kg bw per day), based on reduced body weight and effects on the thyroid in males at 4500 ppm (equal to 277 mg/kg bw per day). The NOAEL for reproductive toxicity was 1500 ppm (equal to 115 mg/kg bw per day), the highest dose tested. The NOAEL for offspring toxicity was 450 ppm (equal to 33.7 mg/kg bw per day), based on reduced pup body weight before weaning in the F_1 generation at 1500 ppm (equal to 115 mg/kg bw per day) (Hackford, 2015).

(b) Developmental toxicity

Rats

In a dose range–finding study of developmental toxicity in rats, mated female Crl:CD (SD) rats (six per dose) were administered pydiflumetofen (lot/batch no. 2491-DC/110; purity 98.6%) in 1% aqueous carboxymethylcellulose by oral gavage at a dose of 0, 100, 200, 500 or 1000 mg/kg bw per day from GD 6 to GD 19, then terminated on day 20 for caesarean section and examination of the fetuses. Mortality, body weight, clinical signs and feed consumption were monitored regularly during the study. At termination, gravid uterus weights were measured, and the fetuses were examined for viability, visceral and skeletal malformations and variations, weighed and sexed. Preimplantation and postimplantation losses were calculated.

There were no effects on mortality, clinical signs, maternal gross pathology, implantation loss, number of live fetuses, sex ratio, fetal weights, gravid uterus weights or the incidence of malformations or variations. Body weight gains were slightly reduced at 500 mg/kg bw per day (control: 6 g; 500 mg/kg bw per day: 0.2 g). Body weight loss was observed at 1000 mg/kg bw per day (3 g) between days 6 and 7, but body weights were comparable to control values by the end of the study.

There were no treatment-related fetal abnormalities in any group. Variations were similar in incidence across all dose groups (Manton, 2011).

A developmental toxicity study was conducted in which mated female Sprague Dawley rats (24 per dose) were administered pydiflumetofen (lot/batch no. SMU2EP12007; purity 98.5%) in 1% aqueous carboxymethylcellulose by oral gavage at a dose of 0, 10, 30 or 100 mg/kg bw per day from GD 6 to GD 19. On day 20, following a caesarean section, all fetuses were examined.

There were two unscheduled deaths, with both rats being terminated prematurely because of clinical signs of morbidity: one dam at 10 mg/kg bw per day as a result of dosing injury on GD 15, and one dam at 100 mg/kg bw per day from unknown causes on GD 6 after the first dose. The dam at 100 mg/kg bw per day had clinical signs of decreased activity, cold body surface, unsteady and abnormal gait, hunched posture, partially closed eyes and piloerection, but necropsy was unable to identify any macroscopic changes indicating the causes of these signs. Given the absence of similar mortality in other rat studies at similar or higher doses, neither death is considered to be toxicologically relevant.

There were no effects of treatment on mortality, clinical signs, terminal body weights and body weight gains, gravid uterus weights, feed consumption, maternal gross pathology, implantations and implantation loss, and fetal, litter or placental weights. Body weight gains were reduced at 100 mg/kg bw per day between days 6 and 10, associated with a slightly lower feed intake, but returned to control values from day 11. Major fetal abnormalities were noted in one fetus each at 0 and 10 mg/kg bw per day, two fetuses from two litters at 30 mg/kg bw per day and two fetuses from the same dam at 100 mg/kg bw per day.

The summary of findings from the developmental toxicity study in rats is shown in Table 20.

Table 20. Summary of findings from the developmental toxicity study of pydiflumetofen in rats

Parameters	0 mg/kg bw per day	10 mg/kg bw per day	30 mg/kg bw per day	100 mg/kg bw per day
Body weight gain (g), F_0 generation				
GDs 0–6				
Mean	27.6	30.0	27.0	28.1
SD	7.8	6.8	6.7	6.2
GDs 6–7				
Mean	6.1	3.5	3.4	0.5**
SD	3.5	4.0	4.3	6.5
GDs 6–10				
Mean	24.1	22.1	22.8	19.7*
SD	4.8	5.3	5.2	6.9
GDs 6–15				
Mean	59.5	59.5	59.9	57.0
SD	6.4	7.8	7.9	10.7
GDs 6–20				
Mean	132.8	135.7	132.3	128.4
SD	14.3	14.4	14.7	19.3
Feed consumption (g/rat per day), F_0 generation				
GDs 6–9				
Mean	26.5	26.5	26.1	24.1*

Parameters	0 mg/kg bw per day	10 mg/kg bw per day	30 mg/kg bw per day	100 mg/kg bw per day
SD	2.3	3.8	3.0	3.0
GDs 9–12				
Mean	27.6	27.7	28.0	26.8
SD	2.2	2.7	3.1	3.5
GDs 6–20				
Mean	28.7	29.5	29.2	28.2
SD	2.11	2.21	2.68	2.91
Identification number of dams and fetuses with fetal abnormalities and their findings				
Diaphragmatic hernia	16/L5[a]	–	–	–
Multiply malformed fetus	–	48/L8	–	–
Anophthalmia; orbital cavity reduced in size; malformed cervical neural arch; absent cervical neural arches; bent scapula	–	–	66/L4	–
Scapulae severely bent; humeri malformed; femurs bowed	–	–	69/L12	–
Exencephaly; open eye; cleft palate; malformed interparietals, parietals, frontals and nasals; cleft palatine	–	–	–	82/L1
Exencephaly; open eye; malformed palate; malformed parietals and frontals; absent interparietals; cleft palatine	–	–	–	82/R3

GD: gestation day; SD: standard deviation; *: $P < 0.05$; **: $P < 0.01$

[a] Dam identification number/fetus identification number.

Source: Davies (2015)

Historical control data from Charles River Laboratories, which supplied the animals for the current study, demonstrated the sporadic occurrence of various abnormalities, such as those observed in this study (Barnes, 2015; Davies, 2016). The single severely malformed fetus at 10 mg/kg bw per day was concluded to be incidental, given the lack of similarly affected fetuses at 30 and 100 mg/kg bw per day. The two affected fetuses at 30 mg/kg bw per day had different malformations that were not observed at 100 mg/kg bw per day and again are concluded to be incidental.

The sponsor argued that the exencephaly in two fetuses from the same litter at 100 mg/kg bw per day was incidental based on historical control incidences at the testing laboratory (and other Charles River facilities) and the lack of statistical significance. The historical control data provided for exencephaly indicated a very low frequency of occurrence and only one fetus per litter affected in each case. Exencephaly is known to occur spontaneously at low litter incidences, and it has previously been reported to occur not uncommonly, with more than one fetus per litter affected (Lang, 1993); the effect is confined to a single litter in the current study. On this basis, although the finding is notable, the incidence at 100 mg/kg bw per day was concluded to be likely to be incidental. The absence of similar findings at 1000 mg/kg bw per day in the dose range–finding study is supportive of this conclusion.

The NOAEL for maternal toxicity was 30 mg/kg bw per day, based on the reduction in body weight gain and lower feed consumption in dams at the beginning of treatment at 100 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 100 mg/kg bw per day, the highest dose tested (Davies, 2015).

Rabbits

In a non-GLP-compliant dose range–finding developmental toxicity study, mated female New Zealand white rabbits (10 per dose) were administered pydiflumetofen (lot/batch no. 2637-AA/110, SMU2EP; purity 98.5–99.3%) by oral gavage at a dose of 0, 250, 500 or 1000 mg/kg bw per day from GD 6 to GD 27 at a dosing volume of 5 mL/kg bw. Additional groups of mated rabbits at 0 and 1000 mg/kg bw per day (10 per dose) were added to the study when it became apparent that only half the does were pregnant in the initial 1000 mg/kg bw per day group. Animals were checked daily for mortality and clinical signs of toxicity and weighed. Feed consumption was measured. At necropsy of the dams on GD 28, fetuses were examined.

There were no effects of treatment on feed consumption, gross pathology, clinical signs, placental or gravid uterus weights, fetal weights, sex ratio, and preimplantation or postimplantation losses. A single animal at 1000 mg/kg bw per day was found dead on GD 12, with dark lungs, pale kidneys, an accentuated lobular pattern of the liver and cream material in the abdominal cavity found at necropsy. Given the absence of clinical signs or gross pathology in other dams at this dose, this death was concluded to be a treatment-related technical error. Pregnancy was confirmed in 10, nine, nine and five does at 0, 250, 500 and 1000 mg/kg bw per day in the initial dose groups, respectively. In the additional dose groups, pregnancy was confirmed in nine and eight does at 0 and 1000 mg/kg bw per day, respectively. Although body weight gains in the first 1000 mg/kg bw per day group were unaffected, body weight gain during GDs 6–28 in the second 1000 mg/kg bw per day group was approximately 30% lower than that in the concurrent control group. There were no treatment-related adverse effects on the fetuses (Penn, 2015b).

In a developmental toxicity study, mated female New Zealand white rabbits (24 per dose) were administered pydiflumetofen (lot/batch no. SMU2EP12007; purity 98.5%) in 1% aqueous carboxymethylcellulose by oral gavage at a dose of 0, 10, 100 or 500 mg/kg bw per day from GD 6 to GD 27 at a dosing volume of 5 mL/kg bw. Blood samples (0.1 mL) were taken from six animals per group on GD 27 at 2, 6 and 12 hours post-dosing for analysis of toxicokinetics. Terminal blood samples (GD 28) were taken immediately after each animal was killed.

There were no effects of treatment on mortality, clinical signs, body weight, body weight gain, feed consumption, maternal gross necropsy, preimplantation or postimplantation losses, live fetuses, sex ratio or fetal, litter or placental weights. There were two early deaths during the study, one at 100 mg/kg bw per day on day 12 as a result of dosing trauma and another at 500 mg/kg bw per day, with termination on day 19 after observed signs of abortion. Both deaths were considered incidental to treatment.

Major fetal abnormalities were observed in all groups, including controls: in five fetuses from three litters in the control group, four fetuses from three litters in the group given 10 mg/kg bw per day, three fetuses from three litters in the group given 100 mg/kg bw per day and two fetuses from two litters in the group given 500 mg/kg bw per day.

Historical control data were supplied for this laboratory and for this strain and source of rabbit. With the exception of diaphragmatic hernia, these major abnormalities have been noted at low incidence in this strain of rabbit in the conducting laboratory. Although diaphragmatic hernia has not been seen within the background data for this strain of rabbit from this supplier, it has been shown to occur spontaneously in rabbits of this strain from other suppliers, and the study report includes information indicating a low level of spontaneous occurrence. The single instance of diaphragmatic hernia at 500 mg/kg bw per day is concluded to be incidental, and this conclusion is supported by the results of the dose range–finding study, in which similar or related abnormalities were not observed at 1000 mg/kg bw per day. Because the nature and intergroup distribution of the fetal abnormalities are consistent with the historical control ranges, they were concluded to be incidental.

The toxicokinetics of pydiflumetofen was characterized by a highly sub-dose-proportional increase in peak concentration and total systemic exposure across the 50-fold dose range. Mean AUC

increased only 4.2-fold across this dose range, and toxicokinetic parameters varied markedly across individuals in each dose group, with a within-group variation of 6- or 7-fold between individuals. As a result, there was considerable overlap in values across dose groups, with individuals in the 10 mg/kg bw per day group achieving AUCs comparable to or higher than those of individuals in the 100 mg/kg bw per day group.

The NOAEL for maternal and embryo/fetal toxicity was 500 mg/kg bw per day, the highest dose tested (Penn, 2015c).

2.6 Special studies

(a) Neurotoxicity

Acute neurotoxicity

In an acute neurotoxicity study, Han Wistar rats (10 of each sex per dose) were administered pydiflumetofen (lot/batch no. SMU2EP12007; purity 98.5%) in aqueous 1% carboxymethylcellulose by oral gavage as a single dose of 0, 300, 1000 or 2000 mg/kg bw for males and 0, 100, 1000 or 2000 mg/kg bw for females. Parameters to investigate neurotoxicity included a FOB, quantitative assessment of landing foot splay, body temperature, grip strength and locomotor activity. Fifteen days after treatment (i.e. day 16), the animals were necropsied. Nervous system tissues were fixed by perfusion in situ and were examined for histopathological changes in the control and high-dose groups only.

For males, treatment-related findings were limited to slight decreases in body weight gain over the first day at 1000 and 2000 mg/kg bw. Various clinical signs of toxicity were confined to the females, mainly at 1000 and 2000 mg/kg bw (Table 21). The slightly increased severity grade for piloerection at 1000 mg/kg bw and above might be associated with a worsened general condition after dosing. The statistically significant decrease in body temperature at 1000 mg/kg bw was considered a treatment-related adverse effect.

Table 21. Clinical observations and body temperature in female rats treated with pydiflumetofen in an acute neurotoxicity study

Parameter	0 mg/kg bw	100 mg/kg bw	1 000 mg/kg bw	2 000 mg/kg bw
Clinical observations (maximum severity grade)				
Recumbency (1)	0	0	1/9 (1.0)	0
Hunched posture (1)	0	1/10 (1.0)	0	2/10 (1.0)
Piloerection (2)	2/10 (1.0)	3/10 (1.0)	4/9 (1.3)	4/10 (1.3)
Vocalization (2)	3/10 (1.0)	0	1/9 (1.0)	2/10 (1.0)
Reduced muscle tone (1)	0	0	1/9 (1.0)	0
Reduced activity (2)	0	1/10 (1.0)	2/9 (1.5)	1/10 (1.0)
Decreased rearing (1)	2/10 (1.0)	1/10 (1.0)	2/9 (1.0)	1/10 (1.0)
Abnormal gait (2)	0	0	1/9 (1.0)	1/10 (1.0)
Skin cold on touch (1)	0	0	1/9 (1.0)	0
Pupillary reflex impaired (1)	0	0	1/9 (1.0)	0
Mydriasis (1)	0	0	1/9 (1.0)	0
Body temperature at 6 hours post-dosing on day 1				
Mean body temperature (°C) ± SD (% change from control)	38.2 ± 0.7 (100)	37.9 ± 0.5 (−0.8)	37.2 ± 1.3 (−2.6)	37.0 ± 1.3* (−3.1)

Parameter	0 mg/kg bw	100 mg/kg bw	1 000 mg/kg bw	2 000 mg/kg bw
Locomotor activity over a 30-minute session at 6 hours post-dosing on day 1				
Total distance (cm)	2 581	1 767	1 346**	1 060
Total centre time (s)	129	82	101	34
Total number of rearings	47	25	16**	9**

bw: body weight; SD: standard deviation; *: $P < 0.05$; **: $P < 0.01$
Source: Broich (2015a)

Locomotor activity was statistically significantly reduced in females at 1000 and 2000 mg/kg bw 6 hours after dosing on day 1. Male values were within the historical control range and not statistically significantly different from control values.

There were no treatment-related effects on feed consumption, organ weights, gross pathology or histopathology in either sex. The findings (reduced locomotor activity) in females at 1000 mg/kg bw were considered treatment related and of a general toxicity nature; however, there was no clear evidence indicating neurotoxicity in these findings.

The NOAEL for systemic toxicity was 100 mg/kg bw, based on altered locomotor activity, clinical signs and reduced body temperature in females at 1000 mg/kg bw. The NOAEL for neurotoxicity was 2000 mg/kg bw per day, the highest dose tested (Broich, 2015a).

A follow-up acute neurotoxicity study was conducted using female rats only. Female Han Wistar rats (10 per dose) were administered a single dose of pydiflumetofen (lot/batch no. SMU2EP12007; purity 98.5%) in aqueous 1% carboxymethylcellulose by oral gavage at a dose of 0, 100, 300 or 1000 mg/kg bw and observed for 8 days. Animals were observed for clinical signs and mortality at least daily. Body weights and feed consumption were measured daily. The FOB was conducted after 6 hours on day 1 and on day 8, including detailed clinical observation and tests for reflexes, hearing, grip strength, landing foot splay, body temperature and locomotor activity. The animals were necropsied for gross pathology. As the previous full study had not identified effects on organ weights or histopathology, these parameters were not assessed in this study.

There were no treatment-related effects on mortality, grip strength, foot splay, body weight, feed consumption or gross pathology. Detailed clinical examination on the day of dosing (as part of the FOB) identified a number of effects across all groups (e.g. vocalization, reduced activity, decreased rearing and tremor). There were no definitive dose–response relationships for any of the parameters assessed, but some of the signs in treated animals did correlate with findings in other stages of the FOB. Two animals at 100 mg/kg bw (animal numbers 15 and 17) and one animal at 1000 mg/kg bw (animal number 34) showed markedly lower body temperatures (34.3, 33.9 and 33.9 °C), but this was not observed in the 300 mg/kg bw group. Therefore, the lower temperature without a dose–response relationship at 100 mg/kg bw was considered to be incidental. The mean body temperature was recalculated excluding these three animals. The mean body temperature 6 hours post-dosing was slightly, but statistically significantly, lower at 300 mg/kg bw and above. This finding was considered treatment related because the results are consistent with those of the previous study (Table 22).

Grip strength in general was unaffected by treatment. Although hindlimb grip strength was slightly decreased on day 8 at 1000 mg/kg bw compared with controls, the results were similar to the pretest data. The deviation from the control mean was only slight, and there were no findings indicative of neurotoxicity at the detailed clinical examinations or on assessment of locomotor activity. Consequently, the observation was concluded to be incidental. Locomotor activity was slightly decreased at 300 and 1000 mg/kg bw at the 6-hour examination. Although the difference was not statistically significant, the decrease was consistent with the results from the previous study and considered to be treatment related (Table 22). The marked decrease in locomotor activity observed in

two animals at 100 mg/kg bw was considered incidental because there was no enhancement of the severity at higher doses. There was no evidence of overt neurotoxicity, with all treatment-related effects consistent with discomfort or other acute effects of dosing related to general toxicity.

Table 22. Clinical observations, body temperature and locomotor activity in abbreviated acute neurotoxicity study of pydiflumetofen in female rats

Parameter	0 mg/kg bw	100 mg/kg bw	300 mg/kg bw	1 000 mg/kg bw
Clinical observations (maximum severity grade)				
Piloerection (2)	0	1/10 (2.0)	0	1/10 (2.0)
Vocalization (2)	1/10 (1.0)	1/10 (1.0)	1/10 (1.0)	2/10 (1.0)
Reduced activity (2)	1/10 (1.0)	4/10 (1.0)	1/10 (1.0)	3/10 (1.0)
Decreased rearing (1)	4/10 (1.0)	7/10 (1.0)	4/10 (1.0)	5/10 (1.0)
Tremor (2)	0	0	0	1/10 (1.0)
Skin cold on touch (1)	0	2/10 (1.0)	0	1/10 (1.0)
Pupillary reflex impaired (1)	0	0	1/10 (1.0)	0
Miosis (1)	0	0	1/10 (1.0)	0
Extensor thrust reflex impaired (1)	0	2/10 (1.0)	0	1/10 (1.0)
Body temperature at 6 hours post-dosing on day 1				
Mean body temperature (°C) ± SD[a]	38.0 ± 0.3	37.7 ± 0.3	37.6 ± 0.2*	37.6 ± 0.3*
Locomotor activity over a 30-minute session at 6 hours post-dosing on day 1				
Total distance (cm)	2 533	2 444	1 874	1 821
Total centre time (s)	103	169	70	117
Total number of rears	51	51	32	30

bw: body weight; SD: standard deviation; *: $P < 0.05$

[a] Exclusion of animal nos 15, 17 and 34 as outliers.

Source: Broich (2015b)

The NOAEL for systemic toxicity was 100 mg/kg bw, based on altered locomotor activity, clinical signs and reduced body temperature at 300 mg/kg bw. The NOAEL for neurotoxicity was 1000 mg/kg bw, the highest dose tested (Broich, 2015b).

Subacute neurotoxicity

Subacute neurotoxicity studies were not conducted, but detailed FOB examinations were conducted in the short-term toxicity studies in mice, rats and dogs and the long-term toxicity study in rats. No changes associated with neurotoxicity were observed in those studies, suggesting that pydiflumetofen is not neurotoxic at the doses tested in those studies.

(b) Immunotoxicity

No study for the assessment of immunotoxicity was provided. No findings indicating immunotoxicity were observed in any of the submitted studies, suggesting that pydiflumetofen is not immunotoxic.

(c) Toxicity of metabolites

A number of toxicity studies have been conducted on metabolites of pydiflumetofen that were detected in livestock and rats after exposure to pydiflumetofen. Summaries of the data are presented below. The metabolites CSAA798670 and SYN508272 are common to a number of other succinate dehydrogenase inhibitors.

2,4,6-Trichlorophenol (2,4,6-TCP)

2,4,6-TCP was the major circulating metabolite of pydiflumetofen in rats (representing up to 5.3% in plasma) and is identified in animal commodities only. 2,4,6-TCP sulfate was identified in plasma at 32–44% (MacDonald & Jewkes, 2015; Tomlinson et al., 2015).

No studies on the toxicity of 2,4,6-TCP were submitted. Oral exposure to 2,4,6-TCP has been shown to cause tumours in two rodent species. When administered in the diet, 2,4,6-TCP caused hepatocellular adenomas or carcinomas in both male and female mice and lymphoma or mononuclear cell leukaemia in male rats (NCI, 1979; IARC, 1982). In the NCI (1979) study, the number of F344 rats and B6C3F1 mice in the control groups was only 20 (the number in treated groups was 50). In addition, the given doses were high (in rats: 5000 and 10 000 ppm; in mice: 5000, 10 000 and 20 000 ppm for the first 38 weeks, reduced to 2500, 5000 and 10 000 ppm thereafter). These doses were considered to exceed the maximum tolerable dose. The data quality, including histopathological criteria, was considered inadequate when compared with current toxicological evaluations. However, increased incidences of leukaemia/lymphoma in male F344 rats at 5000 and 10 000 ppm and liver tumours in both sexes of B6C3F1 mice at 5000 and 10 000 ppm suggested the carcinogenic potential of this compound in rodents at very high doses (NCI, 1979).

WHO has established a guideline value of 0.2 mg/L 2,4,6-TCP in drinking-water, although the compound is generally found in drinking-water at concentrations below 1 µg/L. Like other chlorophenols (e.g. 2-chlorophenol and 2,4-dichlorophenol), 2,4,6-TCP is most likely to occur in drinking-water as a by-product of chlorination (WHO, 2017).

It was concluded that the present mammalian toxicity data on pydiflumetofen have sufficiently assessed the toxicity of 2,4,6-TCP and its conjugates and that the toxicity of 2,4,6-TCP and its conjugates would be covered by the parent compound.

SYN508272

SYN508272 is an identified metabolite in goats, hens and rats. A synonym for this metabolite is Reg. No. 5621781.

Acute oral toxicity. Groups of three female fasted Crl:WI (Han) rats were dosed sequentially with SYN508272 (lot/batch no. L81-108; purity 99.4%) in 0.5% aqueous carboxymethylcellulose by gavage at a dose of 2000 mg/kg bw (one group) or 500 mg/kg bw (two groups) and observed for 14 days.

All animals in the 2000 mg/kg bw test group were found dead within 24 hours after administration. Animals showed impaired and poor general state, dyspnoea, ataxia, tremor, staggering, twitching, abdominal position and piloerection.

No mortality occurred in the six females administered 500 mg/kg bw. In the first group, no adverse clinical signs were noted. However, in the second group, one animal showed impaired general state, dyspnoea, piloerection, chromodacryorrhoea and reduced faeces from the fourth hour after treatment until the next day.

There were no treatment-related effects on body weight in surviving animals up to 14 days. There were no significant pathological findings in either the surviving or dead animals.

The oral LD_{50} was therefore concluded to be greater than 500 mg/kg bw and less than 2000 mg/kg bw (Cords & Lammer, 2009).

Short-term studies of toxicity. In a 28-day toxicity study, Crl:WI (Han) rats (five of each sex per dose) were administered SYN508272 (lot/batch no. MES114/2; purity 94%) in the diet at a concentration of 0, 100, 500 or 2000 (males) / 4000 (females) ppm (equal to 0, 7.3, 37.4 and 143 mg/kg bw per day for males and 0, 7.8, 42.5 and 244 mg/kg bw per day for females, respectively). Study parameters included mortality and clinical signs at least once daily, body weight and feed consumption weekly, ophthalmoscopy pretrial in all animals and in control and high-dose animals in week 4, FOB pretrial and in week 4, clinical pathology prior to termination (haematology, clinical chemistry, urine analysis, coagulation), and organ weights (all), gross pathology (all) and histopathology (control and high-dose rats only) at termination.

There were no effects of treatment on mortality, clinical signs, FOB, urine analysis, organ weights, gross pathology or histopathology. In females at 4000 ppm, body weight and body weight gain were reduced throughout the treatment period. Body weight gain was 78% lower than control values by the end of the study. In males at 2000 ppm, body weight gain after 12 days was 40.6% lower than control values, but body weight recovered during the remainder of the study, and overall body weight gain at the end of the study was only 5.5% lower than control values (not statistically significant). Feed consumption was lower throughout treatment in females at 4000 ppm and slightly lower for the first 8 days in males at 2000 ppm, but then similar to control values, indicating lower palatability.

White blood cell counts were raised in males at 2000 ppm and in females at 4000 ppm. The value for males at 2000 ppm was outside the historical control range, but that for females at 4000 ppm was within the historical control range. The differential white blood cell counts were correspondingly higher for both neutrophil and lymphocytes (nonsignificant) in males at 2000 ppm and for neutrophil and lymphocytes in females at 4000 ppm. Although the pattern of effects is consistent with a treatment relationship at the high dose, there were no histological or clinical correlates indicative of either infection or inflammation that would support the findings. No supporting findings related to an increase in white blood cell count in either sex at the high doses were observed, indicating that the increase was not toxicologically relevant.

Reticulocyte counts were elevated at the high doses in both sexes. However, they were within the historical control range, although close to the upper bound, and were without any correlating changes in other parameters related to tissues in monocyte–macrophage systems or anaemia. Therefore, this change was not considered to be toxicologically relevant.

Globulin levels were lower and the albumin/globulin ratios were higher in both sexes at the high doses, and both were outside the historical control ranges. However, the lack of corresponding findings related to immunosuppression, including histopathology in the immune system, suggests that the changes were not treatment related. Creatinine levels were higher in females at 4000 ppm, again above the historical control range. In males at 2000 ppm, urea values were reduced and below the historical control range. AST activity was higher in both sexes at the high doses, but the increase was of small magnitude, and values were within historical control ranges and not correlated with effects on either the other liver enzymes or histopathology. The increase in AST activity was considered not to be toxicologically relevant. Phosphate levels in plasma were elevated in both sexes at the high doses but were within the historical control range, and the differences were of small magnitude. Given the consistency across sexes and the statistical significance, the increase in phosphate levels is concluded to be potentially treatment related, but not of toxicological relevance.

The absolute thymus weights were higher in both sexes at 500 and 2000/4000 ppm and were above the historical control range. However, the male control absolute thymus weight was lower than the historical control range, and the female control absolute thymus weight was close to the bottom of the historical control range. Thymus weights are known to vary greatly in young rats, depending on age

and associated rapid increases in body weight. The study author concluded that the higher thymus weights at 500 ppm and 2000/4000 ppm in males and females were within the historical control ranges as a percentage of body weight and, furthermore, were not treatment related due to normal variation in thymus weights in young rats and the lack of any histopathological changes in the thymus. Given the lack of a dose–response relationship at the two highest doses, the lack of histological correlates and the fact that the control values were at or below the lower bound of the historical control range, the study author's conclusion is considered reasonable.

The NOAEL was 2000 ppm (equal to 143 mg/kg bw per day), the highest dose tested. Several findings observed in both sexes at the high doses were not considered adverse due to palatability, small magnitude or lack of toxicological relevance (Dymarkowska, 2015).

This short-term study of SYN508272 in rats (Dymarkowska, 2015) indicated that the key toxicological end-point, reduced body weight gain, was the same as for the parent compound, pydiflumetofen, although the NOAEL of the metabolite was lower than that of the parent compound, for which a NOAEL of 8000 ppm (equal to 619 mg/kg bw per day) was identified (Strepka, 2012b).

Genotoxicity. Three genotoxicity studies of SYN508272, in vitro (two tests) and in vivo (one test), were conducted (summarized in Table 23).

Table 23. Summary of genotoxicity assays with SYN508272

				Results		
Test system	**Test object**	**Concentration/ dose**	**Purity (%)**	**Without S9**	**With S9**	**Reference**
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537; *E. coli* WP2*uvr*A/pKM101, WP2pKM101	5 000 µg/plate	94	Negative	Negative	Sokolowski (2014b)
Chromosomal aberration, mammalian (in vitro)	Human lymphocytes	1 860 µg/mL	94	Positive[a]	Negative[a]	Bohnenberger (2013b)
Point mutation (*tk* locus) assay	Mouse lymphoma L5178Y cells	1 860 µg/mL	94	Negative	Negative	Wollny (2013b)
Micronucleus assay in rat	Rat bone marrow cells	1 250 mg/kg bw	94	Negative	NA	Dony (2014b)

bw: body weight; NA: not applicable; S9: 9000 × *g* supernatant fraction from rat liver homogenate; *tk*: thymidine kinase

[a] Precipitation observed at and above 1062.9 µg/plate.

An in vitro chromosomal aberration test in human lymphocytes was conducted to assess the potential of SYN508272 (lot/batch no. MES114/2; purity 94%) to induce structural chromosomal aberrations in cultured human lymphocytes in the absence and presence of an exogenous metabolic activation system (liver S9 mix from phenobarbital/β-naphthoflavone-treated male rats). In each experimental group, two parallel cultures were analysed. Per culture, at least 100 metaphases were evaluated for structural chromosomal aberrations, except for the positive controls in experiment II

without S9 mix, where only 50 metaphases were evaluated. The highest applied concentration of the test substance in this study was 1860.0 µg/mL, approximately 10 mmol/L.

In experiment I, in the absence and presence of S9 mix, and in experiment II, in the presence of S9 mix, no cytotoxicity was observed up to the highest applied concentration. In experiment II, in the absence of S9 mix, and under long exposure conditions, concentrations showing clear cytotoxic effects were not evaluable for cytogenetic damage. However, the mitotic index was markedly reduced to 53.9% and 57.3% of control values at the two highest evaluated concentrations.

In experiment I, either with or without metabolic activation, no clastogenicity was observed at the concentrations evaluated. In experiment II, in the absence of S9 mix, one single statistically significant increase in chromosomal aberrations (4.5% aberrant cells, excluding gaps) was observed after treatment with 1062.9 µg/mL. Results at this concentration clearly exceeded the historical control range (0.0–2.5% aberrant cells, excluding gaps). In experiment II, in the presence of S9 mix, one single statistically significant increase in chromosomal aberrations (4.3% aberrant cells, excluding gaps) was observed after treatment with 347.1 µg/mL. Results at this concentration slightly exceeded the historical control range (0.0–3.5% aberrant cells, excluding gaps). However, in the presence of S9 mix, the effect was not reproducible in the two independent experiments performed. The response seen at 347.1 µg/mL, in the presence of S9 mix, was not part of a concentration-related response.

No evidence of an increase in polyploid metaphases was noticed after treatment with the test substance compared with the control cultures. Appropriate mutagens were used as positive controls. They induced statistically significant increases ($P < 0.05$) in cells with structural chromosomal aberrations.

Under the experimental conditions reported, the test substance induced structural chromosomal aberrations in human lymphocytes in vitro. Therefore, SYN508272 was considered to be clastogenic in this chromosomal aberration test when tested up to precipitating or the highest evaluable concentrations (Bohnenberger, 2013b).

In conclusion, SYN508272, a metabolite in rats, was clastogenic in a chromosomal aberration test at the highest concentrations, but not mutagenic in an Ames test. Negative results in an in vivo genotoxicity test were obtained, indicating that this metabolite is not genotoxic in vivo.

It was concluded that the toxicity of SYN508272 would be covered by the parent compound.

CSAA798670

Metabolite CSAA798670 is also known as DF-pyrazole acid, CA4312, NOA449410 and M700F001 (Reg. No. 5069089, a metabolite of BAS 700 F). This metabolite has been previously identified in the rat biotransformation pathway for fluxapyroxad (BAS 700 F) (Annex 1, reference *127*). This chemical was also evaluated as part of the 2013 JMPR assessment of benzovindiflupyr as a general metabolite (Annex 1, reference *130*), although it is not identified in the rat biotransformation pathway. Similarly, this metabolite is associated with pydiflumetofen, but is not explicitly identified in either the mouse or rat biotransformation pathways provided for pydiflumetofen (see Figs 3 and 4, respectively).

Acute oral toxicity. An acute oral toxicity study of DF-pyrazole acid was conducted. Four fasted Sprague Dawley CD strain rats (two males and two females) were treated with DF-pyrazole acid (CA4312) (lot/batch no. and purity not reported) at a dose of 2000 mg/kg bw. Clinical signs and body weight were monitored during the study. All animals were subjected to gross necropsy.

There were no deaths and no clinical signs of systemic toxicity. All animals gained weight, and no effect on body weight was observed in surviving animals. There were no abnormalities seen at termination. The acute oral LD_{50} of DF-pyrazole acid (CA4312) in the Sprague Dawley CD strain rat was greater than 2000 mg/kg bw (Pooles, 2008).

Short-term studies of toxicity. In a short-term toxicity study, groups of five male and five female RccHan:Wistar (SPF) rats were administered CSAA798670 in the diet at a concentration of 0, 2000, 6000 or 12 000 ppm (equal to 0, 167, 511 and 1007 mg/kg bw per day for males and 0, 175, 572 and 1043 mg/kg bw per day for females, respectively) for at least 28 days. In addition to test guideline parameters, detailed clinical observations comprising open-field evaluation of clinical signs were performed once prior to treatment start and once weekly (during weeks 1–3) thereafter. FOBs, including the quantitative assessment of grip strength in the forelimbs and hindlimbs, were performed on all animals in randomized order in week 4. Locomotor activities were assessed after the FOB evaluation during week 4. Feed consumption values were recorded once prior to treatment start and daily throughout the study. Body weights were recorded twice prior to treatment start, weekly thereafter and before necropsy. Ophthalmoscopic examinations were performed in all animals once prior to treatment start and in all animals in week 4. At the end of the treatment period, all surviving animals were necropsied. Selected organs and tissues were processed and examined microscopically.

All animals survived. There were no treatment-related effects on clinical observations, ophthalmoscopic findings, FOB findings, including grip strength and locomotor activity, feed consumption, feed utilization, body weight, body weight gain, clinical pathology, macroscopic findings or organ weights. Hepatocellular hypertrophy was noted in livers of males and females at 6000 and 12 000 ppm, and this was accompanied by a lower incidence of glycogen deposition at 12 000 ppm. Considering the absence of any effect on liver weight or clinical biochemistry parameters related to liver function, it was considered that minimal hepatocellular hypertrophy is an adaptive response that does not represent an adverse effect. No effects on liver histopathology or any other parameters measured in this study were observed at 2000 ppm.

The NOAEL was 12 000 ppm (equal to 1007 mg/kg bw per day), the highest dose tested (Sommer, 2010).

In a 13-week oral toxicity study in rats, M700F001, Reg. No. 5069089 (metabolite of BAS 700 F) (lot/batch no. L80-68; purity 99.2%) was administered to Crl:WI (Han) rats at a nominal dose of 0, 100, 300 or 1000 mg/kg bw per day for at least 91 days. Dietary concentrations of the test substance were adjusted weekly based on actual body weight and feed consumption to meet the intended nominal doses.

No treatment-related changes or any indications of adverse effects were observed in any parameters examined.

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

Developmental toxicity. In a developmental toxicity study, presumably pregnant Crl:KBL New Zealand white rabbits (31 does for treated groups and 32 does for control group) were administered the BAS 700 F metabolite M700F001 = Reg. No. 5069089 (lot/batch no. L80-68; purity 99.2%) by gavage at a daily dose of 0, 40, 100 or 250 mg/kg bw from GD 6 to GD 28.

No effects on maternal feed consumption or body weight development were observed. Likewise, no treatment-related necropsy findings were noted. Even though the conception rate of the time-mated rabbits was relatively low (72%, 87%, 90% and 84% at 0, 40, 100 and 250 mg/kg bw per day, respectively), a sufficient number of litters for evaluation was available (20, 25, 26 and 25 at 0, 40, 100 and 250 mg/kg bw per day, respectively). No treatment-related effects on gestational parameters (number of corpora lutea, implantation sites, preimplantation and postimplantation losses, early and late resorptions, number of dead and viable fetuses, placental or fetal weights, or sex ratio) were observed.

Fetal examination did not reveal any treatment-related external, visceral or skeletal malformations, variations or unclassified observations.

The NOAEL for maternal and embryo/fetal toxicity was 250 mg/kg bw per day, the highest dose tested (Schneider, Fabian & Mellert, 2009).

Genotoxicity. Genotoxicity assays conducted with CSAA798670 are summarized in Table 24. Overall, CSAA798670 was negative in all genotoxicity studies in vitro.

Table 24. Summary of genotoxicity assays with CSAA798670

Test system	Test object	Concentration/ dose	Purity (%)	Results		Reference
				Without S9	With S9	
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537; *E. coli* WP2*uvr*A/pKM101, WP2pKM101	Exp. I/IA: 3, 10, 33, 100, 333, 1 000, 2 500 and 5 000 µg/plate Exp. II: 33, 100, 333, 1 000, 2 500 and 5 000 µg/plate	99.6	Negative	Negative	Sokolowski (2007)
Chromosomal aberration, mammalian (in vitro)	Human lymphocytes	11.8, 20.6, 36.0, 63.0, 110.3, 193.0, 337.7, 591.0, 1 034.3, 1 810.0 µg/mL	97.4	Negative	Negative	Bohnenberger (2009)
Point mutation (*tk* locus) assay	Mouse lymphoma L5178Y cells	113.1, 226.3, 425.5, 905.0 and 1 810.0 µg/mL	97.4	Negative	Negative	Wollny (2009)

Exp.: Experiment; S9: 9000 × *g* supernatant fraction from rat liver homogenate; *tk*: thymidine kinase

The toxicological profile of CSAA798670 indicated that it was less potent than pydiflumetofen.

SYN547897

SYN547897 is present at greater than 10% in rat faeces (from bile). It was concluded that this metabolite and its conjugates would be covered by the parent compound.

Other metabolites

No toxicity data were provided for the metabolites SYN547948, SYN547890, SYN545547, SYN547891, SYN548265, SYN548263, SYN548264, SYN547893 or SYN547894. These metabolites were identified in the rat metabolism study.

(d) Mechanistic studies

A number of studies were conducted to investigate the MOA of liver tumour induction in male mice in the carcinogenicity study (Robertson, 2015) and follicular cell hypertrophy in the thyroid of male rats (Hackford, 2015; Shearer & Robertson, 2015).

Liver effects in mice

In a non-GLP-compliant study to examine liver effects, including induction of drug metabolism enzymes, histopathological changes and cell proliferation activity, male CD-1 mice (30 males per dose) were treated with pydiflumetofen (lot/batch no. SMU2EP12007; purity 98.5%) at a dietary concentration of 0, 75 or 2250 ppm (equal to 0, 10.0 and 324.0 mg/kg bw per day, respectively) for up to 28 days. The animals were killed (10 per dose) on days 2, 7 and 28 after commencement of the treatment. The histopathological analysis was subjected to internal and external peer review.

There was no effect of pydiflumetofen on terminal body weights in either treatment group. Absolute and relative liver weights were increased at 2250 ppm after 7 and 28 days. AST levels in blood were statistically significantly lower than the control values, reaching 70% and 60% of control values at 75 ppm and 2250 ppm after 28 days of administration. This decrease was considered not to be toxicologically relevant. No other blood biochemistry parameter was changed. Total cytochrome P450 levels were unaffected by treatment at 75 ppm at all time points. Pentoxyresorufin *O*-depentylation (PROD) activities were minimally (1.6- to 2.4-fold), but not statistically significantly, increased at 75 ppm. At 2250 ppm, pydiflumetofen treatment statistically significantly elevated total cytochrome P450 levels (up to 2.1-fold) and PROD activity (up to 37-fold) after all three treatment durations. 7-Benzyloxyresorufin *O*-debenzylation (BROD) activity was not affected by the treatment. No morphological changes were found in the liver stained by haematoxylin and eosin at any time point at 75 ppm. At 2250 ppm, centrilobular hypertrophy was induced and its severity was enhanced with time up to the terminal time point. After 2 days of treatment at 2250 ppm, an increase in mitotic figures in the liver was also observed, but the increase was not observed following 7 or 28 days of dietary administration. The hepatocellular labelling index (S-phase) using uptake of 5-bromo-2′-deoxyuridine (BrdU) in the liver was slightly increased at 75 ppm by 3.1-, 2.3- and 5.6-fold relative to time-matched controls after 2, 7 and 28 days, respectively. These differences were statistically significant after 7 and 28 days. At 2250 ppm, substantial and statistically significant increases in the labelling index were observed. A maximal increase (13.6-fold) was observed after 2 days. After 7 and 28 days, the labelling index was increased to a lesser extent (5.4- and 5.6-fold relative to control values).

In conclusion, pydiflumetofen induced liver hypertrophy, increased cytochrome P450 levels and increased PROD activity after 2, 7 and 28 days of treatment. In addition, DNA synthesis (S-phase) was maximal after 2 days of treatment and accompanied by increased mitosis of hepatocytes. The treatment induced minimal impact on the liver at 75 ppm (Elcombe, 2015).

Cell proliferation and enzyme induction in mouse hepatocytes in vitro

Effects on cytotoxicity, PROD and BROD enzyme activities and hepatocyte proliferation were investigated in isolated male CD-1 mouse hepatocyte cultures in a non-GLP-compliant study. Phenobarbital sodium salt and epidermal growth factor were used as positive controls. Primary monolayer cultures of hepatocytes were prepared in 25 cm^2 flasks and 96-well and six-well plastic tissue culture plates in Leibowitz CL15 medium. Hepatocytes were exposed to pydiflumetofen (lot/batch no. SMU2EP12007; purity 98.5%) at four concentrations (5, 10, 25 and 35 µmol/L), phenobarbital sodium salt at two concentrations (100 and 1000 µmol/L) or vehicle (0.5% v/v DMSO) alone for 96 hours. There were three replicates for each concentration in 25 cm^2 flasks for the measurement of PROD and BROD activities, five replicates for each concentration in six-well plates for replicative DNA synthesis analysis, and six replicates for each concentration in 96-well plates for cytotoxicity measurements as the change in cellular adenosine-5′-triphosphate (ATP).

Treatment with 35 µmol/L pydiflumetofen showed no cytotoxicity and no statistical reductions in intracellular ATP levels. PROD and BROD enzyme activities were increased at 5 and 10 µmol/L pydiflumetofen (up to 1.98-fold). In contrast, both PROD and BROD activities were lower than the vehicle control values following treatment with 25 and 35 µmol/L pydiflumetofen. The decreased PROD and BROD activities observed at higher concentrations were considered to be substrate competition between residual pydiflumetofen in the cell lysate preparation and pentoxyresorufin and benzyloxyresorufin, resulting in decreased enzymatic conversion of these substrates to form resorufin. Treatment of hepatocytes with 25 and 35 µmol/L pydiflumetofen resulted in a significant increase in hepatocyte proliferation as measured by replicative DNA synthesis (maximal at 25 µmol/L, resulting in a 1.90-fold increase relative to controls). The positive controls showed expected responses.

This study indicated that the treatment of cultured male CD-1 mouse hepatocytes with pydiflumetofen increased cell proliferation, measured as the change in replicative DNA synthesis (S-phase of the cell cycle), and increased PROD/BROD activity as an indicator of increased CYP2B/CYP3A activity in mouse hepatocytes, although dose dependency was not clear (Lowes, 2015a).

Cell proliferation and enzyme induction in human hepatocytes in vitro

Effects on cytotoxicity, PROD and BROD activities and replicative DNA synthesis (S-phase of the cell cycle) were investigated in cryopreserved male human hepatocyte cultures in a non-GLP-compliant study. Phenobarbital sodium salt and epidermal growth factor were used as positive controls. Primary monolayer cultures of hepatocytes were prepared in 25 cm^2 flasks and 96-well and six-well plastic tissue culture plates in Leibowitz HCL15 medium. Hepatocytes were exposed to pydiflumetofen (lot/batch no. SMU2EP12007; purity 98.5%) at four concentrations (5, 10, 25 and 35 µmol/L), phenobarbital sodium salt at two concentrations (100 and 1000 µmol/L) or vehicle (0.5% v/v DMSO) alone for 96 hours. There were three replicates for each concentration in 25 cm^2 flasks for measurement of PROD and BROD activities, five replicates for each concentration in six-well plates for replicative DNA synthesis analysis and six replicates for each concentration in 96-well plates for cytotoxicity measurements, measured as the change in cellular ATP.

The treatment resulted in cytotoxicity at 25 µmol/L and above due to a statistically significant reduction of intracellular ATP levels (58.5% of the vehicle control value). The treatment with 5, 10, 25 and 35 µmol/L pydiflumetofen statistically significantly increased PROD activities in hepatocytes by 2.9-, 3.3-, 2.5- and 2.7-fold, respectively. Phenobarbital sodium salt treatment resulted in statistically significant increases in PROD activity. The treatment with 5, 10 and 25 µmol/L pydiflumetofen statistically significantly increased BROD activities by 3.4-, 5.9-, and 4.3-fold, respectively. Phenobarbital sodium salt treatment resulted in statistically significant increases in BROD activity. There were no statistically significant increases in cell proliferation, measured as the change in replicative DNA synthesis, following the treatment at any concentration. Positive control compounds showed expected results.

This study indicated that the treatment of cultured male human hepatocytes with pydiflumetofen had no effect on cell proliferation, measured as the change in replicative DNA synthesis (S-phase of the cell cycle). PROD and BROD activities were increased by the treatment, and these were an indicator of increased CYP2B6/CYP3A4 activity in human hepatocytes, although dose dependencies were not clear (Lowes, 2015b).

In vitro cell proliferation and enzyme induction activity in male human hepatocytes and male CD-1 mouse hepatocytes are compared in Table 25.

Table 25. Comparison of in vitro cell proliferation and enzyme induction activity in male human hepatocytes and male CD-1 mouse hepatocytes treated with pydiflumetofen[a]

	Male CD-1 mouse hepatocytes		Male human hepatocytes	
Treatment	**ATP (luminescence units released)[b]**	**S-phase labelling index (%)[c]**	**ATP (luminescence units released)[b]**	**S-phase labelling index (%)[c]**
Vehicle control (0.5% v/v DMSO)	674 253 ± 68 995 (100 ± 10.2)	2.65 ± 0.1 (100 ± 3.77)	268 057 ± 19 089 (100 ± 7.1)	0.27 ± 0.06 (100 ± 23.08)
PB				
100 µmol/L	595 367 ± 16 637* (88.0 ± 2.5)	3.39 ± 0.27** (127.93 ± 10.34)	352 620 ± 47 400* (132 ± 17.7)	0.30 ± 0.09 (109.38 ± 33.37)
1 000 µmol/L	547 991 ± 27 707** (81.0 ± 4.1)	4.41 ± 0.43** (166.48 ± 16.39)	291 060 ± 70 476 (109 ± 26.3)	0.31 ± 0.06 (116.01 ± 24.01)
Pydiflumetofen				
5 µmol/L	588 475 ± 21 538** (87 ± 3.2)	3.54 ± 0.81 (133.92 ± 30.55)	329 623 ± 44 433** (123 ± 16.6)	0.26 ± 0.06 (97.90 ± 21.65)
10 µmol/L	611 652 ± 24 815* (91.0 ± 3.7)	3.54 ± 0.57 (133.89 ± 21.64)	284 562 ± 29 279 (106 ± 10.9)	0.32 ± 0.14 (120.27 ± 50.20)
25 µmol/L	567 202 ± 19 849** (84.0 ± 2.9)	5.03 ± 0.51** (189.86 ± 19.38)	204 833 ± 25 385** (76 ± 9.5)	0.10 ± 0.06** (36.14 ± 23.31)
35 µmol/L	555 771 ± 23 090** (82.0 ± 3.4)	3.99 ± 0.50** (150.90 ± 18.84)	178 281 ± 18 062** (67 ± 6.7)	0.07 ± 0.05** (25.58 ± 17.88)
EGF				
25 ng/mL	–	19.69 ± 3.14** (743.73 ± 118.49)	–	1.76 ± 0.24** (650.01 ± 89.84)
	Male CD-1 mouse hepatocytes		**Male human hepatocytes**	
Treatment	**PROD (pmol resorufin/mg per minute)[d]**	**BROD (pmol resorufin/mg per minute)[d]**	**PROD (pmol resorufin/mg per minute)[d]**	**BROD (pmol resorufin/mg per minute)[d]**
Vehicle control (0.5% v/v DMSO)	21.26 ± 3.35 (100 ± 15.8)	86.40 ± 36.82 (100 ± 42.6)	0.11 ± 0.017 (100.0 ± 15.3)	1.15 ± 0.062 (100 ± 5.4)
PB				
100 µmol/L	40.36 ± 4.65** (189.9 ± 21.9)	160.93 ± 19.54* (186.3 ± 22.6)	0.21 ± 0.025** (189.9 ± 22.8)	3.05 ± 0.16** (265.5 ± 14.2)
1 000 µmol/L	77.13 ± 8.76** (362.9 ± 41.2)	274.3 ± 25.49** (317.5 ± 29.5)	0.37 ± 0.045** (332.4 ± 41.4)	8.53 ± 0.65** (743.7 ± 56.4)
Pydiflumetofen				
5 µmol/L	39.25 ± 10.23** (184.7 ± 48.1)	171.48 ± 46.89* (198.5 ± 54.3)	0.32 ± 0.086* (293.6 ± 78.0)	3.87 ± 1.05* (336.9 ± 91.7)
10 µmol/L	36.41 ± 5.22* (171.3 ± 24.5)	170.23 ± 32.23* (197.3 ± 37.3)	0.37 ± 0.054** (332.3 ± 48.9)	6.80 ± 0.79** (593.1 ± 69.0)

Treatment	Male CD-1 mouse hepatocytes		Male human hepatocytes	
	ATP (luminescence units released)[b]	S-phase labelling index (%)[c]	ATP (luminescence units released)[b]	S-phase labelling index (%)[c]
25 μmol/L	11.54 ± 3.23 (54.3 ± 15.2)	63.12 ± 7.74 (73.1 ± 9.0)	0.28 ± 0.055** (254.8 ± 49.9)	4.90 ± 0.67** (427.4 ± 57.9)
35 μmol/L	3.34 ± 2.01 (15.7 ± 9.5)	16.78 ± 11.20 (19.4 ± 13.0)	0.29 ± 0.018** (267.2 ± 16.1)	3.66 ± 1.70 (318.9 ± 148.0)

ATP: adenosine 5′-triphosphate; BROD: benzyloxyresorufin *O*-debenzylation; DMSO: dimethyl sulfoxide; EGF: epidermal growth factor; PB: phenobarbital sodium salt; PROD: pentoxyresorufin *O*-depentylation; S-phase: replicative DNA synthesis; SD: standard deviation; v/v: volume per volume; *: $P < 0.05$; **: $P < 0.01$ (analysis of variance with Dunnett's post-test)

[a] Values are mean ± SD. Values in parentheses are mean % control ± SD.

[b] $n = 6$ per group.

[c] $n = 5$ per group.

[d] $n = 3$ per group.

Source: Lowes (2015a,b)

Transactivation assay with mouse, rat and human constitutive androstane receptors

The ability to directly activate the constitutive androstane receptor (CAR, also known as nuclear receptor subfamily 1, group I, member 3, or NR1I3) by pydiflumetofen treatment was investigated in a non-GLP-compliant study using a reporter assay. Briefly, complementary DNA expression vectors for constitutive androstane receptor-3 (CAR3) variants of mouse, rat and human CAR were transfected into COS-1 cells, along with necessary cofactors and a *Cyp2b6* response element–luciferase reporter construct. After a suitable expression time, the cells were incubated with pydiflumetofen (lot/batch no. SMU2EP12007; purity 98.5%) at a concentration of 1, 3, 10 or 30 μmol/L. The direct CAR activator artemisinin was also incubated at these same concentrations, and model direct-acting substrates of CAR3 for mouse (1,4-bis[2-(3,5-dichloropyridyloxy)]benzene, or TCPOBOP), rat (clotrimazole) or human ((6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-*O*-(3,4-dichlorobenzyl) oxime), or CITCO) were each incubated at a single concentration (0.5, 10 and 5 μmol/L for TCPOBOP, clotrimazole and CITCO, respectively). Light emission from the luciferase reporter was quantified to indicate the extent of CAR activation upon incubation with suspected ligands, including pydiflumetofen. Results were reported as normalized luciferase activity and fold change compared with a DMSO solvent control. The CAR3 reporter assay is accepted as a sensitive method for detecting direct activators of CAR, and it does not respond to indirect activators of CAR such as phenobarbital salt, because the second messenger systems that are believed to be responsible for indirect activation are not present in COS-1 cells.

In the human, mouse and rat assays using the respective species' CAR3 reporter constructs, a strong concentration-dependent activation of rodent and human CAR3 was observed. In rodents, pydiflumetofen produced up to 42-fold activation of rat CAR3 and up to 34-fold activation of mouse CAR3. The response in mouse CAR3 approached a maximum at the lower test concentrations of 1 μmol/L (24-fold) to 3 μmol/L (34-fold) and was slightly lower at the highest mouse test concentration of 30 μmol/L (20-fold), which correlated with decreased viability in the COS-1 cells at 10–30 μmol/L. The treatment activated human CAR3 in a concentration-responsive manner, and the maximal response (15-fold) at 30 μmol/L was higher than that of the human CAR positive control compound, CITCO (10-fold). The model activators CITCO, TCPOBOP and clotrimazole produced robust responses in human, mouse and rat CAR3 constructs, respectively. Artemisinin produced a concentration-dependent response that was much more marked with rat CAR3 than with human or mouse CAR3.

This study showed that pydiflumetofen was a direct activator of CAR from mice, rats and humans with high efficacy (i.e. maximal fold change compared with model activators) in all three species.

The summary of this study is shown in Table 26 (Omiecinski, 2014).

Table 26. Comparison of CAR3 transactivation assays among mice, rats and humans treated with pydiflumetofen

Construct	Treatment	Dose	Normalized luciferase activity	Fold change
Empty vector	DMSO	–	0.011 60	1.000
	Phenobarbital	1 mmol/L	0.010 39	0.895 7
	CITCO	5 µmol/L	0.010 29	0.886 6
	TCPOBOP	0.5 µmol/L	0.008 662	0.746 6
	Clotrimazole	10 µmol/L	0.016 25	1.400
	Pydiflumetofen	30 µmol/L	0.008 446	0.727 9
	Artemisinin	30 µmol/L	0.010 04	0.865 6
Human CAR3	DMSO	–	0.006 284	1.000
	Phenobarbital	1 mmol/L	0.005 459	0.868 7
	CITCO	5 µmol/L	0.064 98**	10.34
	Pydiflumetofen	1.0 µmol/L	0.009 235	1.470
		3.0 µmol/L	0.030 06	4.784
		10 µmol/L	0.079 40**	12.64
		30 µmol/L	0.093 05**	14.81
	Artemisinin	1.0 µmol/L	0.007 721	1.229
		3.0 µmol/L	0.010 19	1.621
		10 µmol/L	0.017 77	2.829
		30 µmol/L	0.051 29**	8.162
Mouse CAR3	DMSO	–	0.011 44	1.000
	Phenobarbital	1 mmol/L	0.023 63**	2.065
	TCPOBOP	0.5 µmol/L	0.518 7**	45.33
	Pydiflumetofen	1.0 µmol/L	0.275 0**	24.03
		3.0 µmol/L	0.385 7**	33.70
		10 µmol/L	0.363 9**	31.80
		30 µmol/L	0.229 4**	20.05
	Artemisinin	1.0 µmol/L	0.021 70	1.897
		3.0 µmol/L	0.045 02**	3.934
		10 µmol/L	0.125 8**	11.00
		30 µmol/L	0.147 7**	12.91
Rat CAR3	DMSO	–	0.005 455	1.000
	Phenobarbital	1 mmol/L	0.032 47**	5.953
	Clotrimazole	10 µmol/L	0.520 5**	95.43
	Pydiflumetofen	1.0 µmol/L	0.015 53	2.847
		3.0 µmol/L	0.077 95**	14.29
		10 µmol/L	0.020 08**	36.82

Construct	Treatment	Dose	Normalized luciferase activity	Fold change
		30 µmol/L	0.228 8**	41.95
	Artemisinin	1.0 µmol/L	0.015 97	2.927
		3.0 µmol/L	0.061 15	11.21
		10 µmol/L	0.206 8**	37.91
		30 µmol/L	0.286 0**	52.44

CAR: constitutive androstane receptor; CITCO: 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-*O*-(3,4-dichlorobenzyl) oxime; DMSO: dimethyl sulfoxide; TCPOBOP: 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene; **: $P < 0.01$
Source: Omiecinski (2014)

Ex vivo enzyme analysis of mouse liver from the 28-day dietary study

Effects of pydiflumetofen on liver peroxisomal and microsomal enzyme expression in male and female mice were investigated in a non-GLP-compliant study. Male and female mice were administered diet containing pydiflumetofen (lot/batch no. SMU2EP12007; purity 98.5%) at 500–7000 ppm for 28 days. Animals in the satellite study group were administered pydiflumetofen at 7000 ppm for 3 or 7 days. At termination, liver tissue was dissected, snap frozen and dispatched to CXR Biosciences (Dundee, United Kingdom) for analysis. The frozen liver chunks were processed to heavy pellets and microsomes. The heavy pellet samples prepared from these livers were assayed for total protein content and the activity of CN^--insensitive palmitoyl coenzyme A (CoA) oxidase. The microsomal samples prepared from these livers were assayed for total protein content, total cytochrome P450 content, the activities of ethoxyresorufin *O*-deethylation (EROD), PROD, benzyloxyquinoline *O*-debenzylation (BQ) and lauric acid 12-hydroxylation (LAH).

Administration of pydiflumetofen at 500–7000 ppm did not stimulate hepatic peroxisomal palmitoyl CoA oxidation and minimally increased (less than 4-fold) microsomal LAH in both sexes. This indicates that pydiflumetofen is not a peroxisome proliferator. Pydiflumetofen induced total cytochrome P450, PROD and BQ in a dose-dependent manner. A marked induction (up to 20-fold) of PROD, a marker for CYP2B enzymes, was observed. Increases in EROD, a marker for CYP1A enzymes, were minimal (up to 1.4-fold). Similar dose–response relationships were seen in both sexes except in the case of BQ, where much greater fold increases were found in females. This was considered to be due to lower constitutive CYP3A activities, which were more commonly seen in female mice than in male mice. Cytochrome P450, EROD, PROD and BQ activities increased time dependently in both sexes.

A summary of the enzyme analysis is shown in Table 27.

Table 27. Summary of ex vivo hepatic enzyme analysis in mice treated with pydiflumetofen

	Males					Females				
Enzymes	**0 ppm**	**500 ppm**	**1 500 ppm**	**4 000 ppm**	**7 000 ppm**	**0 ppm**	**500 ppm**	**1 500 ppm**	**4 000 ppm**	**7 000 ppm**
PCO (nmol NAD^+ reduced/min per mg protein)	15.47	9.97**	12.95	11.22*	10.69**	14.10	8.31	7.16**	7.97*	6.78*
Total P450 (nmol/mg protein)	0.51	0.78**	0.84**	0.84**	0.90**	0.55	0.65	0.74*	0.95**	0.87**
EROD (pmol resorufin	25.5	18.02*	18.36	21.22	34.77	23.79	26.22	19.14	32.84*	33.06**

Enzymes	Males					Females				
	0 ppm	500 ppm	1 500 ppm	4 000 ppm	7 000 ppm	0 ppm	500 ppm	1 500 ppm	4 000 ppm	7 000 ppm
formed/min per mg protein)										
PROD (pmol resorufin formed/min per mg protein)	2.77	32.68**	25.55**	24.97**	42.50**	7.50	38.77**	40.30**	31.70**	23.57**
BQ (nmol 7-hydroxy-quinoline formed/min per mg protein)	1.73	2.58**	2.05	4.63**	6.13**	4.31	4.28	6.38	10.79**	10.82**
LAH (nmol 12-OH lauric acid formed/10 min per mg protein)	4.12	5.39	10.35**	10.16**	14.46**	4.19	2.91	4.13	2.14**	4.22

BQ: benzyloxyquinoline *O*-debenzylation; EROD: ethoxyresorufin *O*-deethylation; LAH: lauric acid 12-hydroxylation; NAD^+: nicotinamide adenine dinucleotide (oxidized); P450: cytochrome P450; PCO: CN^--insensitive palmitoyl coenzyme A oxidation; ppm: parts per million; PROD: pentoxyresorufin *O*-depentylation; *: $P < 0.05$; **: $P < 0.01$
Source: Haines (2012)

This study indicated that pydiflumetofen did not demonstrate the prototypical properties of peroxisome proliferators in male or female mice. Pydiflumetofen was considered to have similar properties to those of phenobarbital (Haines, 2012).

Proposed MOA for the formation of mouse liver tumours

MOA and relevance to humans for liver tumour induction in mice by pydiflumetofen were postulated based on the international framework of the International Programme on Chemical Safety (IPCS) of WHO and the International Life Sciences Institute (ILSI). The data, including mechanistic studies and all toxicity studies of pydiflumetofen provided in the present evaluation, as well as current knowledge regarding liver tumour induction, support a proposed MOA involving the following key events:

- activation of CAR;
- an early, transient increase in hepatocellular proliferation;
- increased hepatocellular foci as a result of clonal expansion of spontaneously mutated (initiated) cells; and
- eventual progression to liver tumours.

Based on the qualitative species differences in the hepatocellular proliferation response to pydiflumetofen, it has been established that this MOA is not relevant to humans. In conclusion, these data support the conclusion that pydiflumetofen does not pose a hepatocarcinogenic hazard to humans.

Details of this MOA are described in Appendix 2 (Cowie, 2015).

Rat thyroid peroxidase activity

Effects on rat thyroid peroxidase activity were investigated in vitro. The aim of this study was to evaluate the effect of pydiflumetofen (lot/batch no. SMU2EP12007; purity 98.5%) at 0, 0.007, 0.1,

1.5 and 10 µmol/L on rat thyroid peroxidase activity in vitro. A pooled thyroid gland microsomal preparation from five rats was assayed for thyroid peroxidase activity by determining the monoiodination of L-tyrosine using a method based on studies by Doerge et al. (1998) and Freyberger & Ahr (2006). As a positive control, the effect of 6-propyl-2-thiouracil (PTU) at 10 µmol/L on rat thyroid peroxidase activity was also determined.

Preparations of thyroid gland microsomes from male Wistar Han rats (age range 67–74 days) were obtained from Charles River UK Ltd. A whole homogenate of the pooled thyroid glands from five rats was prepared in 0.154 mol/L potassium chloride containing 50 mmol/L Tris-hydrochloride (pH 7.4).

Pydiflumetofen at 0.007, 0.1, 1.5 and 10 µmol/L had no significant effect on thyroid peroxidase activity. In contrast, the addition of 10 µmol/L PTU, the positive control compound, resulted in a 99.9% inhibition of thyroid peroxidase activity (Table 28).

Table 28. Effects of pydiflumetofen and PTU on rat thyroid peroxidase activity

Addition[a]	Thyroid peroxidase activity (nmol/min per mg protein)[b,c]
Control (DMSO only)	7.07 ± 0.546 (100)
Pydiflumetofen 0.007 µmol/L	6.81 ± 0.329 (96)
Pydiflumetofen 0.1 µmol/L	6.72 ± 0.699 (95)
Pydiflumetofen 1.5 µmol/L	6.76 ± 0.217 (96)
Pydiflumetofen 10 µmol/L	7.05 ± 0.197 (100)
PTU 10 µmol/L	0.007 ± 0.001 5** (0.1)

DMSO: dimethyl sulfoxide; PTU: 6-propyl-2-thiouracil; SD: standard deviation; **: $P < 0.01$
[a] Pydiflumetofen and PTU were added in DMSO (2.5 µL/incubation).
[b] Results are presented as mean ± SD of either quadruplicate (control) or triplicate (all concentrations of pydiflumetofen and PTU) incubations.
[c] Values in parentheses are percentage of control levels.
Source: Lake (2014)

Pydiflumetofen was not an inhibitor of rat thyroid peroxidase activity in vitro (Lake, 2014).

Rat hepatic microsomal protein content and UGT activity

Using the liver samples of male rats obtained from the 91-day toxicity study (Shearer & Robertson, 2015), hepatic microsomal protein content and hepatic microsomal UGT activity were measured.

The treatment of male rats with 1500 or 8000 ppm pydiflumetofen for 91 days statistically significantly increased hepatic microsomal protein content. The treatment of male rats with pydiflumetofen induced hepatic microsomal UGT activity at all treated doses in a dose-dependent manner.

This study demonstrated that pydiflumetofen was an inducer of hepatic microsomal UGT activity towards thyroxine as a substrate in male rats. Treatment with 250 ppm pydiflumetofen for 91 days resulted in statistically significant increases in hepatic microsomal UGT activity towards thyroxine as substrate expressed as specific activity and per gram of liver, whereas treatment with 1500 and 8000 ppm pydiflumetofen resulted in statistically significant increases in enzyme activity expressed as specific activity, per gram of liver, per total liver weight and per relative liver weight (Table 29).

Table 29. Effects of pydiflumetofen on hepatic microsomal protein content and UGT activity in rats

	Males					Females				
Parameter	**0 ppm**	**250 ppm**	**1 500 ppm**	**8 000 ppm**	**16 000 ppm**	**0 ppm**	**250 ppm**	**1 500 ppm**	**8 000 ppm**	**16 000 ppm**
Hepatic microsomal protein content (mg/g liver)										
Mean	33.4	35.8	38.0	40.8**	NE	NE	NE	NE	NE	NE
(%)	(100)	(107)	(114)	(122)						
SD	3.82	3.43	2.89**	2.96						
UGT in the liver[a]										
A, Mean	5.6	8.5*	9.6**	16.1**	NE	NE	NE	NE	NE	NE
SD	1.4	2.8	2.71	2.37						
B, Mean	189	306*	366**	656**	NE	NE	NE	NE	NE	NE
SD	58.9	115	117	111						
C, Mean	2.77	4.39	6.20	11.66**	NE	NE	NE	NE	NE	NE
SD	1.03	1.66	1.57	2.21						
D, Mean	6.34	10.46	15.2	30.8**	NE	NE	NE	NE	NE	NE
SD	2.16	3.46	4.45	5.40						

bw: body weight; NE: not examined; ppm: parts per million; SD: standard deviation; UGT: uridine diphosphate glucuronosyltransferase activity (substrate thyroxine); *: $P < 0.05$; **: $P < 0.01$

[a] Units are as follows: A, pmol/min per mg protein; B, pmol/min per g liver; C, nmol/min per total liver weight; D, nmol/min per total liver weight per kg bw.

Source: Lake (2015)

These increases in microsome protein and UGT activity in the rat liver obtained from the present study indicated that the thyroid finding was secondary to phase II enzyme induction of metabolism in the liver (Lake, 2015). Whereas thyroxine and thyroid stimulating hormone levels in blood were not measured, the evidence suggested that follicular cell hypertrophy found in males at 1500 ppm and above and in females at 8000 ppm and above was a secondary effect on the thyroid resulting from overexcretion of thyroxine from blood due to liver phase II enzyme induction.

(e) Microbiological effects

The fungicide pydiflumetofen was evaluated at the 2018 JMPR to determine its impact on microbiota in the gastrointestinal tract. As no data were submitted by the sponsors, a literature search was performed using a number of search engines. These included BioOne (http://www.bioone.org/), Google (https://www.google.com/), Google Scholar (http://scholar.google.com/), PubMed (http://www.ncbi.nlm.nih.gov/pubmed), ScienceDirect (http://www.sciencedirect.com/) and Web of Science (https://apps.webofknowledge.com).

The search strategy used the input keywords of the fungicide chemical name (pydiflumetofen), chemical structure, antimicrobial mode of action, antimicrobial spectrum of activity, antimicrobial resistance, resistance mechanisms and genetics, microbiome, microbiota, gut microbiota, gut microbiome, gastrointestinal microbiota, gastrointestinal microbiome, etc., as well as the Boolean operators AND, OR and NOT.

The extensive search and review of the scientific literature did not find any reports on the effects of pydiflumetofen on the intestinal microbiome to include in the toxicological risk assessment.

3. Observations in humans

In a database of incidents involving chemical exposure of workers (2002–2017), no records of adverse health effects were reported during synthesis or formulation activities (Syngenta, 2018). Furthermore, no epidemiological studies have been conducted with pydiflumetofen.

Comments

Biochemical aspects

In rats administered radiolabelled pydiflumetofen by gavage, the oral absorption of total radioactivity was 85–90% at a dose of 5 mg/kg bw. Absorption became limited at 100 mg/kg bw (females: 50–55%) and 300 mg/kg bw (males: 19–24%). Pydiflumetofen was widely distributed, with highest concentrations of radioactivity observed in the liver and kidney. There was no evidence for accumulation. Following a single oral gavage dose of 5 mg/kg bw, excretion was rapid (at least 91% within 48 hours). Excretion was predominantly in faeces via the bile. Systemic exposure (AUC_{0-t}) of pydiflumetofen was nonlinear from 300 mg/kg bw in males and from 100 mg/kg bw in females. Following a single oral administration of radiolabelled pydiflumetofen, C_{max} was observed at 0.5–2 hours at 5 mg/kg bw and at 8 hours at 100 mg/kg bw and above (Punler & Harris, 2014b; Hutton, 2015a,b; Hutton & O'Hagan, 2015; MacDonald & Jewkes, 2015; Penn, 2015a; Webbley & Williams, 2015).

In rats, unchanged parent accounted for less than 4% of the 5 mg/kg bw dose by oral administration. The primary metabolic routes included demethoxylation, *N*-dealkylation, monohydroxylation and dihydroxylation, *O*-demethylation, and oxidative and reductive dechlorination. Numerous metabolites were detected, with only two metabolites each accounting for between 10% and 15% of the dose: 1) 2,4,6-TCP sulfate (phenyl label) and 2) SYN548263 (pyrazole label). The majority of these metabolites were also monohydroxylated and dihydroxylated and in many cases were conjugated with glucuronic acid or sulfate (MacDonald & Jewkes, 2015).

Studies in mice demonstrated similar toxicokinetics and biotransformation as were found in rats (Punler & Harris, 2014a; Tomlinson et al., 2015). The toxicokinetics of pydiflumetofen in pregnant rabbits indicated a subproportional increase in systemic exposure with dose, with no increase in systemic exposure with doses of 750 mg/kg bw or higher (Penn, 2015a).

Toxicological data

In rats, the acute oral LD_{50} of pydiflumetofen was greater than 5000 mg/kg bw (Petus-Árpásy, 2012), the acute dermal LD_{50} was greater than 5000 mg/kg bw (Petus-Árpásy, 2013) and the 4-hour acute inhalation LC_{50} was greater than 5.11 mg/L (Nagy, 2013). Pydiflumetofen showed no skin irritation in rabbits (Török-Bathó, 2012a), showed mild irritation to rabbit eyes (Török-Bathó, 2012b) and was not sensitizing in mice (Hargitai, 2013).

The main toxic effects of pydiflumetofen in short- and long-term toxicity studies were reduced body weight and effects in the liver in mice, rats and dogs.

In a 91-day toxicity study in mice fed pydiflumetofen at a dietary concentration of 0, 100, 500, 4000 or 7000 ppm (equal to 0, 17.5, 81.6, 630 and 1158 mg/kg bw per day for males and 0, 20.4, 106, 846 and 1483 mg/kg bw per day for females, respectively), the NOAEL was 500 ppm (equal to 81.6 mg/kg bw per day), based on increased cholesterol concentrations associated with liver hypertrophy at 4000 ppm (equal to 630 mg/kg bw per day) (Shearer, 2015).

In a 28-day toxicity study in rats administered pydiflumetofen at a dietary concentration of 0, 500, 4000, 8000 or 16 000 ppm (equal to 0, 43, 343, 677 and 1322 mg/kg bw per day for males and 0, 40, 322, 619 and 1174 mg/kg bw per day for females, respectively), the NOAEL was 8000 ppm (equal to 619 mg/kg bw per day), based on reduction of body weight gain at 16 000 ppm (equal to 1174 mg/kg bw per day) (Strepka, 2012b).

In a 91-day toxicity study in rats administered pydiflumetofen in the diet at a concentration of 0, 250, 1500, 8000 or 16 000 ppm (equal to 0, 18.6, 111, 587 and 1187 mg/kg bw per day for males and 0, 21.6, 127, 727 and 1325 mg/kg bw per day for females, respectively), the NOAEL was 250 ppm (equal to 18.6 mg/kg bw per day), based on follicular cell hypertrophy in the thyroid at 1500 ppm (equal to 111 mg/kg bw per day) (Shearer & Robertson, 2015). The Meeting noted that MOA studies for the effect of pydiflumetofen on the thyroid indicated that the induction of follicular cell hypertrophy by pydiflumetofen at high doses was secondary to increased hepatic microsomal UGT activity, not a direct effect on the thyroid. Therefore, the Meeting concluded that the follicular cell hypertrophy in the thyroid of rats would not be relevant to humans (Lake, 2014, 2015).

In a 13-week oral toxicity study in dogs treated with pydiflumetofen in capsules at 0, 30, 300 or 1000 mg/kg bw per day, the NOAEL was 300 mg/kg bw per day, based on reduced body weight gain and increased triglycerides at 1000 mg/kg bw per day (Blunt, 2015a).

In a 1-year oral toxicity study in dogs administered pydiflumetofen in capsules at a dose of 0, 30, 100 or 300 mg/kg bw per day, the NOAEL was 300 mg/kg bw per day, the highest dose tested (Blunt, 2015b).

The overall NOAEL for pydiflumetofen in dogs was 300 mg/kg bw per day, and the overall lowest-observed-adverse-effect level (LOAEL) was 1000 mg/kg bw per day.

In an 18-month carcinogenicity study in mice administered pydiflumetofen at a dietary concentration of 0, 75, 375 or 2250 ppm (equal to 0, 9.2, 45.4 and 287.9 mg/kg bw per day for males and 0, 9.7, 48.4 and 306.2 mg/kg bw per day for females, respectively), the NOAEL for toxicity was 375 ppm (equal to 45.4 mg/kg bw per day), based on reduced body weight gain in males at 2250 ppm (equal to 287.9 mg/kg bw per day). The NOAEL for carcinogenicity was 75 ppm (equal to 9.2 mg/kg bw per day), based on an increased incidence of liver tumours in male mice at 375 ppm (equal to 45.4 mg/kg bw per day) (Robertson, 2015).

The results of several mechanistic studies of the liver effects of pydiflumetofen in mice (Cowie, 2015; Elcombe, 2015; Lowes, 2015a) indicate that pydiflumetofen has a CAR-mediated MOA in the induction of liver tumours. The MOA for these tumours was assessed using the IPCS/ILSI human relevance framework. It was concluded that these carcinogenic responses are not relevant to humans.

In a 2-year toxicity and carcinogenicity study in rats administered pydiflumetofen at a dietary concentration of 0, 200, 1000 or 6000 ppm for males (equal to 0, 9.9, 51.0 and 319 mg/kg bw per day, respectively) or 0, 150, 450 or 1500 ppm for females (equal to 0, 10.2, 31.0 and 102 mg/kg bw per day, respectively), the NOAEL for toxicity was 200 ppm (equal to 9.9 mg/kg bw per day), based on lower body weight and reduced body weight gain in males at 1000 ppm (equal to 51.0 mg/kg bw per day). No treatment-related increases in tumour incidence were observed (Robertson, 2016).

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

Pydiflumetofen was tested for genotoxicity in an adequate range of in vitro and in vivo assays (Roth, 2012; Sokolowski, 2012, 2014a; Bohnenberger, 2013a; Wollny, 2013a; Dony, 2014a). It gave a positive response in a chromosomal aberration mammalian test using human lymphocytes without S9 (Bohnenberger, 2013a), but a negative response in the micronucleus test in vivo (Roth, 2012; Dony, 2014a).

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

In view of the lack of genotoxicity in vivo, the absence of carcinogenicity in rats and female mice and evidence that the tumours in male mice are not relevant to humans, the Meeting concluded that pydiflumetofen is unlikely to pose a carcinogenic risk to humans.

In a two-generation reproductive toxicity study, rats were given pydiflumetofen in the diet at a concentration of 0, 150, 750 or 4500 ppm (equal to 0, 9.1, 46.1 and 277 mg/kg bw per day, respectively) for males and 0, 150, 450 or 1500 ppm (equal to 0, 12.6, 33.7 and 115 mg/kg bw per day, respectively)

for females. The NOAEL for parental toxicity was 750 ppm (equal to 46.1 mg/kg bw per day), based on reduced body weight and effects on the thyroid (increased thyroid weight and minimal thyroid follicular cell hypertrophy) in males at 4500 ppm (equal to 277 mg/kg bw per day). The NOAEL for offspring toxicity was 450 ppm (equal to 33.7 mg/kg bw per day), based on reduced pup body weight before weaning in the F_1 generation at 1500 ppm (equal to 115 mg/kg bw per day). The NOAEL for reproductive toxicity was 1500 ppm (equal to 115 mg/kg bw per day), the highest dose tested (Hackford, 2015).

In a developmental toxicity study, pregnant rats were administered pydiflumetofen by gavage at a dose of 0, 10, 30 or 100 mg/kg bw per day during GDs 6–19. The NOAEL for maternal toxicity was 30 mg/kg bw per day, based on reductions in body weight gain and feed consumption at the beginning of dosing at 100 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 100 mg/kg bw per day, the highest dose tested (Davies, 2015).

In a developmental toxicity study in which rabbits were administered pydiflumetofen by gavage at a dose of 0, 10, 100 or 500 mg/kg bw per day during GDs 6–27, the NOAEL for maternal and embryo/fetal toxicity was 500 mg/kg bw per day, the highest dose tested (Penn, 2015c).

The Meeting concluded that pydiflumetofen is not teratogenic.

In an acute neurotoxicity study in rats administered pydiflumetofen by gavage at a dose of 0, 100 (females) or 300 (males), 1000 or 2000 mg/kg bw, the NOAEL for systemic toxicity was 100 mg/kg bw, based on clinical signs, lower body temperature and decreased locomotor activity in females at 1000 mg/kg bw. There was no evidence of neuropathological changes. The NOAEL for neurotoxicity was 2000 mg/kg bw, the highest dose tested (Broich, 2015a).

In an additional acute neurotoxicity study in female rats administered pydiflumetofen by gavage at a dose of 0, 100, 300 or 1000 mg/kg bw, the NOAEL for systemic toxicity was 100 mg/kg bw, based on clinical signs, lower body weight and decreased locomotor activity at 300 mg/kg bw. The NOAEL for neurotoxicity was 1000 mg/kg bw, the highest dose tested (Broich, 2015b).

The overall NOAEL for systemic toxicity in the two acute neurotoxicity studies in rats was 100 mg/kg bw.

Although a subchronic neurotoxicity study was not submitted, the Meeting noted that FOBs were performed in repeated-dose studies in mice, rats and dogs. No effects were observed.

The Meeting concluded that pydiflumetofen is not neurotoxic.

Although no immunotoxicity study was submitted, there was no indication of immunotoxicity in the submitted toxicity studies. The Meeting concluded that pydiflumetofen is not immunotoxic.

There was no information about the effect of pydiflumetofen on the microbiome of the human gastrointestinal tract based on a search of the literature.

Toxicological data on metabolites and/or degradates

2,4,6-Trichlorophenol (2,4,6-TCP)

2,4,6-TCP was the major circulating metabolite of pydiflumetofen in rats (representing up to 5.3% in plasma) and is identified in animal commodities only. 2,4,6-TCP sulfate was identified in plasma at 32–44% (MacDonald & Jewkes, 2015; Tomlinson et al., 2015).

WHO has established a drinking-water guideline value of 0.2 mg/L for 2,4,6-TCP, although it is generally found in drinking-water at concentrations below 0.001 mg/L. Like other chlorophenols (e.g. 2-chlorophenol and 2,4-dichlorophenol), 2,4,6-TCP is most likely to occur in drinking-water as a by-product of chlorination (WHO, 2017).

The Meeting noted that this metabolite was identified in animal commodities and concluded that the toxicity of 2,4,6-TCP and its conjugates would be covered by the parent compound.

SYN547897 (livestock and rat metabolite)

SYN547897 is present at greater than 10% in rat faeces (from bile). Therefore, the Meeting concluded that this metabolite and its conjugates would be covered by the parent compound.

SYN508272 (Reg. No. 5621781)

SYN508272 was found in rats. The precursor of this metabolite was SYN548263, a major metabolite in rats. The acute oral LD_{50} of SYN508272 was greater than 500 mg/kg bw and less than 2000 mg/kg bw (Cords & Lammer, 2009).

In a 28-day toxicity study in which rats were administered diets containing SYN508272 at a concentration of 0, 100, 500 or 2000 ppm (males) / 4000 ppm (females) (equal to 0, 7.3, 37.4 and 143 mg/kg bw per day for males and 0, 7.8, 42.5 and 244 mg/kg bw per day for females, respectively), marginally lower body weights and lower feed consumption were observed in females only during the first part of the study at 4000 ppm (equal to 244 mg/kg bw per day). The Meeting noted that these effects on body weight and feed consumption may have been due to palatability issues. The NOAEL was 2000 ppm (equal to 143 mg/kg bw per day), the highest dose tested (Dymarkowska, 2015).

The toxicological profile of this metabolite was the same as that of pydiflumetofen. The NOAEL of the metabolite in the 28-day rat study (2000 ppm, equal to 143 mg/kg bw per day, the highest dose tested) (Dymarkowska, 2015) was slightly lower than that of the parent compound in a similar study (NOAEL of 8000 ppm, equal to 619 mg/kg bw per day) (Strepka, 2012b).

SYN508272 showed one positive result in an in vitro genotoxicity study and negative results in two other in vitro studies and an in vivo study (Bohnenberger, 2013b; Wollny, 2013b; Dony, 2014b; Sokolowski, 2014b).

Human data

In reports on manufacturing plant personnel, no adverse health effects during the manufacture of pydiflumetofen or its product formulation were noted by the sponsor (Syngenta, 2018).

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

Toxicological evaluation

The Meeting established an ADI of 0–0.1 mg/kg bw on the basis of the NOAEL of 9.9 mg/kg bw per day in a 2-year study in rats for decreased body weight in males observed at 51.0 mg/kg bw per day. A safety factor of 100 was applied.

The Meeting established an ARfD of 0.3 mg/kg bw on the basis of the NOAEL of 30 mg/kg bw per day in a rat developmental toxicity study for reduction in maternal body weight gain and feed consumption early during treatment, observed at 100 mg/kg bw per day. A safety factor of 100 was applied.

The ADI and the ARfD apply to the metabolites 2,4,6-TCP and SYN547897 and their conjugates, expressed as pydiflumetofen.

Levels relevant to risk assessment of pydiflumetofen

Species	Study	Effect	NOAEL	LOAEL
Mouse	Eighteen-month study of carcinogenicity[a]	Toxicity	375 ppm, equal to 45.4 mg/kg bw per day	2 250 ppm, equal to 287.9 mg/kg bw per day
		Carcinogenicity	75 ppm, equal to 9.2 mg/kg bw per day	375 ppm, equal to 45.4 mg/kg bw per day
Rat	Two-year study of toxicity and carcinogenicity[a]	Toxicity	200 ppm, equal to 9.9 mg/kg bw per day	1 000 ppm, equal to 51.0 mg/kg bw per day
		Carcinogenicity	1 500 ppm, equal to 102 mg/kg bw per day[b]	–
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	1 500 ppm, equal to 115 mg/kg bw per day[b]	–
		Parental toxicity	750 ppm, equal to 46.1 mg/kg bw per day	4 500 ppm, equal to 277 mg/kg bw per day
		Offspring toxicity	450 ppm, equal to 33.7 mg/kg bw per day	1 500 ppm, equal to 115 mg/kg bw per day
	Developmental toxicity study[c]	Maternal toxicity	30 mg/kg bw per day	100 mg/kg bw per day
		Embryo and fetal toxicity	100 mg/kg bw per day[b]	–
Rabbit	Developmental toxicity study[c]	Maternal toxicity	500 mg/kg bw per day[b]	–
		Embryo and fetal toxicity	500 mg/kg bw per day[b]	–
Dog	Three-month and 1-year studies of toxicity[d,e]	Toxicity	300 mg/kg bw per day	1 000 mg/kg bw per day

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

Acceptable daily intake (ADI) (applies to pydiflumetofen and the metabolites 2,4,6-TCP and SYN547897 and their conjugates, expressed as pydiflumetofen)

0–0.1 mg/kg bw

Acute reference dose (ARfD) (applies to pydiflumetofen and the metabolites 2,4,6-TCP and SYN547897 and their conjugates, expressed as pydiflumetofen)

0.3 mg/kg bw

Information that would be useful for the continued evaluation of pydiflumetofen

Further characterization of the toxicity of metabolites found in plant and animal commodities; results from epidemiological, occupational health and other such observational studies of human exposure

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

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	85–90% absorbed; T_{max} within 2 hours at low dose
Distribution	Widely distributed; highest levels in liver and kidney
Potential for accumulation	No indication for accumulation in tissues
Rate and extent of excretion	Rapidly excreted (>90% within 48 hours)
Metabolism in animals	Demethoxylation, *N*-dealkylation, monohydroxylation and dihydroxylation, *O*-demethylation, oxidative and reductive dechlorination
Toxicologically significant compounds in animals and plants	Pydiflumetofen, 2,4,6-trichlorophenol (2,4,6-TCP)
Acute toxicity	
Rat, LD_{50}, oral	>5 000 mg/kg bw
Rat, LD_{50}, dermal	>5 000 mg/kg bw
Rat, LC_{50}, inhalation	>5.11 mg/L
Rabbit, dermal irritation	Not irritating to skin
Rabbit, ocular irritation	Slightly irritating to eye
Mouse, dermal sensitization	Not sensitizing (LLNA)
Short-term studies of toxicity	
Target/critical effect	Thyroid follicular cell hyperplasia (rat)
Lowest relevant oral NOAEL	18.6 mg/kg bw per day (rat)
Lowest relevant dermal NOAEL	1 000 mg/kg bw per day, highest dose tested (rat)
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Reduced body weight and body weight gain (rat)
Lowest relevant NOAEL	9.9 mg/kg bw per day (rat)
Carcinogenicity	Carcinogenic in male mice, but not in female mice or rats[a]
Genotoxicity	
	No evidence of genotoxicity in vivo[a]
Reproductive toxicity	
Target/critical effect	Thyroid follicular cell hypertrophy (rat)
Lowest relevant parental NOAEL	46.1 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	33.7 mg/kg bw per day (rat)
Lowest relevant reproductive NOAEL	115 mg/kg bw per day, highest dose tested (rat)

Developmental toxicity	
Target/critical effect	Reduced body weight gain and feed consumption (rat)
Lowest relevant maternal NOAEL	30 mg/kg bw per day (rat)
Lowest relevant embryo/fetal NOAEL	100 mg/kg bw per day, highest dose tested (rat)
Neurotoxicity	
Acute neurotoxicity NOAEL	1 000 mg/kg bw, highest dose tested (rat)
Subchronic neurotoxicity NOAEL	No evidence for neurotoxicity in repeated-dose studies in mice, rats and dogs
Other toxicological studies	
Immunotoxicity NOAEL	No evidence for immunotoxicity in repeated-dose toxicity studies
Studies on toxicologically relevant metabolites	
2,4,6-Trichlorophenol (2,4,6-TCP)	Major metabolite (rat)
	WHO drinking-water guideline value: 0.2 mg/L
SYN508272	500 mg/kg bw < LD_{50} < 2 000 mg/kg bw
	28-day study NOAEL 143 mg/kg bw per day, highest dose tested (rat)
	No evidence of genotoxicity in vivo
Human data	
	No adverse health effects on manufacturing plant personnel have been reported

[a] Unlikely to pose a carcinogenic risk to humans.

Summary

	Value	Study	Safety factor
ADI	0–0.1 mg/kg bw[a]	Two-year study of toxicity and carcinogenicity in rats	100
ARfD	0.3 mg/kg bw[a]	Developmental toxicity study in rats	100

[a] Applies to pydiflumetofen and the metabolites 2,4,6-TCP and SYN547897 and their conjugates, expressed as pydiflumetofen.

References

Barnes E (2015). SYN545974: additional historical control data to support developmental toxicity studies in the rat. Unpublished report no. TK0103655. Syngenta Ltd, Jealott's Hill International Research Centre, Bracknell, Berkshire, United Kingdom. Syngenta File No. SYN545974_10244. Submitted to WHO by Syngenta Ltd.

Blunt H (2015a). SYN545974 – 90 day oral (capsule) study in the dog. Unpublished report no. BFI0113. Sequani Ltd, Ledbury, Herefordshire, United Kingdom. Syngenta File No. SYN545974_10207. Submitted to WHO by Syngenta Ltd.

Blunt H (2015b). SYN545974 – 52 week oral (capsule) toxicity study in the dog. Unpublished report no. BFI0114. Sequani Ltd, Ledbury, Herefordshire, United Kingdom. Syngenta File No. SYN545974_10205. Submitted to WHO by Syngenta Ltd.

Bohnenberger S (2009). CSAA798670: chromosome aberration test in human lymphocytes in vitro. Unpublished report no. 1266902. Harlan Cytotest Cell Research GmbH (Harlan CCR), Rossdorf, Germany. Syngenta File No. NOA449410_10001. Submitted to WHO by Syngenta Ltd.

Bohnenberger S (2013a). SYN545974 – chromosome aberration test in human lymphocytes in vitro. Unpublished report no. 1498902. Harlan Cytotest Cell Research GmbH (Harlan CCR), Rossdorf, Germany. Syngenta File No. SYN545974_10048. Submitted to WHO by Syngenta Ltd.

Bohnenberger S (2013b). SYN508272: chromosome aberration test in human lymphocytes in vitro. Unpublished report no. 1555902. Harlan Cytotest Cell Research GmbH (Harlan CCR), Rossdorf, Germany. Syngenta File No. SYN508272_10904. Submitted to WHO by Syngenta Ltd.

Broich K (2015a). SYN545974 – acute oral (gavage) neurotoxicity study in the Wistar rat (report amendment 1). Unpublished report no. D84504. Harlan Laboratories Ltd, Itingen, Switzerland. Syngenta File No. SYN545974_10198. Submitted to WHO by Syngenta Ltd.

Broich K (2015b). SYN545974 – an abbreviated acute oral (gavage) neurotoxicity study in the female Wistar rat (report amendment 1). Unpublished report no. D97813. Harlan Laboratories Ltd, Itingen, Switzerland. Syngenta File No. SYN545974_10197. Submitted to WHO by Syngenta Ltd.

Cords S, Lammer E (2009). Reg. No. 5621781 (metabolite of BAS 700 F): acute oral toxicity study in rats. Unpublished report no. 2009/1084176. Bioassay – Labor für Biologische Analytik GmbH, Heidelberg, Germany. Syngenta File No. SYN508272_10903. Submitted to WHO by Syngenta Ltd.

Cowie D (2015). SYN545974 – human relevance framework assessment of liver tumour induction in CD-1 mice. Unpublished report no. TK0258437. Syngenta Crop Protection Ltd, Bracknell, Berkshire, United Kingdom. Syngenta File No. SYN545974_10290. Submitted to WHO by Syngenta Ltd.

Davies S (2015). SYN545974: oral (gavage) prenatal developmental toxicity study in the rat. Unpublished report no. BFI0118. Sequani Ltd, Ledbury, Herefordshire, United Kingdom. Syngenta File No. SYN545974_10190. Submitted to WHO by Syngenta Ltd.

Davies S (2016). SYN545974 – historical control data for SYN545974_10190. Syngenta, Bracknell, Berkshire, United Kingdom. Syngenta File No. SYN545974_10453. Submitted to WHO by Syngenta Ltd.

Doerge D, Chang H, Divi R, Churchwell M (1998). Mechanism for inhibition of thyroid peroxidase by leucomalachite green. Chem Res Toxicol. 11:1098–104.

Dony E (2014a). SYN545974 – micronucleus assay in bone marrow cells of the mouse. Unpublished report no. 1648702. Harlan Cytotest Cell Research GmbH (Harlan CCR), Rossdorf, Germany. Syngenta File No. SYN545974_10141. Submitted to WHO by Syngenta Ltd.

Dony E (2014b). SYN508272: micronucleus assay in bone marrow cells of the rat. Unpublished report no. 1602600. Harlan Cytotest Cell Research GmbH (Harlan CCR), Rossdorf, Germany. Syngenta File No. SYN508272_10910. Submitted to WHO by Syngenta Ltd.

Dymarkowska K (2015). SYN508272: a 28 day dietary toxicity study in rats. Unpublished report no. 35015. Charles River Laboratories, Preclinical Services, Tranent, Edinburgh, United Kingdom. Syngenta File No. SYN508272_10919. Submitted to WHO by Syngenta Ltd.

Elcombe B (2015). SYN545974: a 28-day dietary liver mode of action study in male CD-1 mice. Unpublished report no. CXR1508. CXR Biosciences, Dundee, United Kingdom. Syngenta File No. SYN545974_10267. Submitted to WHO by Syngenta Ltd.

European Commission (2018). Pydiflumetofen. Volume 1. Combined draft assessment report prepared according to Regulation (EC) No. 1107/2009 and proposal for harmonized classification and labelling (CLH report) according to Regulation (EC) No. 1272/2008 (https://echa.europa.eu/documents/10162/aeb9f88b-912e-4228-af6c-d75a2ce217ce, accessed 2 February 2019).

Freyberger A, Ahr HJ (2006). Studies on the goitrogenic mechanism of action of *N*,*N*,*N'*,*N'*-tetramethylthiourea. Toxicology. 217(2–3):169–75.

Hackford S (2015). SYN545974: oral (dietary) two-generation reproduction toxicity study in the rat. Unpublished report no. BFI0171. Sequani Ltd, Ledbury, Herefordshire, United Kingdom. Syngenta File No. SYN545974_10246. Submitted to WHO by Syngenta Ltd.

Haines C (2012). SYN545974: ex-vivo enzyme analysis of liver samples taken at termination of a 28 day dietary study of SYN545974 and SYN546022 in the mouse. Unpublished report no. CXR1117. CXR Biosciences, Dundee, United Kingdom. Syngenta File No. SYN545974_10209. Submitted to WHO by Syngenta Ltd.

Hargitai J (2013). SYN545974 – local lymph node assay in the mouse. Unpublished report no. 12/344-037E. CiToxLAB Hungary Ltd, Szabadságpuszta, Hungary. Syngenta File No. SYN545974_10052. Submitted to WHO by Syngenta Ltd.

Hutton E (2015a). SYN545974 – the absorption and excretion of [phenyl U ^{14}C] and [pyrazole 5 ^{14}C] SYN545974 following single oral administration in the rat. Unpublished report no. 34214. Charles River, Tranent, Edinburgh, United Kingdom. Syngenta File No. SYN545974_10248. Submitted to WHO by Syngenta Ltd.

Hutton E (2015b). SYN545974 – tissue depletion of [phenyl U ^{14}C] and [pyrazole 5 ^{14}C] SYN545974 following single oral administration in the rat. Unpublished report no. 34340. Charles River, Tranent, Edinburgh, United Kingdom. Syngenta File No. SYN545974_10252. Submitted to WHO by Syngenta Ltd.

Hutton E, O'Hagan P (2015). SYN545974 – the pharmacokinetics of [phenyl U ^{14}C] and [pyrazole 5 ^{14}C] SYN545974 following single oral and intravenous administration in the rat. Unpublished report no. 34107. Charles River, Tranent, Edinburgh, United Kingdom. Syngenta File No. SYN545974_10250. Submitted to WHO by Syngenta Ltd.

IARC (1982). 2,4,5-Trichlorophenol and 2,4,6-trichlophenol. In: Chemicals, industrial processes and industries associated with cancer in humans: an updating of IARC Monographs Volumes 1 to 29. IARC Monogr Eval Carcinogen Risk Chem Hum. 4(Suppl):249–50.

Kaspers U, Strauss V, Graters S, Fabian E, van Ravenzwaay B (2009). Reg. No. 5069089 (metabolite of BAS 700 F): repeated dose 90-day oral toxicity study in Wistar rats – administration in the diet. Unpublished report no. 50S0451/07119. BASF SE, Ludwigshafen/Rhein, Germany. Syngenta File No. R958945_11273. Submitted to WHO by Syngenta Ltd.

Lake B (2014). SYN545974: effect on rat thyroid peroxidase activity in vitro. Unpublished report no. 5523/1/2/2014 (amendment 1). Molecular Sciences Department, Leatherhead Food Research (LFR), Leatherhead, Surrey, United Kingdom. Syngenta File No. SYN545974_10120. Submitted to WHO by Syngenta Ltd.

Lake B (2015). SYN545974: effect on hepatic UDPglucuronosyltransferase activity towards thyroxine as substrate after dietary administration for 90 days to male rats. Unpublished report no. 5522/1/2/2014 (amendment 1). Molecular Sciences Department, Leatherhead Food Research (LFR), Leatherhead, Surrey, United Kingdom. Syngenta File No. SYN545974_10264. Submitted to WHO by Syngenta Ltd.

Lowes D (2015a). SYN545974: in vitro hepatocyte proliferation indexing and enzyme activity measurements in male CD-1 mouse hepatocyte cultures. Unpublished report no. CXR1503. CXR Biosciences, Dundee, United Kingdom. Syngenta File No. SYN545974_10272. Submitted to WHO by Syngenta Ltd.

Lowes D (2015b). SYN545974: in vitro hepatocyte proliferation indexing and enzyme activity measurements in male human hepatocyte cultures. Unpublished report no. CXR1504. CXR Biosciences, Dundee, United Kingdom. Syngenta File No. SYN545974_10270. Submitted to WHO by Syngenta Ltd.

MacDonald M, Jewkes Y (2015). SYN545974 – biotransformation of [^{14}C]-SYN545974 in rat. Unpublished report no. 34216. Charles River, Tranent, Edinburgh, United Kingdom. Syngenta File No. SYN545974_10259. Submitted to WHO by Syngenta Ltd.

Manton J (2011). SYN545974, SYN546022: preliminary oral (gavage) prenatal developmental toxicity dose range finding study in the rat. Unpublished report no. BFI0031. Sequani Ltd, Bromyard Road, Ledbury, Herefordshire, United Kingdom. Syngenta File No. SYN545974_10003. Submitted to WHO by Syngenta Ltd.

Nagy K (2013). SYN545974 – acute inhalation toxicity study (nose-only) in the rat. Unpublished report no. 12/334-004P. CiToxLAB Hungary Ltd, Szabadságpuszta, Hungary. Syngenta File No. SYN545974_10051. Submitted to WHO by Syngenta Ltd.

NCI (1979). Bioassay of 2,4,6-trichlorophenol for possible carcinogenicity. CAS No. 88-06-2. NCI-CG-TR 155. Technical Report Series No. 155; DHEW Publications No. (NIH) 79-1711. Bethesda (MD): United States Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, National Cancer Institute (https://ntp.niehs.nih.gov/ntp/htdocs/lt_rpts/tr155.pdf, accessed September 2018).

Omiecinski C (2014). SYN545974: CAR3 transactivation assay with mouse, rat and human CAR. Unpublished report no. TK0219831. Department of Veterinary & Biomedical Sciences, Penn State University, University Park, PA, USA. Syngenta File No. SYN545974_50044. Submitted to WHO by Syngenta Ltd.

Penn L (2015a). SYN545974 – oral (gavage) toxicokinetic study in the pregnant rabbit (final report amendment 3). Unpublished report no. BFI0126. Sequani Ltd, Ledbury, Herefordshire, United Kingdom. Syngenta File No. SYN545974_10125. Submitted to WHO by Syngenta Ltd.

Penn L (2015b). SYN545974: preliminary oral (gavage) prenatal developmental toxicity study in the rabbit (final report amendment 2). Unpublished report no. BFI0046. Sequani Ltd, Ledbury, Herefordshire, United Kingdom. Syngenta File No. SYN545974_10192. Submitted to WHO by Syngenta Ltd.

Penn L (2015c). SYN545974: oral (gavage) prenatal developmental toxicity study in the rabbit. Unpublished report no. BFI0116. Sequani Ltd, Ledbury, Herefordshire, United Kingdom. Syngenta File No. SYN545974_10177. Submitted to WHO by Syngenta Ltd.

Petus-Árpásy M (2012). SYN545974 – acute oral toxicity study in the rat (up and down procedure). Unpublished report no. 12/344-001P. CiToxLAB Hungary Ltd, Szabadságpuszta, Hungary. Syngenta File No. SYN545974_10043. Submitted to WHO by Syngenta Ltd.

Petus-Árpásy M (2013). SYN545974 – acute dermal toxicity study in rats. Unpublished report no. 12/344-002P. CiToxLAB Hungary Ltd, Szabadságpuszta, Hungary. Syngenta File No. SYN545974_10046. Submitted to WHO by Syngenta Ltd.

Pooles A (2008). DF-pyrazole acid (CA4312): screening acute oral toxicity study in the rat. Unpublished report no. 2364/0169. SafePharm Laboratories Ltd, Shardlow, Derbyshire, United Kingdom. Syngenta File No. CA4312/0003. Submitted to WHO by Syngenta Ltd.

Punler M, Harris J (2014a). SYN545974 – pharmacokinetics of SYN545974 in the mouse following multiple oral and single intravenous administration. Unpublished report no. 33408. Charles River, Tranent, Edinburgh, United Kingdom. Syngenta File No. SYN545974_10124. Submitted to WHO by Syngenta Ltd.

Punler M, Harris J (2014b). SYN545974 – pharmacokinetics of SYN545974 in the rat following multiple oral and single intravenous administration. Unpublished report no. 33409. Charles River, Tranent, Edinburgh, United Kingdom. Syngenta File No. SYN545974_10104. Submitted to WHO by Syngenta Ltd.

Robertson B (2015). SYN545974: 80 week mouse dietary carcinogenicity study (report amendment 1). Unpublished report no. 35914. Charles River Laboratories Edinburgh Ltd, Tranent, Edinburgh, United Kingdom. Syngenta File No. SYN545974_10237. Submitted to WHO by Syngenta Ltd.

Robertson B (2016). SYN545974: 104 week rat dietary carcinogenicity study with a combined 52 week toxicity study (report amendment 1). Unpublished report no. 36248. Charles River Laboratories Edinburgh Ltd, Tranent, Edinburgh, United Kingdom. Syngenta File No. SYN545974_10245. Submitted to WHO by Syngenta Ltd.

Roth M (2012). SYN545974 – micronucleus assay in bone marrow cells of the mouse. Unpublished report no. 1498904. Harlan Cytotest Cell Research GmbH (Harlan CCR), Rossdorf, Germany. Syngenta File No. SYN545974_10045. Submitted to WHO by Syngenta Ltd.

Sakamoto Y, Inoue K, Takahashi M, Taketa Y, Kodama Y, Nemoto K et al. (2013). Different pathways of constitutive androstane receptor–mediated liver hypertrophy and hepatocarcinogenesis in mice treated with piperonyl butoxide or decabromodiphenyl ether. Toxicol Pathol. 41(8):1078–92.

Schneider S, Fabian E, Mellert W (2009). Reg. No. 5069089 (metabolite of BAS 700 F) – prenatal developmental toxicity study in New Zealand white rabbits – oral administration (gavage). Unpublished report no. 2009/1072507. BASF SE, Ludwigshafen/Rhein, Germany. Syngenta File No. NOA449410_10005. Submitted to WHO by Syngenta Ltd.

Shearer J (2015). SYN545974: a 13 week toxicity study of SYN545974 by oral (dietary) administration in mice (final report amendment 2). Unpublished report no. 33011. Charles River, Tranent, Edinburgh, United Kingdom. Syngenta File No. SYN545974_10211. Submitted to WHO by Syngenta Ltd.

Shearer J, Robertson B (2015). SYN545974: a 13 week toxicity study of SYN545974 by oral (dietary) administration in rats (final report amendment 2). Unpublished report no. 33012. Charles River, Tranent, Edinburgh, United Kingdom. Syngenta File No. SYN545974_10210. Submitted to WHO by Syngenta Ltd.

Shearer J, Robertson B (2016). SYN545974 – historical control data for SYN545974_10210 and SYN545974_10211. Unpublished report. Syngenta, Jealott's Hill, Bracknell, United Kingdom. Syngenta File No. SYN545974_10448. Submitted to WHO by Syngenta Ltd.

Sieber M (2013). SYN545974: 28-day dermal toxicity study in the Wistar rat (final report amendment 1). Unpublished report no. D62072. Harlan Laboratories Ltd, Itingen, Switzerland. Syngenta File No. SYN545974_10047. Submitted to WHO by Syngenta Ltd.

Sokolowski A (2007). *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay with DF-pyrazole acid (CA4312). Unpublished report no. 1077403. RCC, Cytotest Cell Research GmbH (RCC-CCR), Rossdorf, Germany. Syngenta File No. SYN520453/0096. Submitted to WHO by Syngenta Ltd.

Sokolowski A (2012). SYN545974: *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay. Unpublished report no. 1498901. Harlan, Cytotest Cell Research GmbH (Harlan CCR), Rossdorf, Germany. Syngenta File No. SYN545974_10018. Submitted to WHO by Syngenta Ltd.

Sokolowski A (2014a). SYN545974 – *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay. Unpublished report no. 1648701. Harlan Cytotest Cell Research GmbH, Rossdorf, Germany. Syngenta File No. SYN545974_10127. Submitted to WHO by Syngenta Ltd.

Sokolowski A (2014b). SYN508272: *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay. Unpublished report no. 1555901. Harlan Cytotest Cell Research GmbH, Rossdorf, Germany. Syngenta File No. SYN508272_10908. Submitted to WHO by Syngenta Ltd.

Sommer E (2010). CSAA798670: 28-day oral (dietary) toxicity study in the Wistar rat. Unpublished report no. C57852. Harlan Laboratories Ltd, Itingen, Switzerland. Syngenta File No. NOA449410_10003. Submitted to WHO by Syngenta Ltd.

Strepka C (2012a). SYN545974, SYN546022: 28 day dietary toxicity study in mice. Unpublished report no. 32153. Charles River Laboratories Edinburgh Ltd, Tranent, Edinburgh, United Kingdom. Syngenta File No. SYN545974_10042. Submitted to WHO by Syngenta Ltd.

Strepka C (2012b). SYN545974, SYN546022: 28 day dietary toxicity study in rats. Unpublished report no. 32168. Charles River Laboratories Edinburgh Ltd, Tranent, Edinburgh, United Kingdom. Syngenta File No. SYN545974_10044. Submitted to WHO by Syngenta Ltd. Submitted to WHO by Syngenta Ltd.

Strepka C, Shearer J (2015). SYN545974 – a 28 day toxicity study of SYN545974 by the oral (capsule) route in dogs. Unpublished report no. 33167. Charles River Laboratories Edinburgh Ltd, Tranent, Edinburgh, United Kingdom. Submitted to WHO by Syngenta Ltd.

Sun G, Thai SF, Lambert GR, Wolf DC, Tully DB, Goetz AK et al. (2006). Fluconazole-induced hepatic cytochrome P450 gene expression and enzymatic activities in rats and mice. Toxicol Lett. 164(1):44–53.

Syngenta (2018). Pydiflumetofen. SYN545974. JMPR 2018. Information on animal metabolism and toxicology for evaluation. Working paper. Submission to the WHO Panel of the JMPR for review as new compound submission to the WHO for the review of pydiflumetofen. Submitted to WHO by Syngenta Ltd.

Tomlinson J, Hutton E, Green M, Allan G (2015). SYN545974 – the excretion and biotransformation of [phenyl-U-^{14}C] and [pyrazole-5-^{14}C]-SYN545974 following single oral administration in the mouse. Unpublished report no. 35415. Charles River Laboratories Edinburgh Ltd, Tranent, Edinburgh, United Kingdom. Syngenta File No. SYN545974_10257. Submitted to WHO by Syngenta Ltd.

Török-Bathó M (2012a). SYN545974 – primary skin irritation study in rabbits. Unpublished report no. 12/344-006N. CiToxLAB Hungary Ltd, Szabadságpuszta Hungary. Syngenta File No. SYN545974_10035. Submitted to WHO by Syngenta Ltd.

Török-Bathó M (2012b). SYN545974 – acute eye irritation study in rabbits. Unpublished report no. 12/344-005N. CiToxLAB Hungary Ltd, Szabadságpuszta, Hungary. Syngenta File No. SYN545974_10033. Submitted to WHO by Syngenta Ltd.

Webbley K, Williams D (2015). SYN545974 – a preliminary study of pharmacokinetics, absorption, metabolism and excretion in rats following single oral and intravenous administration of ^{14}C-SYN545974. Unpublished report no. SGA-64. Quotient Bioresearch (Rushden) Ltd, Rushden, Northamptonshire, United Kingdom. Syngenta File No. SYN545974_10188. Submitted to WHO by Syngenta Ltd.

WHO (2017). Guidelines for drinking-water quality, 4th edition, incorporating the first addendum. Geneva: World Health Organization (http://www.who.int/water_sanitation_health/publications/drinking-water-quality-guidelines-4-including-1st-addendum/en/, accessed September 2018).

Wollny H (2009). CSAA798670: cell mutation assay at the thymidine kinase locus ($TK^{+/-}$) in mouse lymphoma L5178Y cells. Unpublished report no. 1266901. Harlan Cytotest Cell Research GmbH (Harlan CCR), Rossdorf, Germany. Syngenta File No. NOA449410_10000. Submitted to WHO by Syngenta Ltd.

Wollny H-E (2013a). SYN545974 – cell mutation assay at the thymidine kinase locus (*TK*$^{+/-}$) in mouse lymphoma L5178Y cells. Unpublished report no. 1498903. Harlan Cytotest Cell Research GmbH (Harlan CCR), Rossdorf, Germany. Syngenta File No. SYN545974_10049. Submitted to WHO by Syngenta Ltd.

Wollny H (2013b). SYN508272: cell mutation assay at the thymidine kinase locus (*TK*$^{+/-}$) in mouse lymphoma L5178Y cells. Unpublished report no. 1555903. Harlan Cytotest Cell Research GmbH (Harlan CCR), Rossdorf, Germany. Syngenta File No. SYN508272_10906. Submitted to WHO by Syngenta Ltd.

Yamamoto Y, Moore R, Goldsworthy TL, Negishi M, Maronpot RR (2004). The orphan nuclear receptor constitutive active/androstane receptor is essential for liver tumor promotion by phenobarbital in mice. Cancer Res. 64(20):7197–200.

Appendix 1: List of animal metabolites

All metabolites of pydiflumetofen that have been identified in the rat are listed in Table A1-1.

Table A1-1. Summary of metabolites found in various matrices (structures, codes and synonyms)

Code number (synonyms)	Description	Compound found in:	Structure
Pydiflumetofen SYN545974 CSCD678790	(*S*)-3-Difluoromethyl-1-methyl-1*H*-pyrazole-4-carboxylic acid methoxy-[1-methyl-2-(2,4,6-trichlorophenyl)-ethyl]-amide (*R*)-3-Difluoromethyl-1-methyl-1*H*-pyrazole-4-carboxylic acid methoxy-[1-methyl-2-(2,4,6-trichlorophenyl)-ethyl]-amide	Soil (sterile, aerobic, anaerobic, photolysis) Water (photolysis) Water–sediment systems Crops (wheat, oilseed rape, tomato, rotated crops) Livestock (hen, goat) Rat	Cl Cl Cl F F O N O N N

Code number (synonyms)	Description	Compound found in:	Structure
SYN545547 CSCD550897	3-(Difluoromethyl)-1-methyl-*N*-[1-methyl-2-(2,4,6-trichlorophenyl)ethyl]pyrazole-4-carboxamide	Soil (sterile, aerobic, anaerobic, photolysis) Water (photolysis) Water–sediment systems Crops (wheat, oilseed rape, tomato, rotated crops) Livestock (hen, goat) Rat	
SYN547891 CSCV764139	3-(Difluoromethyl)-*N*-methoxy-*N*-[1-methyl-2-(2,4,6-trichlorophenyl)ethyl]-1*H*-pyrazole-4-carboxamide	Crops (wheat, oilseed rape, tomato, rotated crops) Livestock (hen, goat) Rat	

Code number (synonyms)	Description	Compound found in:	Structure
2,4,6-Trichlorophenol 2,4,6-TCP	2,4,6-Trichlorophenol	Livestock (hen, goat) Rat	OH, Cl, Cl, Cl
2,4,6-TCP sulfate	Sulfate conjugate of 2,4,6-trichlorophenol	Livestock (hen, goat) Rat	O, OH, S, O, O, Cl, Cl, Cl
SYN548264 CSCD548196 *N*-Desmethoxy SYN548263	2-[[3-(Difluoromethyl)-1-methyl-pyrazole-4-carbonyl]amino]propanoic acid	Livestock (goat) Rat	F, F, O, N H, OH, O, N, N

Code number (synonyms)	Description	Compound found in:	Structure
SYN508272 CSCC210616 R423363	3-(Difluoromethyl)-1-methyl-pyrazole-4-carboxamide	Livestock (hen, goat) Rat	
SYN547897 CSCV764146	3-(Difluoromethyl)-*N*-methoxy-1-methyl-*N*-[1-methyl-2-(2,4,6-trichloro-3-hydroxy-phenyl)ethyl]pyrazole-4-carboxamide	Livestock (hen, goat) Rat	
SYN548263 CSCZ159698	2-[[3-(Difluoromethyl)-1-methyl-pyrazole-4-carbonyl]-methoxy-amino]propanoic acid	Livestock (goat) Rat	

Code number (synonyms)	Description	Compound found in:	Structure
SYN547948 CSCY608054	3-(Difluoromethyl)-*N*-[2-hydroxy-1-methyl-2-(2,4,6-trichlorophenyl)ethyl]-*N*-methoxy-1-methyl-pyrazole-4-carboxamide	Livestock (hen, goat) Rat	
Hydroxylated SYN545974	Hydroxylated *N*-methoxy-*N*-[1-methyl-2-(2,4,6-trichlorophenyl)-ethyl]-3-(difluoromethyl)-1-methylpyrazole-4-carboxamide	Livestock (goat) Rat	

Code number (synonyms)	Description	Compound found in:	Structure
N-Desmethyl SYN547890	3-(Difluoromethyl)-*N*-hydroxy-*N*-[1-methyl-2-(2,4,6-trichlorophenyl)ethyl]-1*H*-pyrazole-4-carboxamide	Rat	
Desmethyl SYN548263 CSCZ159698	Desmethyl 2-[[3-(difluoromethyl)-1-methyl-pyrazole-4-carbonyl]-methoxy-amino]propanoic acid	Rat	
SYN548265	3-(Difluoromethyl)-*N*-(2-hydroxy-1-methyl-ethyl)-*N*-methoxy-1-methyl-pyrazole-4-carboxamide	Rat	

Code number (synonyms)	Description	Compound found in:	Structure
SYN547893 CSCD677133	*N*-[2-(2,4-Dichlorophenyl)-1-methyl-ethyl]-3-(difluoromethyl)-*N*-methoxy-1-methyl-pyrazole-4-carboxamide	Rat	
SYN547894 CSCV764141	*N*-[2-(2,6-Dichloro-4-hydroxy-phenyl)-1-methyl-ethyl]-3-(difluoromethyl)-*N*-methoxy-1-methyl-pyrazole-4-carboxamide	Rat	

Code number (synonyms)	Description	Compound found in:	Structure
SYN547890	3-(Difluoromethyl)-*N*-hydroxy-1-methyl-*N*-[1-methyl-2-(2,4,6-trichlorophenyl)ethyl]pyrazole-4-carboxamide	Rat	
Hydroxylated TCP-sulfate HTCP sulfate	(2,4,6-Trichloro-3-hydroxyphenyl) hydrogen sulfate	Rat	

Code number (synonyms)	Description	Compound found in:	Structure
TCPM glucuronide	2,4,6-Trichloro-3-(methoxymethyl)phenol glucuronide	Rat	
Hydroxylated SYN545974 Sulfate conjugate	Hydroxylated *N*-methoxy-*N*-[1-methyl-2-(2,4,6-trichlorophenyl)-ethyl]-3-(difluoromethyl)-1-methylpyrazole-4-carboxamide Sulfate conjugate	Rat	

Code number (synonyms)	Description	Compound found in:	Structure
Hydroxylated SYN545974 Glucuronide conjugate	Hydroxylated *N*-methoxy-*N*-[1-methyl-2-(2,4,6-trichlorophenyl)-ethyl]-3-(difluoromethyl)-1-methylpyrazole-4-carboxamide Glucuronide conjugate	Rat	HO; glucuronide; F; F; O; N; O; N; N; Cl; Cl; Cl
Dihydroxy SYN545974	Dihydroxylated *N*-methoxy-*N*-[1-methyl-2-(2,4,6-trichlorophenyl)-ethyl]-3-(difluoromethyl)-1-methylpyrazole-4-carboxamide	Rat	HO; HO; F; F; O; N; O; N; N; Cl; Cl; Cl

Code number (synonyms)	Description	Compound found in:	Structure
Dihydroxy SYN545974 Glucuronide conjugate	Dihydroxylated *N*-methoxy-*N*-[1-methyl-2-(2,4,6-trichlorophenyl)-ethyl]-3-(difluoromethyl)-1-methylpyrazole-4-carboxamide Glucuronide conjugate	Rat	
Dechlorinated hydroxy SYN545974 Glucuronide conjugate	Dechlorinated hydroxylated *N*-methoxy-*N*-[1-methyl-2-(2,4,6-trichlorophenyl)-ethyl]-3-(difluoromethyl)-1-methylpyrazole-4-carboxamide Glucuronide conjugate	Rat	

Code number (synonyms)	Description	Compound found in:	Structure
[Code not provided]	[Description not provided]	Rat	
Hydroxy SYN545547	Hydroxylated 3-(difluoromethyl)-1-methyl-*N*-[1-methyl-2-(2,4,6-trichlorophenyl)ethyl]pyrazole-4-carboxamide	Rat	

Code number (synonyms)	Description	Compound found in:	Structure
Hydroxy SYN547891	Hydroxylated 3-(difluoromethyl)-*N*-methoxy-*N*-[1-methyl-2-(2,4,6-trichlorophenyl)ethyl]-1*H*-pyrazole-4-carboxamide	Rat	
SYN547891 Glucuronide conjugate	3-(Difluoromethyl)-*N*-methoxy-*N*-[1-methyl-2-(2,4,6-trichlorophenyl)ethyl]-1*H*-pyrazole-4-carboxamide Glucuronide conjugate	Rat	

Code number (synonyms)	Description	Compound found in:	Structure
Desmethyl hydroxy SYN545974	Hydroxylated *N*-methoxy-*N*-[1-methyl-2-(2,4,6-trichlorophenyl)-ethyl]-3-(difluoromethyl)-1-methylpyrazole-4-carboxamide	Rat	
Desmethyl SYN545974 Glucuronide conjugate	Desmethyl *N*-methoxy-*N*-[1-methyl-2-(2,4,6-trichlorophenyl)-ethyl]-3-(difluoromethyl)-1-methylpyrazole-4-carboxamide Glucuronide conjugate	Rat	

Code number (synonyms)	Description	Compound found in:	Structure
Desmethyl hydroxy SYN545974 Sulfate conjugate	3-(Difluoromethyl)-*N*-methoxy-*N*-[1-methyl-2-(2,4,6-trichlorophenyl)ethyl]-1*H*-pyrazole-4-carboxamide Sulfate conjugate	Rat	
Desmethyl hydroxy SYN545974 Glucuronide conjugate	Hydroxylated *N*-methoxy-*N*-[1-methyl-2-(2,4,6-trichlorophenyl)-ethyl]-3-(difluoromethyl)-1-methylpyrazole-4-carboxamide Glucuronide conjugate	Rat	

Code number (synonyms)	Description	Compound found in:	Structure
Desmethyl SYN545974 Glucuronide conjugate	3-(Difluoromethyl)-*N*-methoxy-*N*-[1-methyl-2-(2,4,6-trichlorophenyl)ethyl]-1*H*-pyrazole-4-carboxamide Glucuronide conjugate	Rat	
Desmethyl SYN548265 Glucuronide conjugate	Desmethyl 3-(difluoromethyl)-*N*-(2-hydroxy-1-methyl-ethyl)-*N*-methoxy-1-methyl-pyrazole-4-carboxamide Glucuronide conjugate	Rat	

Code number (synonyms)	Description	Compound found in:	Structure
SYN548265 Glucuronide conjugate	3-(Difluoromethyl)-*N*-(2-hydroxy-1-methyl-ethyl)-*N*-methoxy-1-methyl-pyrazole-4-carboxamide Glucuronide conjugate	Rat	F F O OH N O N N glucuronide
Dechlorinated hydroxy thiomethyl SYN545974	Dechlorinated hydroxy thiomethyl *N*-methoxy-*N*-[1-methyl-2-(2,4,6-trichlorophenyl)-ethyl]-3-(difluoromethyl)-1-methylpyrazole-4-carboxamide	Rat	Cl SCH_3 OH F O Cl F N O N N

Code number (synonyms)	Description	Compound found in:	Structure
Dechlorinated hydroxy thiomethyl SYN545974 Glucuronide conjugate	Dechlorinated hydroxy thiomethyl *N*-methoxy-*N*-[1-methyl-2-(2,4,6-trichlorophenyl)-ethyl]-3-(difluoromethyl)-1-methylpyrazole-4-carboxamide Glucuronide conjugate	Rat	
Dechlorinated dihydroxy thiomethyl SYN545974	Dechlorinated dihydroxy thiomethyl *N*-methoxy-*N*-[1-methyl-2-(2,4,6-trichlorophenyl)-ethyl]-3-(difluoromethyl)-1-methylpyrazole-4-carboxamide	Rat	

Code number (synonyms)	Description	Compound found in:	Structure
Dechlorinated hydroxy SYN545974 Cysteine conjugate	Dechlorinated hydroxy *N*-methoxy-*N*-[1-methyl-2-(2,4,6-trichlorophenyl)-ethyl]-3-(difluoromethyl)-1-methylpyrazole-4-carboxamide Cysteine conjugate	Rat	
Hydroxy SYN545974 Sulfate conjugate	Hydroxy *N*-methoxy-*N*-[1-methyl-2-(2,4,6-trichlorophenyl)-ethyl]-3-(difluoromethyl)-1-methylpyrazole-4-carboxamide Sulfate conjugate	Rat	

Code number (synonyms)	Description	Compound found in:	Structure
Hydroxy SYN547891 Sulfate conjugate	Hydroxy 3-(difluoromethyl)-*N*-methoxy-*N*-[1-methyl-2-(2,4,6-trichlorophenyl)ethyl]-1*H*-pyrazole-4-carboxamide Sulfate conjugate	Rat	SO_4H – [structure: F, F, O, N, O, N, N, H, Cl, Cl, Cl]

Note: The majority of the nomenclature used in the table above was provided by the sponsor.

Appendix 2: Mode of action of pydiflumetofen-induced mouse liver tumours: analysis of their human relevance using the IPCS/ILSI framework[1]

The hypothesized MOA for pydiflumetofen-induced liver tumours and their relevance to humans have been assessed using an established international framework: that of WHO's IPCS and ILSI.

Carcinogenicity of pydiflumetofen

The carcinogenicity of pydiflumetofen has been evaluated in 104- and 80-week bioassays in the rat and mouse, respectively (Robertson, 2015, 2016). The treatment-related neoplastic finding was liver tumours in male mice at 375 ppm and higher (Table A2-1). No treatment-related neoplastic findings were observed in rats of either sex or in female mice. The NOAEL for liver tumours in male mice was 75 ppm.

Table A2-1. Liver carcinogenicity[a] of pydiflumetofen in the male CD-1 mouse

Tumour type	0 ppm	75 ppm	375 ppm	2 250 ppm	Charles River Laboratories (Edinburgh) historical control data
No. of animals examined	*50*	*50*	*49*	*50*	*247*
Hepatocellular adenoma	4 (8%) [0]	6 (12%) [0]	9 (18.4%) [7]**	22**, $$$ (44%) [14]**	45 (18.2%) (range = 10.0–28.0%)
Hepatocellular carcinoma	2 (4%) [0]	3 (6%) [0]	4 (8.2%) [0]	10* (20%) [2]	19 (7.7%) (range = 6.0–10.0%)

ppm: parts per million; *: $P \leq 0.05$; **: $P \leq 0.01$ (Fisher's exact test); $$$: $P \leq 0.001$ (Peto trend test)

[a] Tumour incidences are expressed as total per group (percentage incidence) [number with multiple tumours].

Source: Robertson (2015)

Postulated mode of action and key events

A number of time- and dose-related key events have been identified to characterize the MOA for pydiflumetofen-induced liver tumours in male mice. Integrating all evidence, the proposed non-genotoxic MOA is initiated by CAR activation.

The essential key event, CAR activation, in male mice results in altered expression of CAR-responsive genes, leading to CAR-mediated stimulation of cell proliferation, including replicative DNA synthesis. This promotes an environment permissive for increased cell replication, which can result in a higher rate of spontaneous mutations due to normal replication errors. Suppression of apoptosis promotes an alteration of environment that would allow a spontaneous cell mutation to clonally expand before it could be removed by the normal control process of apoptosis. Over time, transformed cells progress to eosinophilic foci of cellular alteration, a preneoplastic change, with clonal expansion, eventually leading to the development of liver tumours. The eosinophilic foci of hepatocellular alteration or eosinophilic adenomas were reported to be induced in mice treated with phenobarbital or chemicals that activate mouse CAR (Yamamoto et al., 2004; Sakamoto et al., 2013). The activation of CAR and subsequent burst of cellular proliferation are considered to be key events in the tumour MOA, being necessary and directly resulting in the induction of liver tumours in the mouse.

[1] Adapted from Cowie (2015).

In addition to the CAR activation in male mice, a number of other genes, including some coding for members of specific cytochrome P450 families of isozymes, particularly those of *Cyp2b* and, to a lesser extent, *Cyp3a* and *Cyp2a* subfamilies, were induced. The effects on cytochrome P450s are a characteristic hallmark of CAR activation, but associative events of the proposed MOA – that is, they are not central to the induction of liver tumours. A further associative event is liver hepatocellular hypertrophy, which is caused by proliferation of the smooth endoplasmic reticulum as a consequence of cytochrome P450 induction. This hypertrophy, in combination with the increased hepatocyte proliferation, in turn results in an increase in liver weight.

The MOA outlining the key and associative events is shown in Fig. A2-1.

Fig. A2-1. MOA hypothesis for pydiflumetofen-induced liver tumour formation in male mice

Exposure of Liver to SYN545794

Activation of CAR/PXR

Increased expression of cytochrome P450 (*Cyp2b*/*Cyp3a*)

Transiently increased hepatocellular proliferation

Hepatocellular hypertrophy

Spontaneous initiation

Increased liver weight

Hepatocellular foci

Potential for increased incidence of Hepatocellular adenoma/carcinoma

Legend: Key Event; Associative Event; Tumour Outcome

CAR: constitutive androstane receptor; PXR: pregnane X receptor
Source: Cowie (2015)

After the key events within a postulated MOA have been described, the IPCS/ILSI framework and Hill criteria require that they be evaluated by a standardized weight of evidence evaluation. In this process, the key events that are causally related to the formation of tumours must be shown to be required steps that lead to the tumour, and the findings must be shown to be reproducible. A weight of evidence analysis for pydiflumetofen-induced liver tumour formation is described in the following sections.

Relevant data for the assessment of the MOA hypothesis for pydiflumetofen

The following studies were considered when evaluating the MOA hypothesis for pydiflumetofen:

- an assessment of the ability of pydiflumetofen to activate CAR (Omiecinski, 2014);
- three studies with MOA end-points that assess the dietary toxicity of pydiflumetofen over a period of 28 days (Haines, 2012; Strepka, 2012a; Elcombe, 2015);

- an assessment of the subchronic (13-week) toxicity in the CD-1 mouse (Shearer, 2015);
- an 80-week carcinogenicity study in the CD-1 mouse (Robertson, 2015); and
- investigative studies in primary cultures of mouse and human hepatocytes to assess mechanistic end-points with respect to human relevance (Lowes, 2015a,b).

Concordance of dose–response relationships

For the key events in a proposed MOA to be causally related to the formation of tumours, they must show concordance with the doses that produce tumours (dose concordance). Table A2-2 summarizes the dose concordance of the key and associative events (as defined in the hypothesized MOA) with the tumour outcome. This table considers concordance with the following doses:

- 75 ppm as the NOAEL for liver tumours in male CD-1 mice; and
- 2250 ppm as a carcinogenic concentration that induces liver tumours in male CD-1 mice.

Table A2-2 demonstrates that it is only at the tumorigenic dose (2250 ppm) that all of the key (and associative) events are observed. Early effects at the non-tumorigenic dose (75 ppm) are confined to a small increase in hepatocellular proliferation, which is probably indicative of a low level of CAR activation of insufficient magnitude to stimulate any of the other key or associative events. This is supported by the lack of any other key or associative events occurring at the non-tumorigenic dose, including no effects on liver weight or liver histopathology findings at any study duration. The absence of any other histopathology changes, including no evidence of increased adenomas or carcinomas, and in light of the CAR-mediated MOA for liver tumours that is fully described in this MOA framework appendix, the marginal increase in cell proliferation at 75 ppm is considered not to be a human-relevant adverse effect. Taken together, it can be concluded that there is strong concordance between key (and associative) events and doses that produce tumours.

Table A2-2. Dose concordance of key and associative events obtained from all data on pydiflumetofen in mice

Event	**75 ppm**	**2 250 ppm**
CAR activation (in vivo)[a]	No	Yes
Induction of *Cyp2b/Cyp3a* (PROD activity)	No	Yes
Increase in hepatocellular replicative DNA synthesis (2–28 days)	Yes (slight)	Yes
Increased mitosis[b]	No	Yes
Increased hepatocellular hypertrophy[b]	No	Yes
Increased liver weight	No	Yes
Increased incidence of hepatocellular foci[b]	No	Yes
Increased incidence of liver tumours[b]	No	Yes

CAR: constitutive androstane receptor; Cyp: cytochrome P450; DNA: deoxyribonucleic acid; ppm: parts per million; PROD: pentoxyresorufin *O*-depentylation

[a] CAR activation as assessed by surrogate markers of CAR activation from in vivo studies (e.g. *Cyp2b*-mediated PROD activity). The intrinsic ability of pydiflumetofen to directly activate mouse CAR also has been demonstrated using an in vitro transactivation assay.

[b] Effects observed in histopathology examinations of the liver.

Source: Cowie (2015)

Temporal association

Liver tumours were clearly induced at 2250 ppm. The observed effects on parameters associated with the key and associative events must occur in a logical, time-dependent manner

consistent with the proposed MOA (see Fig. A2-1). The temporal concordance for pydiflumetofen-induced liver tumours in the male CD-1 mouse database is outlined below:

- Activation of CAR was evident after 2 days of exposure (based on increases in *Cyp2b10* [PROD] activity) and likely occurred as soon as pydiflumetofen was absorbed and transported to the liver. Surrogate markers of specific CAR activity were only measured to day 28 of exposure; however, other, less specific markers of CAR activation, such as liver weight increases, were evident in treatment durations of up to 80 weeks.
- Increases in cell proliferation of hepatocytes were evident from day 2 through day 28 (the last time point tested). Proliferative indices were maximal after day 2, when cell proliferation was associated with increased mitosis, and decreased with time. Consistent with the typical pattern of CAR activation, the histopathological evidence of increased mitosis was not present at day 7 and beyond, indicating a transient stimulation of cell division.
- Elevated cytochrome P450, measured as both total cytochrome content and PROD activity, was evident after 2 days of exposure and likely occurred throughout the exposure period (day 28 was the latest time point tested).
- Elevation of centrilobular hypertrophy was evident after 2 days of exposure to pydiflumetofen, and the corresponding increase in liver weight was statistically significant after 7 days of treatment. These increases persisted throughout the duration of both the subchronic and chronic mouse studies.
- Increased hepatocellular foci and hepatocellular adenomas and carcinomas were evident after 80 weeks of treatment.

Overall, the progression of changes following CAR activation in the liver by pydiflumetofen followed a logical time course, with the earlier key events preceding the ultimate progression to adenomas and carcinomas in the liver (Table A2-3).

Table A2-3. Temporal concordance of key and associative events obtained from all data on pydiflumetofen (2250 ppm) in mice

Key/associative event	Day 2	Day 3	Day 7	Day 28	Day 90	Day 560
CAR activation	Yes	Yes	Yes	Yes	–[a]	–[a]
Hepatocellular replicative DNA synthesis	Yes	–[a]	Yes	Yes	–	–
Increased mitosis	Yes	–	No	No	–	–
Elevated *Cyp2b/Cyp3a* levels (PROD activity)	Yes	Yes	Yes	Yes	–[a]	–[a]
Elevated liver weight	No	Yes	Yes	Yes	Yes	Yes
Centrilobular hypertrophy	No	No	Yes	Yes	Yes	Yes
Hepatocellular foci	No	No	No	No	No	Yes
Tumours	No	No	No	No	No	Yes

–: no data; No: key/associative event not occurring; Yes: key/associative event occurring
[a] Presumed to still be occurring due to effects observed at earlier times and other associative events occurring.
Source: Cowie (2015)

Strength, consistency and specificity of association of tumour response with key events

All data related to the toxicity or carcinogenicity of pydiflumetofen have been integrated. There is clear consistency across the database. The activation of CAR was measured either directly or via alterations in its surrogate markers of enzyme induction, hepatocyte proliferation, hypertrophy and

hepatomegaly and was seen consistently, across multiple studies, at several time points before the observation of an increased incidence of liver tumours. CAR activation was confirmed both via a direct in vitro transactivation assay and via surrogate markers across multiple studies, which included increased PROD activity. PROD activity is catalysed primarily by CYP2B family enzymes in mice or rats, but with some contribution from other cytochrome P450s, including those in the CYP3A subfamily. In mice, CAR activation has been shown to produce a very large (>100-fold) induction of *Cyp2b10* in particular, with lesser induction of *Cyp3a11* and other cytochrome P450s; therefore, the consistent large increases in PROD activity in multiple mouse studies with pydiflumetofen help confirm the sustained activation of CAR in the liver at higher doses.

The specificity of the hepatocellular adenomas and carcinomas induced by pydiflumetofen in male mice was demonstrated (Robertson, 2015). Studies have shown that although certain key and associative events such as CAR activation and increased liver weight do occur in the rat (Omiecinski, 2014), tumours were not observed in the 104-week carcinogenicity study in the rat with pydiflumetofen (Robertson, 2016). This highlights that CAR activation, while being a necessary causal key event in the proposed MOA for mouse liver tumour formation, is not sufficient by itself. Ample evidence in the literature has shown that mice (particularly male mice) are more prone to liver tumours than rats.

In all data integrated, a very weak proliferative signal after exposure to 75 ppm pydiflumetofen was observed in the 2-day investigative study; however, this key event occurred in isolation, and no statistically significant increases in surrogate markers of CAR activation (*Cyp2b10*-mediated PROD activity), no increases in mitosis and no alterations in liver weight or histopathology in studies up to 90 days in duration were observed. In the 80-week carcinogenicity study, consistent with what was observed in the short-term studies, no effects on liver weight were observed, and, ultimately, no increases in tumour incidence were observed at 75 ppm. This reinforces that a small increase in a necessary causal key event in the proposed MOA for mouse liver tumour formation (hepatocellular proliferation) is not sufficient in isolation to result in a tumorigenic response.

Biological plausibility and coherence

The liver is by far the most common target tissue affected in cancer bioassays. When evaluating liver tumour susceptibility, two metrics are typically used: incidence and multiplicity. The former is most often used to characterize spontaneous liver neoplasia, and the latter to assign relative susceptibility to treatment-induced liver neoplasia. The carcinogenicity database for liver tumour induction in the mouse with pydiflumetofen supports the promotor, non-genotoxic MOA, which is known to increase the incidence of spontaneous age-related tumours without necessarily decreasing the tumour latency. Several MOAs have been identified for the induction of liver tumours in rodents, including activation of nuclear receptors such as CAR, peroxisome proliferator–activated receptor (PPAR) and aryl hydrocarbon receptor (AhR).

Integrated data from multiple studies with pydiflumetofen demonstrating the temporal and dose concordance of the key events are summarized in Tables A2-2 and A2-3, respectively. These data are consistent with the current understanding of the necessary key events in the MOA for CAR activators. Thus, the data for pydiflumetofen support a biologically plausible MOA by a mechanism that is well understood and supported by extensive published research.

Other MOAs

Several MOAs have been identified for liver carcinogenesis, both in humans and in rodent models (Table A2-4). Some of the key events described for pydiflumetofen are common to other known MOAs. However, a full examination of the available database for pydiflumetofen can be used to exclude the alternative MOAs.

Table A2-4. Alternative MOAs for hepatocarcinogenesis

Alternative MOA	Pydiflumetofen: reason for exclusion
DNA reactivity	Pydiflumetofen has been demonstrated to be negative in in vitro bacterial and mammalian mutagenicity assays (Sokolowski, 2012, 2014a; Bohnenberger, 2013a; Wollny, 2013a). Pydiflumetofen has been demonstrated to be negative in in vivo assays for clastogenicity and aneugenicity (Roth, 2012; Dony, 2014a). Late onset of tumours is not indicative of a DNA-reactive mechanism.
Peroxisome proliferator	Pydiflumetofen does not induce peroxisomal palmitoyl CoA oxidase or lauric acid 12-hydroxylase (marker for *Cyp4a*) activities in microsomal mouse liver preparations (Haines, 2012). A slight decrease in palmitoyl CoA oxidase activity with increasing dose was observed, and prior work has established that CAR activators suppress the activation of the PPARα receptor. A 2- to 3-fold increase in lauric acid 12-hydroxylase activity was observed with pydiflumetofen, and similar small increases in this enzyme activity by CAR activators have been shown previously in mice, in contrast to very large increases in this marker of *Cyp4a* activity by PPARα activators (12-fold).
Enzyme induction (AhR)	Pydiflumetofen does not induce substantial EROD activity (a marker for *Cyp1a*) in microsomal male mouse liver preparations (Haines, 2012). Pydiflumetofen caused a 2- to 3-fold increase in EROD activity after 3 or 7 days of treatment, but no effects after 28 days. Small increases in EROD by CAR activators have been demonstrated previously (Sun et al., 2006) and are possibly due to some metabolism via induced CYP2B isoenzymes; these minor changes are in contrast to the large increases by AhR activators.
Statins	Pydiflumetofen was not designed to inhibit HMG-CoA reductase, and pydiflumetofen treatment does not result in decreased cholesterol (Shearer, 2015).
Cytotoxicity	Pydiflumetofen is not a hepatic cytotoxicant based on (a) lack of evidence of elevated hepatic damage in clinical chemistry markers and (b) lack of histopathological evidence of sustained inflammation, necrosis or regenerative proliferation in the liver (Strepka, 2012a; Elcombe, 2015; Robertson, 2015; Shearer, 2015).
Infection	Excluded based on lack of evidence of infection, cellular inflammatory response or regenerative proliferation (Robertson, 2015, 2016; Shearer, 2015).
Increased apoptosis	There is no histological evidence that pydiflumetofen increases hepatocyte apoptosis (Robertson, 2015, 2016; Shearer, 2015).
Estrogenic activity	In the large mammalian toxicological database available for pydiflumetofen, including the studies summarized in this appendix, as well as studies of the effects of pydiflumetofen on reproduction and development (Davies, 2015; Hackford, 2015; Penn, 2015c), there is no evidence for estrogenic stimulation.

AhR: aryl hydrocarbon receptor; CAR: constitutive androstane receptor; CoA: coenzyme A; Cyp: cytochrome P450; DNA: deoxyribonucleic acid; EROD: ethoxyresorufin *O*-deethylation; HMG-CoA: 3-hydroxy-3-methyl-glutaryl-coenzyme A; PPARα: peroxisome proliferator–activated receptor-alpha
Source: Cowie (2015)

Liver carcinogens can be divided into those that are DNA reactive and those that are non–DNA reactive, and both produce their carcinogenic effect by increasing cell proliferation. DNA reactivity can be excluded, as pydiflumetofen tested negative in a battery of in vitro and in vivo genetic toxicity studies. Furthermore, the tumour profile observed in pydiflumetofen carcinogenicity bioassays is typical of a non-genotoxic mechanism (single species, single sex, single organ and lack of decreased latency).

MOAs involving induction of other cytochrome P450 isoforms or peroxisome proliferation can also be excluded based on the nature of the liver findings. Increased liver weight and hepatocellular

hypertrophy are not specific surrogate markers for CAR activation, because the induction of other cytochrome P450 isoforms or peroxisome proliferation can also produce these findings. However, these other MOAs can be ruled out because the experimental evidence shows that pydiflumetofen treatment does not result in any observable biochemical evidence for peroxisome proliferation within the liver hepatocytes; and no substantial increase was observed in EROD activity, which is a marker for *Cyp1a* and AhR activation (Haines, 2012). Hepatocellular cytotoxicity with subsequent regenerative proliferation, such as that caused by chloroform, is another mechanism by which carcinogenesis can occur. This mechanism is characterized by sustained diffuse necrosis and cellular proliferation; however, it can be excluded for pydiflumetofen because of the experimental evidence demonstrating a lack of hepatic damage and regenerative proliferation at all time points investigated. The exclusion of other MOAs for pydiflumetofen induction of liver tumours is detailed in Table A2-4**.**

Uncertainties, inconsistencies and data gaps

The available data support the proposed MOA for the increased incidence of liver tumours in the male CD-1 mouse and exclude the alternative MOAs as described. No critical uncertainties, inconsistencies or data gaps have been identified. The only minor inconsistency in the database for pydiflumetofen is in one 28-day dietary study in CD-1 mice, where there was a lack of observed centrilobular hypertrophy in the presence of increased cytochrome P450 activity and hepatomegaly (Strepka, 2012a). This is in contrast to centrilobular hypertrophy being observed, associated with these events, in numerous other studies of similar or longer duration (Elcombe, 2015; Robertson, 2015; Shearer, 2015).

Assessment of the postulated MOA

The concordance analysis presented above has established that the proposed key events resulting in the liver tumour response are well documented and reproducible across many studies and exhibit a strong dose–response relationship and temporal consistency with the tumour end-point. In addition, this is a well-established MOA based on prior publications, and the parameters essential for describing the MOA have been presented for pydiflumetofen.

Therefore, there is a high level of confidence that the postulated MOA was responsible for the tumour outcome in the male CD-1 mouse.

Human relevance of postulated MOA

Once an MOA in mice has been established, the relevance to humans is assessed by examining each key event. Based on experimental data with pydiflumetofen as well as published information, each key event is assessed to determine whether it is plausible in humans based on 1) qualitative differences or 2) quantitative differences between species in toxicokinetics and toxicodynamics. Table A2-5 describes the similarities and differences between mice and humans in relation to each key event.

Table A2-5. Interspecies comparison of key events for pydiflumetofen

	Mouse		Human		
Key event	**In vivo**	**In vitro**	**In vitro**	**In vivo**	**Comments**
Activation of CAR and altered gene expression	Yes	Yes	Yes	Likely	Activation of both human and mouse CAR occurs
Cytochrome P450 induction (*Cyp2b* and *Cyp3a* isoforms)	Yes	Yes	Yes	Likely	Based on large increase in PROD activities

Key event	Mouse		Human		Comments
	In vivo	In vitro	In vitro	In vivo	
Cell proliferation	Yes	Yes	No	Unlikely[a]	[a] Based on in vitro/in vivo correlation
Tumours	Yes	N/A	N/A	Unlikely[b]	[b] Based on lack of cell proliferation and data on human drugs that are CAR activators

N/A: not applicable
Source: Cowie (2015)

PROD activity has been reported to be catalysed primarily by CYP2B family enzymes in mice and rats, but with some contribution from other cytochrome P450 isoforms, including those in the CYP3A subfamily. With human primary hepatocytes and purified cytochrome P450 isoforms, PROD activity has been shown to correlate with human CYP2B6 activity, although other isoforms may also contribute.

CAR activation

The data generated with in vitro CAR reporter gene assays clearly demonstrate that pydiflumetofen was a direct activator of mouse and human CAR. Further, the concentrations at which maximal CAR activation was attained provide evidence that pydiflumetofen may have greater potency with mouse CAR than with rat or human CAR. The downstream effects that follow human CAR activation were also tested in a human hepatocyte system. Testing human male donor hepatocytes in primary culture with pydiflumetofen resulted in elevations of PROD and BROD activities, which are surrogate markers of *Cyp2b/Cyp3a* induction produced via CAR activation (Lowes, 2015b). Similar results were obtained in the mouse (Lowes, 2015a). As activation of CAR and induction of *Cyp2b/Cyp3a* isoforms occurred in both mouse and human hepatocytes, there is no qualitative difference between human and mouse in terms of the ability of pydiflumetofen to activate CAR.

Therefore, the human is not qualitatively different from the mouse in terms of the initial key event, pydiflumetofen-induced CAR activation.

Hepatocyte proliferation

The ability of pydiflumetofen to increase the proliferation of human hepatocytes was evaluated by treating male donor hepatocytes in primary culture with pydiflumetofen and measuring replicative DNA synthesis by BrdU labelling index as the marker of increased S-phase and proliferation (Lowes, 2015b). Similar experiments were performed using mouse hepatocytes in primary culture (Lowes, 2015a). At doses ranging from 5 to 35 µmol/L, pydiflumetofen did not increase BrdU labelling in male human hepatocytes, whereas a robust increase in labelling index was observed in male mouse hepatocytes (Lowes, 2015a,b). In addition to this, the positive control CAR activator phenobarbital (PB) displayed a similar pattern to pydiflumetofen, causing increased BrdU labelling of mouse hepatocytes, but no effect in male human hepatocytes.

This response is typical of that seen with other CAR activators, such as PB, sulfoxaflor and metofluthrin.

Therefore, the human is qualitatively different from the mouse in terms of the second key event in the MOA for pydiflumetofen, hepatocellular proliferation induced by CAR activation.

Statement of confidence, analysis and implications

Based on the above, there is strong evidence to support an MOA for pydiflumetofen-induced hepatocellular tumours in male mice that involves *Cyp2b/Cyp3a* induction through CAR activation and an increase in hepatocyte proliferation that results ultimately in an increase in the incidence of liver tumours. Based on the human evidence presented above, using comparative studies in hepatocytes, there is strong evidence that qualitative species differences exist between mouse and human for the pivotal key event of increased hepatocellular proliferation; pydiflumetofen did not produce cell proliferation in human hepatocytes. Based on these species differences in response, pydiflumetofen is unlikely to cause cell proliferation in humans in vivo, and it is therefore unlikely to cause tumours in humans (Table A2-1; Figs A2-1 and A2-2). This conclusion is supported further by epidemiology studies that show a lack of tumour response in patients treated with known CAR activators, which share the same MOA as pydiflumetofen.

Conclusion

Based on the available data, the MOA for liver tumour induction in male CD-1 mice treated with pydiflumetofen was established. This MOA involves key events that include an initial activation of CAR, altered CAR-dependent gene transcription, and a critical key event of increased cell proliferation. Based on the qualitative species differences in the hepatocellular proliferation response to pydiflumetofen, it has been established that this MOA is not relevant to humans (Fig. A2-2).

Fig. A2-2. Summary of species differences in response to pydiflumetofen

Source: Cowie (2015)

Therefore, these data support the conclusion that pydiflumetofen does not pose a hepatocarcinogenic hazard to humans.

Appendix 3: Blood concentrations of pydiflumetofen in toxicity studies

Thirteen-week study in mice (Shearer, 2015)

Blood samples (approximately 0.1 mL) were obtained from the saphenous vein of all animals (five mice of each sex at each time point) in each group for the analysis of pydiflumetofen in whole blood. The samples were collected at 07:00, 11:00, 15:00 and 18:00 on days 2, 16, 30 and 91. Control group (0 ppm) samples were collected prior to those from the treatment groups. Fifty microlitres of whole blood from each sample was mixed with 50 µL of deionized water and stored at −80 °C prior to analysis. Concentrations of pydiflumetofen were determined using a suitable liquid chromatography–tandem mass spectrometry (LC-MS/MS) analytical method. A research method was established by preparing calibration and quality control samples over the range 5–5000 ng/mL.

The mean concentrations are shown in Table A3-1 and Fig. A3-1.

Table A3-1. Group mean blood/water[a] concentrations of pydiflumetofen in mice over a 13-week period

	Group mean[b] blood/water concentrations (ng/mL)									
	0 ppm		100 ppm		500 ppm		4 000 ppm		7 000 ppm	
Time	M	F	M	F	M	F	M	F	M	F
Day 2										
07:00	<LLOQ	<LLOQ	<LLOQ	12.0	<LLOQ	21.9	<LLOQ	1.9	98.0	103.4
11:00	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	16.3	20.3	73.5	50.9	248.2
15:00	–[c]	<LLOQ	1.1	5.9	3.9	8.5	27.2	116.8	101.5	142.2
18:00	–[c]	–[c]	4.1	5.3	6.2	14.7	31.6	105.1	66.5	390.2
Day 16										
07:00	<LLOQ	<LLOQ	1.3	3.8	8.5	101.7	138.3	335.4	239.2	633.8
11:00	–[c]	–[c]	9.5	4.2	2.4	33.3	256.6	181.5	85.1	329.2
15:00	–[c]	<LLOQ	<LLOQ	13.9	2.8	29.8	39.3	93.2	64.1	182.3
18:00	<LLOQ	–[c]	1.4	3.0	3.6	41.3	100.2	715.2	143.4	705.4
Day 30										
07:00	–[c]	<LLOQ	5.7	<LLOQ	2.2	14.6	21.7	36.2	52.0	108.8
11:00	<LLOQ	–[c]	149.1	3.6	5.4	3.7	30.6	44.5	64.3	310.8
15:00	–[c]	–[c]	<LLOQ	1.5	2.9	15.5	25.6	27.3	43.3	53.1
18:00	<LLOQ	2.8	2.8	1.9	1.9	32.3	17.3	42.2	38.9	217.0
Day 91										
07:00	–[c]	<LLOQ	<LLOQ	1.1	4.3	21.8	65.0	139.8	118.4	770.1
11:00	<LLOQ	<LLOQ	1.7	3.4	<LLOQ	11.1	110.5	138.4	45.2	194.0
15:00	–[c]	<LLOQ	<LLOQ	1.1	3.8	21.5	13.1	46.9	122.4	132.1
18:00	<LLOQ	<LLOQ	<LLOQ	1.3	<LLOQ	8.7	28.4	190.7	60.6	122.2

F: females; LLOQ: lowest limit of quantification (<5.0 ng/mL); M: males; ppm: parts per million

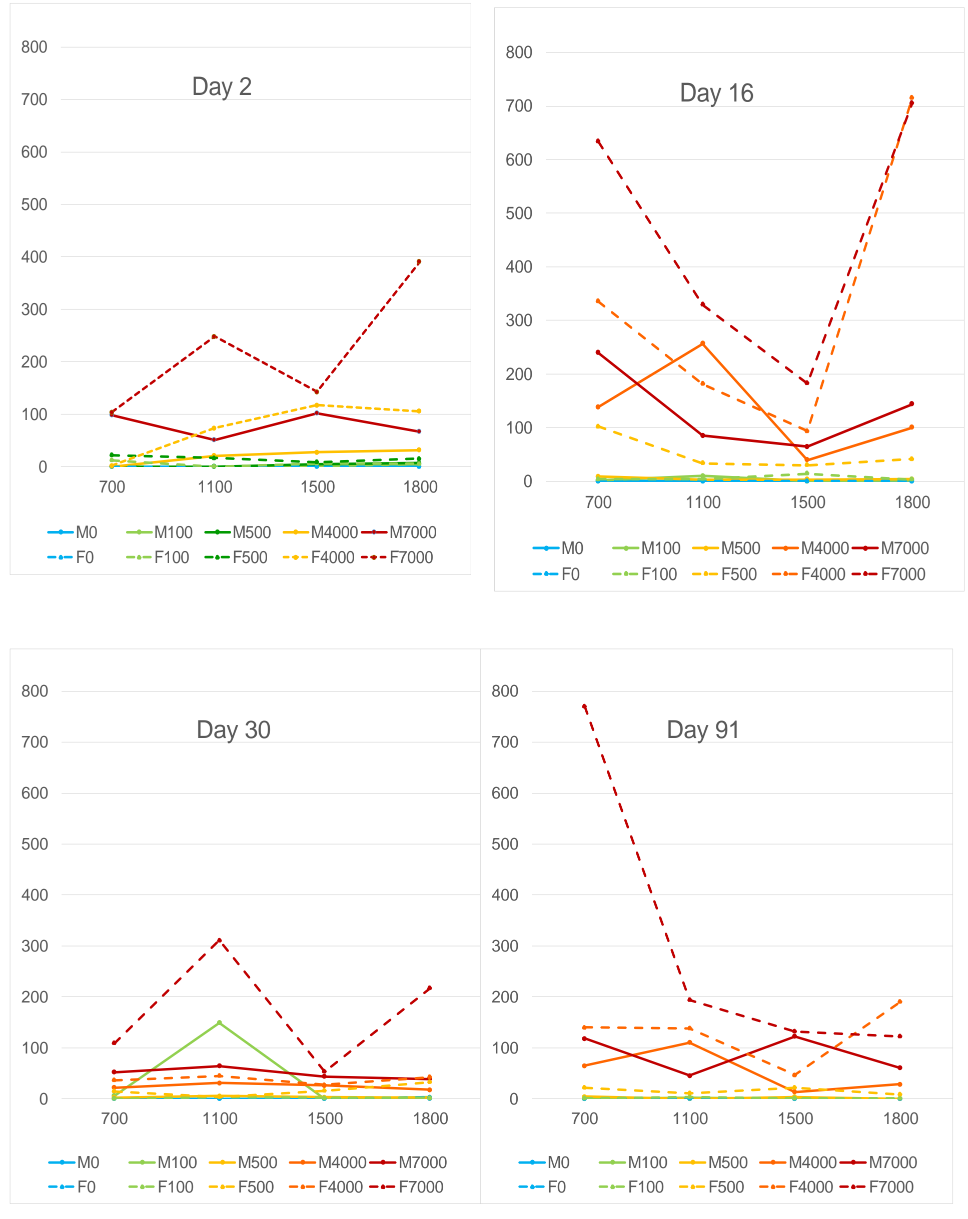
[a] Immediately following collection, 50 µL of whole blood from each sample was measured into a plain plastic tube containing 50 µL of deionized water. Concentrations are based on a whole blood:water (1:1, v/v) solution.

[b] In mean calculations, LLOQ imputed as 0.

[c] Eighteen animals were noted with a blood concentration marginally above the LLOQ.

Source: Shearer (2015)

Fig. A3-1. Group mean blood/water[a] concentrations (ng/mL) of pydiflumetofen in mice over a 13-week period



F: females; M: males
LOQ or LLOQ was expressed as 0.
[a] Immediately following collection, 50 µL of whole blood from each sample was measured into a plain plastic tube containing 50 µL of deionized water. Concentrations are based on a whole blood:water (1:1, v/v) solution.
Source: Shearer (2015)

Thirteen-week study in rats (Shearer & Robertson, 2015)

Blood samples (approximately 0.1 mL) were obtained from the tail vein of all animals (10 rats of each sex at each time point) in each group for the analysis of pydiflumetofen in whole blood. The samples were collected at 07:00, 11:00, 15:00 and 18:00 on days 2, 9, 28 and 91. Samples from the control group (0 ppm) were collected prior to those from the treatment groups. Fifty microlitres of whole blood from each sample was mixed with 50 µL of deionized water and stored at −80 °C prior to analysis. Concentrations of pydiflumetofen were determined using a suitable LC-MS/MS analytical method. A

research method was established by preparing calibration and quality control samples over the range 5–5000 ng/mL.

The mean concentrations are shown in Table A3-2 and Fig. A3-2.

Thirteen-week study in dogs (Blunt, 2015a)

Blood samples (0.3 mL) were taken from all animals by venipuncture of the cephalic veins before dosing and at 0.5, 1, 1.5, 2, 4, 8, 12 and 24 hours after dosing on days 1 and 28 and during week 13 and placed into tubes containing dipotassium ethylenediaminetetraacetic acid (EDTA) as anticoagulant. Whole blood (0.05 mL) was mixed with 0.05 mL of deionized water and stored at −70 °C before analysis. All samples from animals given pydiflumetofen were analysed, with only predosing samples analysed for controls. Concentrations of pydiflumetofen were determined from blood samples using a validated LC-MS/MS bioanalytical method.

Pydiflumetofen was detected in the blood of control animals. Concentrations of pydiflumetofen in blood from treated groups at each time point are shown in Table A3-3.

There was no consistent evidence of accumulation over the treatment period. Exposure to pydiflumetofen tended to be higher in males, most notably at 300 mg/kg bw per day.

Table A3-2. Group mean blood/water[a] concentrations of pydiflumetofen in rats over a 13-week period

	Group mean[b] blood/water concentrations (ng/mL)									
	0 ppm		250 ppm		1 500 ppm		8 000 ppm		16 000 ppm	
Time	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
Day 2										
07:00	<LLOQ	<LLOQ	14.8	23.9	44.1	86.7	47.5	70.6	58.2	107.1
11:00	<LLOQ	–[c]	3.4	28.9	26.5	69.1	31.6	68.4	35.1	77.3
15:00	<LLOQ	<LLOQ	1.6	17.0	16.8	36.9	21.4	42.6	25.7	53.0
18:00	<LLOQ	–[c]	2.4	21.8	15.6	49.1	29.8	43.2	31.3	50.0
Day 16										
07:00	–[c]	–[c]	13.3	46.3	39.5	84.4	50.0	81.3	59.8	94.9
11:00	–[c]	<LLOQ	8.6	26.8	13.0	74.3	46.9	73.9	53.9	70.1
15:00	–[c]	<LLOQ	0.6	17.9	12.1	39.7	45.1	63.5	49.2	94.1
18:00	<LLOQ	<LLOQ	13.7	19.8	19.1	50.0	28.1	89.7	65.9	65.4
Day 30										
07:00	<LLOQ	–[c]	2.4	20.7	15.0	64.6	32.8	83.1	54.1	103.0
11:00	<LLOQ	–[c]	<LLOQ	23.3	10.5	48.6	23.4	84.5	62.3	102.2
15:00	<LLOQ	–[c]	0.9	14.7	9.8	37.7	30.3	63.9	90.2	67.8
18:00	<LLOQ	–[c]	<LLOQ	18.1	8.3	38.2	27.7	73.8	38.3	67.2

LLOQ: lowest limit of quantification (5.0 ng/mL); ppm: parts per million

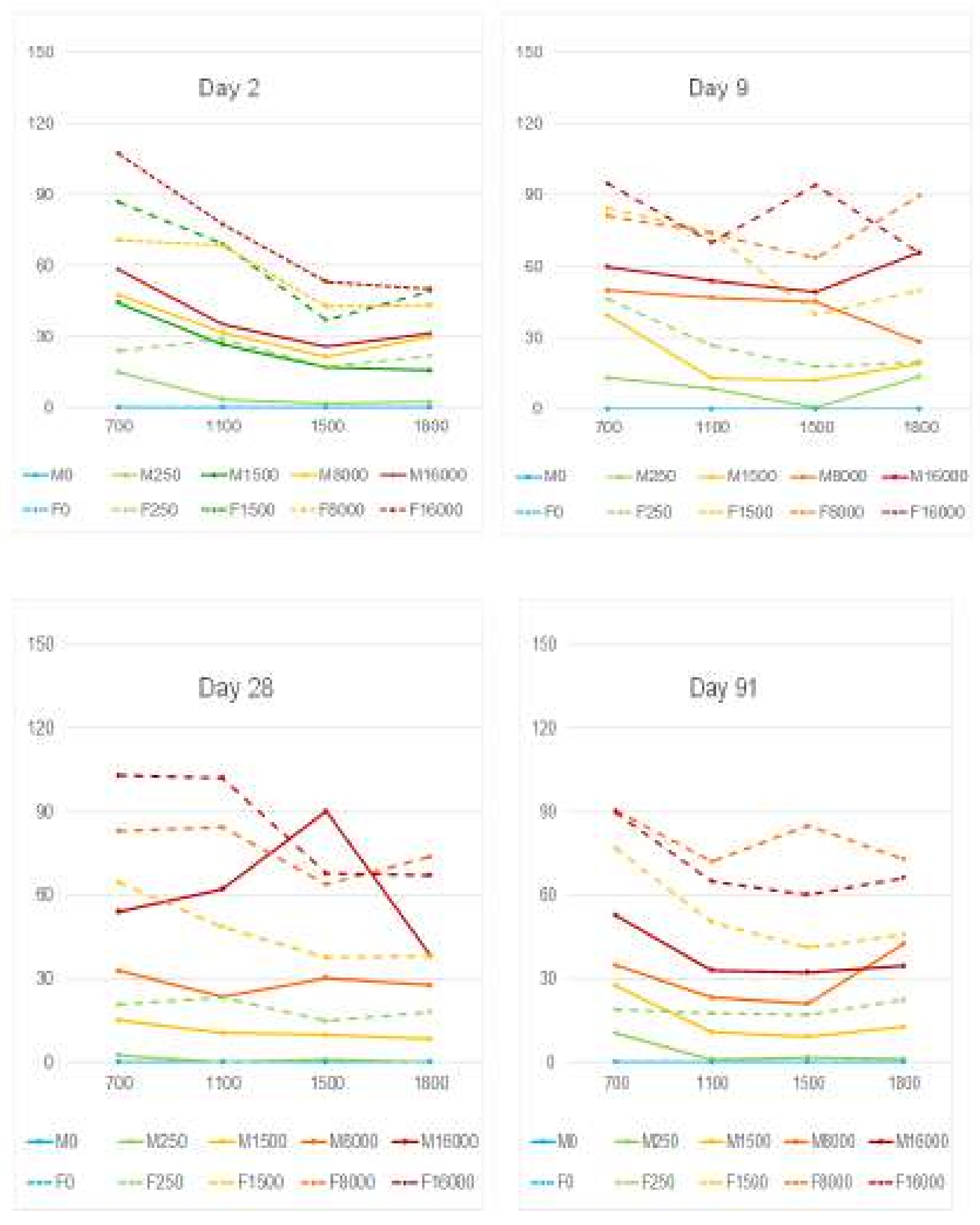
[a] Immediately following collection, 50 µL of whole blood from each sample was measured into a plain plastic tube containing 50 µL of deionized water. Concentrations are based on a whole blood:water (1:1, v/v) solution.

[b] In mean calculations, LLOQ imputed as 0.

[c] Twelve animals were noted with a blood concentration above the LLOQ.

Source: Shearer & Robertson (2015)

Fig. A3-2. Group mean blood/water[a] concentrations (ng/mL) of pydiflumetofen in rats over a 13-week period



F: females; M: males
LOQ or LLOQ was expressed as 0.
[a] Immediately following collection, 50 µL of whole blood from each sample was measured into a plain plastic tube containing 50 µL of deionized water. Concentrations are based on a whole blood:water (1:1, v/v) solution.
Source: Shearer & Robertson (2015)

Table A3-3. Group mean blood/water[a] concentrations (ng/mL) of pydiflumetofen in dogs over a 13-week period

Hours after dosing	Group mean blood/water concentrations (ng/mL)					
	30 mg/kg bw		300 mg/kg bw		1 000 mg/kg bw	
	Males	**Females**	**Males**	**Females**	**Males**	**Females**
Day 1						
0	<5.00	<5.00	<5.00	<5.00	<5.00	<5.00
0.5	4.50	1.68	7.57	6.11	12.8	5.53
1	10.4	2.95	13.4	15.8	27.4	15,6
1.5	15.1	8.15	13.4	15.8	33.9	39.5
2	24.0	16.1	104	133	140	109
4	11.0	13.6	399	494	2 170	2 200
8	1.78	3.18	266	210	3 040	2 300
12	<5.00	<5.00	488	88.6	1 400	1 230
24	<5.00	<5.00	65.8	33.9	231	169
Day 28						
0	2.68	<5.00	14.9	3.76	49.4	29.5
0.5	5.13	1.34	40.0	13.8	60.9	55.1
1	10.1	7.06	75.8	31.9	128	161
1.5	19.0	18.8	127	89.5	269	513
2	34.5	20.8	144	138	501	834
4	15.1	16.0	207	119	1 700	1 740
8	9.01	3.02	362	18.7	160	1 360
12	3.70	0.38	474	9.01	267	259
24	6.33	<5.00	10.0	8.57	32.4	25.5
Day 91						
0	<5.00	<5.00	11.9	5.78	24.6	24.7
0.5	4.94	5.37	45.1	23.5	103	64.0
1	12.1	13.6	89.0	46.1	239	141
1.5	26.0	11.4	258	56.6	689	255
2	34.5	11.2	390	91.7	1 040	638
4	17.3	10.4	510	129	2 040	825
8	4.88	1.66	370	19.8	1 460	295
12	2.73	<5.00	314	11.3	435	270
24	5.52	4.65	11.1	11.5	19.3	28.3

<5.00: below the limit of quantification; bw: body weight

[a] Values based on 1:1 blood:water mixture.

Source: Blunt (2015a)

PYRACLOSTROBIN (addendum)

First draft prepared by
Katsuhiko Yoshizawa,[1] Midori Yoshida[2] and Angelo Moretto[3]

[1] Department of Food Sciences and Nutrition, Mukogawa Women's University, Hyogo, Japan
[2] Food Safety Commission, Cabinet Office, Tokyo, Japan
[3] Department of Biomedical and Clinical Sciences, University of Milan, and International Centre for Pesticides and Health Risk Prevention, ASST Fatebenefratelli Sacco, Milan, Italy

Explanation 585
Evaluation for acceptable intake 586
1. Biochemical aspects 586
1.1 Biotransformation 586
(a) Comparative in vitro metabolism among humans, rats, rabbits and dogs 586
(b) Influence of an inhibitor of human carboxylesterase on the in vitro metabolism of pyraclostrobin in human hepatocytes and human liver cytosol 588
(c) Metabolism of pyraclostrobin in rat plasma 589
(d) Metabolism of pyraclostrobin in rat serum 591
(e) Metabolism of metabolite 500M106 in male rats 593
(f) Summary of biotransformation of pyraclostrobin 596
2. Toxicological studies 597
2.1 Short-term studies of toxicity 597
(a) Exposure by inhalation 597
2.2 Long-term studies of toxicity and carcinogenicity 598
2.3 Reproductive and developmental toxicity 600
(a) Developmental toxicity 600
2.4 Special studies 600
(a) Immunotoxicity 600
(b) Phototoxicity 604
(c) Effects on iron levels in blood serum and urine of rats 604
(d) Effects on oxidative stress in liver 605
(e) In vitro haemolytic potential 606
(f) Combination study of pyraclostrobin with vitamin B_{12} 606
(g) Combination study of dimoxystrobin with iron 606
(h) Effect of dimoxystrobin on iron absorption and transport in the duodenum 607
2.5 Toxicity of metabolites 608
(a) 500M04 (pyrazolon) 608
(b) 500M106 611
(c) Genotoxicity 613
3. Observations in humans 617
Comments 617
Toxicological evaluation 620
References 621
Appendix 1: List of metabolites of pyraclostrobin 627
Appendix 2: Summary of potential direct/secondary local effects induced by pyraclostrobin in toxicity studies 633
Appendix 3: Proposed mode of action for the induction of mucosal hyperplasia in the duodenum by strobilurin fungicides, including pyraclostrobin 634

Explanation

Pyraclostrobin was evaluated previously by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 2003, when an acceptable daily intake (ADI) of 0–0.03 mg/kg body weight (bw) was established based on a no-observed-adverse-effect level (NOAEL) of 3.4 mg/kg bw per day in two 2-year studies in rats with application of a safety factor of 100. An acute reference dose (ARfD) of 0.05

mg/kg bw was established based on a NOAEL of 5 mg/kg bw per day for embryo and fetal toxicity in a developmental toxicity study in rabbits with application of a safety factor of 100. The 2003 Meeting noted that further information on the relationship between local irritation of the gastrointestinal tract and reduced body weight gains in pregnant rabbits and the effect of maternal nutritional deficit on fetal resorptions might allow the ARfD to be refined.

Following a request by the Codex Committee on Pesticide Residues for additional maximum residue levels and an evaluation of metabolites possibly relevant to these new uses, pyraclostrobin was placed on the agenda of the present Meeting, which assessed additional toxicological information available since the last review.

Additional studies on pyraclostrobin (inhalation toxicity in rats, carcinogenicity in rats and the mode of action for the induction of mucosal hyperplasia in the duodenum) and its metabolites (toxicity and genotoxicity studies) were evaluated by the present Meeting. In particular, the present Meeting reviewed the new studies on pyraclostrobin to determine whether they would allow its ARfD to be refined.

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

Evaluation for acceptable intake

1. Biochemical aspects

1.1 Biotransformation

Five new studies on biotransformation were submitted to the present Meeting.

(a) Comparative in vitro metabolism among humans, rats, rabbits and dogs

A comparative in vitro metabolism study was performed using hepatocytes or microsomes from humans, rats, rabbits and dogs. The objective of this study was to compare the metabolism of pyraclostrobin in hepatocytes and microsomes from three animal species used in toxicological studies with the metabolism in human hepatocytes and microsomes in order to determine whether the metabolic profiles were similar among the species and whether a unique human metabolite occurs. To address these aims, pyraclostrobin radiolabelled on the chlorophenyl ([ch-^{14}C]) or tolyl ([tol-^{14}C]) ring at 3 or 10 µmol/L was incubated with hepatocytes from humans, rats or rabbits. Incubations with liver microsomes from humans, rats, rabbits or dogs were performed with the 10 µmol/L concentration only. The viability of the hepatocytes was determined after incubation for up to 180 minutes using a luminescent cell viability assay. After incubation for 10, 30, 60 or 180 minutes, the reaction was terminated. The resulting supernatant after concentration using a centrifugal evaporator was analysed. The pellet after centrifugal evaporation was extracted, and the radioactive residues in the resulting extract and final pellet were determined.

Twelve peaks were identified in human hepatocytes or human liver microsomes, which each represented more than 5% of the applied radioactivity. The peak representing pyraclostrobin and all 11 metabolite peaks were detected in either rat or rabbit hepatocytes. Four peaks each representing more than 5% of the applied radioactivity were found in human liver microsomes, and two additional peaks identified in human hepatocytes were also detected in human liver microsomes at lower concentrations. The peak representing pyraclostrobin and all five metabolite peaks found in human liver microsomes were detected in rat, rabbit or dog liver microsomes. The results indicated that there was no relevant unique human metabolite of pyraclostrobin.

The metabolic degradation of pyraclostrobin was faster in rat and rabbit hepatocytes than in human cells. Metabolites such as 500M04, 500M108, 500M103, 500M104 and 500M88 (see Appendix

1 for structures of metabolites of pyraclostrobin) were common to all species examined. In humans and rabbits, cleavage of the amide bond, resulting in the formation of metabolite 500M106, was a major degradation pathway; 500M106 was subsequently metabolized by conjugation with glucuronic acid to form metabolite 500M107. In humans and rabbits, metabolite 500M02, which is formed by dimerization, was also identified. Hydroxylation and conjugation to form metabolite 500M104 seemed to be more pronounced in rat hepatocytes than in human and rabbit hepatocytes. The investigation with liver microsomes, which included dog as an additional test species, confirmed the picture obtained with hepatocytes. After incubation of pyraclostrobin with dog liver microsomes, four biotransformation products were found, including low amounts of metabolite 500M102.

Table 1 provides an overview on all investigated test systems and incubation periods, including indications of the amounts of detected metabolites.

Table 1. Overview of the occurrence of metabolites of pyraclostrobin after incubation with hepatocytes or liver microsomes from humans, rats, rabbits or dogs

	Humans		**Rats**		**Rabbits**		**Dogs**
Metabolite code	**10 µmol/L**	**3 µmol/L**	**10 µmol/L**	**3 µmol/L[a]**	**10 µmol/L**	**3 µmol/L[a]**	**10 µmol/L**
Hepatocytes ([ch-^{14}C]pyraclostrobin)							
Pyraclostrobin	++	++	++[b]	nd	++[b]	nd	NA
500M03	+[c]	++[d]	nd	nd	nd	++	NA
500M108 (regioisomers)	++	++	+	++	++	++	NA
500M04	+	++	+[b]	nd	++	nd	NA
500M103	+	+	nd	nd	+	+	NA
500M104	++	++	++	+	+[b]	+	NA
500M107	+	++	nd	nd	+[b]	nd	NA
500M88	+	nd	+[b]	nd	+[b]	nd	NA
500M73	+	++	nd	nd	++	+	NA
500M106	++	++	nd	nd	++[b]	+	NA
500M02	++	++	nd	nd	++[b]	nd	NA
Hepatocytes ([tol-^{14}C]pyraclostrobin)							
Pyraclostrobin	++	++	++[b]	nd	++[b]	nd	NA
500M108 (regioisomers)	++	++	+[b]	nd	++[b]	++	NA
500M103	+	++	+[b]	nd	+[b]	++	NA
500M104	++	++	++	+	+[b]	nd	NA
500M107	nd	++	nd	nd	+[b]	nd	NA
500M88	+	nd	+[b]	nd	+[b]	nd	NA
500M73	++	++	nd	nd	++	nd	NA
500M106	++	++	nd	nd	++[b]	nd	NA
500M02	++	++	nd	nd	++	nd	NA
Liver microsomes ([ch-^{14}C]pyraclostrobin, 90 minutes)							
Pyraclostrobin	++	NA	++	NA	+	NA	++

Metabolite code	Humans 10 µmol/L	Humans 3 µmol/L	Rats 10 µmol/L	Rats 3 µmol/L[a]	Rabbits 10 µmol/L	Rabbits 3 µmol/L[a]	Dogs 10 µmol/L
500M04	++	NA	++	NA	++	NA	++
500M88	++	NA	++	NA	+	NA	++
500M73	+	NA	+	NA	++	NA	+
500M106	+	NA	nd	NA	++	NA	nd
500M02	++	NA	nd	NA	++	NA	+

ch: chlorophenyl; NA: not applicable; nd: not detected; tol: tolyl

[a] Investigations performed at one incubation period (180 minutes).

[b] Detected at short incubation periods of 10 and 30 minutes.

[c] +: a mean portion of 0–5% of the applied radioactivity from at least one time interval.

[d] ++: a mean portion above 5% of the applied radioactivity from at least one time interval.

Source: Funk, Glaessgen & Kalyon (2014)

In conclusion, some species differences in metabolites were found. For example, 500M106, a major metabolite in humans and rabbits, was not found in rats. However, no human metabolite was identified that was not also found in rats, rabbits or dogs (Funk, Glaessgen & Kalyon, 2014).

(b) Influence of an inhibitor of human carboxylesterase on the in vitro metabolism of pyraclostrobin in human hepatocytes and human liver cytosol

A study was conducted to investigate the in vitro metabolism of pyraclostrobin labelled with ^{14}C at the tolyl ring (lot/batch no. 556-5501; purity 99.5%; Fig. 1) in human hepatocytes and human liver cytosol and the influence of an inhibitor of human carboxylesterase 1, WWL229 (lot/batch no. 104M4753V; purity 98.8%), on the biotransformation products. To address this issue, [tol-^{14}C]pyraclostrobin at 3 or 6 µmol/L with or without WWL229 at 20 µmol/L was incubated with human hepatocytes or human liver microsomes (both sexes mixed). All experiments were performed in triplicate. The viability of the hepatocytes was determined after an incubation for 180 minutes using a luminescent cell viability assay.

Fig. 1. Chemical structure of pyraclostrobin labelled with ^{14}C at the tolyl ring

Source: Funk & Bellwon (2016a)

For human hepatocytes incubated with pyraclostrobin without WWL229, pyraclostrobin and its biotransformation products 500M103, 500M104, 500M107, 500M73, 500M106 and 500M02 as well as an isomer of 500M02 were identified. The parent compound was time-dependently and completely metabolized after 180 minutes. In the presence of WWL229, biotransformation was slower, as pyraclostrobin was still present after 180 minutes, and metabolites 500M73, 500M107, 500M106 and 500M02 as well as an isomer of 500M02, formed after an initial cleavage of the ether and/or amide bond of the parent pyraclostrobin, were not detected at all. Instead, the formation of low levels of metabolite 500M88 was observed.

For human liver cytosol incubated with pyraclostrobin without WWL229, pyraclostrobin and its biotransformation products 500M73, 500M106 and 500M02 as well as an isomer of 500M02 were identified. Similar to the incubations performed on human hepatocytes, metabolites 500M73, 500M106 and 500M02 and an isomer of 500M02 were not detected in samples obtained after simultaneous incubation of human liver cytosol with pyraclostrobin and WWL229.

These results indicate an inhibition of the biotransformation of pyraclostrobin in the presence of WWL229. This compound especially inhibits the formation of metabolites 500M106, 500M73 and 500M02 (and its isomer) as well as their conjugates, showing the involvement of the enzyme human carboxylesterase 1 in the respective metabolic reactions (Funk & Bellwon, 2016a).

(c) Metabolism of pyraclostrobin in rat plasma

A study was conducted to clarify the fate and kinetic behaviour of pyraclostrobin after administration of a single oral dose of 50 mg/kg bw to rats. In particular, the purpose was to analyse rat plasma samples at different time points after dosing (especially at the time at which the maximum concentration [C_{max}] is reached, or T_{max}) to compare the results with those obtained in the previously evaluated rat metabolism study (Annex 1, reference *100*) and to specifically address metabolites present at short time intervals after dosing. For designated time points (0.25, 0.5, 1, 2, 3, 4, 8 and 16 hours), one female and one male Crl:WI(Han) rat (average body weights 312.1 g and 200.1 g for males and females, respectively) were dosed with a mixture of pyraclostrobin labelled with ^{14}C at the tolyl ring (tolyl-ring-U-^{14}C; batch no. 566-5011; purity 99.3%), pyraclostrobin labelled with ^{13}C at the pyrazole ring (pyrazole-3-^{13}C; batch no. 1026-1018; purity 99.8%; Fig. 2) and unlabelled pyraclostrobin (batch no. 1815-65; purity 95.7%). In addition, unlabelled metabolite 500M106 (batch no. L83-166; purity 98.9%) and ^{14}C-labelled metabolite 500M02 (batch no. 933-2025; purity 98.8%) were used as reference compounds. Blood was sampled in the presence of ethylenediaminetetraacetic acid (EDTA) or lithium heparin (LiHep), and plasma was obtained.

Fig. 2. Chemical structure of pyraclostrobin labelled with ^{13}C at the pyrazole ring

Source: Birk, Lutz & Doebbe (2014)

For the EDTA plasma at time points 0.25 and 0.5 hour, the concentrations of radiolabelled pyraclostrobin were 1.429 mg/kg and 1.343 mg/kg, respectively. From time point 1 hour to 16 hours, the concentration almost reached a steady state and was generally higher than at time points 0.25 and 0.5 hour (3 hours: 2.615 mg/kg; 16 hours: 3.030 mg/kg). Only at time point 4 hours did the concentration decrease to 1.288 mg/kg. The results for the LiHep plasma were comparable with those of the EDTA plasma. The plasma concentrations are in accordance with those noted in the previously evaluated rat metabolism study (Annex 1, reference *100*). Analysis of the EDTA plasma supernatants at the different time intervals using high-performance liquid chromatography (HPLC) led to comparable metabolite patterns. The unchanged parent compound pyraclostrobin was identified at all time points. The concentration of radiolabelled pyraclostrobin ranged from 0.068 mg/kg (0.5 hour) to 0.290 mg/kg (16 hours). HPLC analysis of the LiHep plasma supernatants led to results similar to those with the EDTA plasma samples.

The masses corresponding to the parent pyraclostrobin and the metabolites 500M108, 500M29, 500M15, 500M46, 500M06, 500M104, 500M107, 500M30 and 500M106 were detected using HPLC with mass spectrometric (HPLC-MS) analysis of plasma supernatant samples. All metabolites that were identified in plasma in the previously evaluated rat metabolism study (Annex 1, reference *100*) were confirmed (pyraclostrobin, 500M06, 500M15 and 500M46). Moreover, metabolites that were identified in the previously described in vitro comparative study (Funk, Glaessgen & Kalyon, 2014) were also detected in rat plasma (500M108, 500M104, 500M107 and 500M106).

The identified metabolites were quantified in four samples (EDTA plasma supernatant at 3 hours; concentrated plasma supernatant of the EDTA blood pool sample at 0.25–16 hours; and LiHep plasma supernatants at 0.25 and 0.5 hour). HPLC peaks that contained more than one component were classified as to region (regions 1–4). Region 1 included 500M108 and 500M29, region 2, 500M15 and 500M108, region 3, 500M46 and 500M06, and region 4, 500M104, 500M107 and 500M30. In all four samples, the relative amounts of region 1, region 3 and region 4 were similar, ranging from 0.337 to 0.560 mg/kg for the EDTA plasma supernatant at 3 hours, from 0.172 to 0.268 mg/kg for the concentrated plasma supernatant of the EDTA blood pool sample at 0.25–16 hours, from 0.158 to 0.227 mg/kg for the LiHep plasma supernatant at 0.25 hour and from 0.227 to 0.331 mg/kg for the LiHep plasma supernatant at 0.5 hour. In all four samples, region 2 was the least abundant region and accounted for between 0.044 mg/kg (LiHep at 0.5 hour) and 0.111 mg/kg (EDTA at 3 hours). Pyraclostrobin was identified in all four samples, and the quantities corresponded well to the analyses of the EDTA plasma supernatant samples of the different time intervals (0.25–16 hours). Metabolite 500M106 was detected only in the LiHep plasma supernatant at 0.25 hour, where it was the least abundant component (0.030 mg/kg). The HPLC analyses are summarized in Table 2.

Table 2. Quantification of radioactive residues of pyraclostrobin in EDTA plasma supernatant at 3 hours, concentrated plasma supernatant of the EDTA blood pool sample at 0.25–16 hours and LiHep plasma supernatants at 0.25 and 0.5 hour

		Concentration of radioactivity (mg/kg)[a]			
Region	**Metabolite identified**	**EDTA 3 hours**	**EDTA 0.25–16 hours**	**LiHep 0.25 hour**	**LiHep 0.5 hour**
Identified					
	Pyraclostrobin	0.313	0.086	0.163	0.035
1	500M108 500M29	0.560	0.268	0.227	0.331
2	500M15 500M108	0.111	0.078	0.089	0.044
3	500M46 500M06	0.412	0.226	0.158	0.228
4	500M104 500M107 500M30	0.337	0.172	0.195	0.316
	500M106	nd	nd	0.030	nd
Characterized by HPLC					
Up to 3–5 further peaks (each below or equal to 0.218 mg/kg)		0.465	0.472	0.303	0.223
Total identified and characterized		2.197	1.303	1.164	1.177

EDTA: ethylenediaminetetraacetic acid; HPLC: high-performance liquid chromatography; LiHep: lithium heparin; nd: not detected
[a] Data taken from HPLC analysis.
Source: Birk, Lutz & Doebbe (2014)

The results indicated that pyraclostrobin was metabolized by typical phase I and II reactions. Its proposed metabolic pathway was:

- desmethoxylation of the side-chain;
- hydroxylation of the chlorophenyl pyrazole ring system;
- hydroxylation of the tolyl ring system;
- desmethylation of the side-chain; and
- cleavage of the amide bond in the side-chain.

The combination of these reactions followed by conjugation results in a large number of metabolites. Hydroxylation and glucuronic acid conjugation of the chlorophenyl and pyrazole rings of pyraclostrobin yield metabolite 500M29. Hydroxylation and glucuronic acid conjugation of only the pyrazole ring result in metabolites 500M46 and 500M104. Metabolite 500M30 is a cysteine conjugation product of pyraclostrobin, wherein cysteine is bound via its sulfur to the pyrazole ring. 500M30 is probably generated via conjugation of pyraclostrobin with glutathione, followed by enzymatic cleavage of the glutamic acid and the glycine of glutathione.

Desmethoxylation of pyraclostrobin yields the intermediate 500M07. Hydroxylation of the pyrazole ring of 500M07 results in the intermediate 500M08. Both 500M07 and 500M08 were identified in a previously evaluated rat metabolism study (Annex 1, reference *100*). Glucuronic acid conjugation of 500M08 results in metabolite 500M06. 500M06 might also be generated via *N*-desmethoxylation of 500M46.

Desmethylation of the *N*-methoxy side-chain of pyraclostrobin, followed by hydroxylation of the pyrazole ring and glucuronic acid conjugation, results in metabolite 500M15. Cleavage of the methylcarboxy group of pyraclostrobin yields metabolite 500M106. Conjugation of the secondary amine group of 500M106 with glucuronic acid results in metabolite 500M107. Cleavage of the methoxy moiety of 500M106, followed by hydroxylation and sulfate conjugation of the tolyl moiety, results in metabolite 500M108.

This proposed metabolic pathway of pyraclostrobin in rat plasma is illustrated in Fig. 3 (Birk, Lutz & Doebbe, 2014).

(d) Metabolism of pyraclostrobin in rat serum

A study was conducted to investigate the potential biotransformation of pyraclostrobin by enzymes in rat serum. [tol-^{14}C]Pyraclostrobin (lot/batch no. 566-5501; radiochemical purity 99.5%) at a concentration of 6 µmol/L was incubated with sex-mixed serum of Crl:WI(Han) rats (males aged 27–28 weeks and females aged 14 or 16 weeks). All experiments were performed in triplicate. After incubation for 0, 1, 3, 5 or 20 hours, the reaction was terminated by mixing with ice-cold acetonitrile. The radioactive residues in the supernatant resulting after centrifuged evaporation were determined by liquid scintillation counting, and an aliquot was analysed by HPLC with radiodetection. Selected replicates of the supernatants were analysed by HPLC-MS to investigate the presence of metabolites identified at concentrations above 5% of the applied radioactivity in the previously described comparative in vitro metabolism study (Funk, Glaessgen & Kalyon, 2014) performed on human hepatocytes and human liver microsomes.

Radio-HPLC analyses of the samples after incubation of radiolabelled pyraclostrobin at 6 µmol/L with rat serum allowed the identification of the parent compound and four relevant metabolites known from the previously described comparative in vitro metabolism study (Funk, Glaessgen & Kalyon, 2014). Within the first hour of incubation, no significant differences were observed in comparison with the zero incubation controls. After 3 hours, the concentration of pyraclostrobin started to decrease continuously to 66.29% of the applied radioactivity after 20 hours. The formation of metabolites 500M73, 500M106 and 500M02 and an isomer of 500M02 in trace amounts was observed after 1 hour. The concentration of all metabolites increased time-dependently,

with metabolite 500M106 being the main biotransformation product for all incubation periods (48.81% of the applied radioactivity after 20 hours).

Fig. 3. Proposed metabolic pathway of pyraclostrobin in rat plasma

Source: Birk, Lutz & Doebbe (2014)

A summary of the relevant metabolites of pyraclostrobin following incubation in rat serum is shown Table 3.

Table 3. Summary of relevant metabolites of pyraclostrobin (6 µmol/L) after incubation with rat serum

Incubation time (hours)	Sample description	Component (% of the applied radioactivity)				
		Pyraclostrobin	**500M02**	**Isomer of 500M02**	**500M73**	**500M106**
0	Supernatant	89.14	–	–	–	–
	Extract pellet 1	52.90	–	–	–	–
	Sum	**142.04**	–	–	–	–

Incubation time (hours)	Sample description	Component (% of the applied radioactivity)				
		Pyraclostrobin	500M02	Isomer of 500M02	500M73	500M106
1	Supernatant	79.93	0.29[a]	–	0.32[a]	0.78[a]
	Extract pellet 1	62.59	0.68[a]	0.27[a]	0.30[a]	0.57[a]
	Sum	**142.52**	**0.98[a]**	**0.27[a]**	**0.62[a]**	**1.34[a]**
3	Supernatant	84.90	0.60	–	0.58[a]	5.03[a]
	Extract pellet 1	53.48	1.78	0.69[a]	0.59[a]	1.60[a]
	Sum	**138.38**	**2.38**	**0.69[a]**	**1.17[a]**	**6.63[a]**
5	Supernatant	93.01	2.03	0.37[a]	0.95[a]	8.29[a]
	Extract pellet 1	ne	ne	ne	ne	ne
	Sum	**93.01**	**2.03**	**0.37[a]**	**0.95[a]**	**8.29[a]**
20	Supernatant	52.38	5.98	1.94	0.70	25.55
	Extract pellet 1	13.91	5.05	2.32	0.84	23.26
	Sum	**66.29**	**11.03**	**4.27**	**1.55**	**48.81**

ne: not extracted

[a] Peak assignment was based on comparison of the retention time and the metabolite pattern with the chromatogram obtained from analysis of the supernatants collected from assays incubated for 20 hours.

Source: Funk & Bellwon (2016b)

These results indicate that a significant biotransformation of pyraclostrobin occurred in rat serum within 20 hours, leading to metabolites. These metabolites were also formed in human hepatocytes or human liver microsomes (Funk & Bellwon, 2016b).

(e) Metabolism of metabolite 500M106 in male rats

The metabolism of 500M106, a major pyraclostrobin metabolite in humans, was investigated in four male Crl:WI(Han) rats (10 weeks of age) following the administration of a single oral dose of a mixture of ^{14}C-labelled 500M106 (lot/batch no. 1166-1100; chemical purity 95.2%; radiochemical purity 99.7%) and unlabelled 500M106 (lot/batch no. l83-166; purity 97.1%). The test item preparation was applied at a nominal dose of 10 mg/kg bw. Urine was sampled at time intervals of 0–6, 6–12 and 12–24 hours and afterwards at 24-hour intervals for up to 168 hours after treatment. Faeces were sampled at 24-hour intervals for up to 168 hours after treatment. After 168 hours, the rats were euthanized. In addition to the excreta samples, the total amount of radioactive residue was measured in the carcass of each animal. For balance estimates, the cage wash was also checked for radioactivity. Metabolites were identified by HPLC with tandem mass spectrometry (HPLC-MS/MS) and quantified by radio-HPLC (Thiaener & Bellwon, 2016).

The excretion of 500M106 was complete within the observation period. The main excretion route was via faeces, and detected portions of radioactive residues in faeces ranged from 84.61% to 89.89% of the dose within 168 hours. The excretion via urine ranged from 15.93% to 17.84% of the dose.

The combined faecal samples were extracted 3 times with acetonitrile and twice with water. The residue after acetonitrile and water extraction was further extracted 3 times with a mixture of acetonitrile and acetone.

The extractability of radioactive residues from faeces was moderate, and the main portion of the radioactive residues, ranging from 42.0% to 69.2% of the total radioactive residues (TRR), was extracted with acetonitrile. Low amounts were subsequently extracted with water and with the mixture

of acetonitrile and acetone, leading to radioactive residues after solvent extraction above or equal to 21.7% of the TRR. The residual radioactive residues after solvent extraction were subjected to a sequential solubilization procedure. Amounts up to 4.2% of the TRR were released by enzyme incubation (up to 8% of the TRR in total), and high portions were solubilized by applying hot alkaline treatment, accounting for up to 26.5% of the TRR.

All metabolites were present at 1% or more of the administered dose. Metabolites 500M109, 500M03, 500M05, 500M04 and 500M21 were identified in urine sampled within 48 hours. Metabolites 500M109 and 500M04 were quantified as the main components, accounting for up to 3.82% and 4.63% of the dose, respectively (average of four animals). Additional detected metabolites were present at levels ranging from 0.89% to 1.91% of the dose (average of four animals). In pooled and combined urine sampled within 48–168 hours, metabolites 500M109/500M03, 500M05/500M109, 500M04 and 500M21 were present at similar low levels, ranging from 0.12% to 0.49% of the dose. The administered compound 500M106 was not detected in urine sampled within 168 hours. Metabolites 500M04, 500M117, 500M105, 500M73/500M112, 500M01 and 500M02 as well as isomers of 500M01 and 500M02 were identified in faeces; metabolites 500M73/500M112 accounted for the main fraction (in total, 31.90% of the dose). Metabolites 500M01 and 500M02 were the next most abundant metabolites, accounting for 8.02% and 11.47% of the dose. Metabolite 500M04 was present at 6.18% of the dose, and the remaining metabolites 500M117 and 500M105 accounted for up to 0.39% of the dose. The applied compound 500M106 was not detected in faeces sampled within 168 hours.

The radioactive residues in urine are summarized in Table 4.

The major biotransformation steps in the metabolic pathway of 500M106 in male rats were considered to be as follows:

- cleavage of the N–O bond of the phenylamine moiety, followed by (a) hydroxylation, (b) dimerization with subsequent *N*-oxidation or (c) formation of formaldehyde adducts; and
- cleavage of the ether bridge between the pyrazole and phenylamine moiety, followed by conjugation (sulfation/glucuronidation) or hydroxylation and sulfation.

The proposed metabolic pathway of the metabolite 500M106 in male rats is illustrated in Fig. 4 (Fabian & Landsiedel, 2016).

Table 4. Summary of radioactive residues in urine obtained from rats during 0–168 hours

	Radioactive residues (% of dose)				
	Rat 1	**Rat 2**	**Rat 3**	**Rat 4**	**Rats 1–4**
Metabolite identity	**0–48 hours**	**0–48 hours**	**0–48 hours**	**0–48 hours**	**48–168 hours**
Identified					
500M109[a]	5.16	3.49	3.66	2.95	–
500M109 (RT 16.0–16.2)	0.90	0.90	1.00	1.69	–
500M109 (RT 17.6–18.4)	4.26	2.59	2.66	1.26	–
500M109/500M03	–	–	–	–	0.32
500M03	0.38	2.20	2.44	2.63	–
500M05/500M109	0.92	1.65	0.71	2.60	0.23
500M04	6.10	3.26	3.60	5.55	0.49
500M21	1.08	0.92	0.36	1.19	0.12
Total identified peaks	13.65	11.52	10.77	14.93	1.16

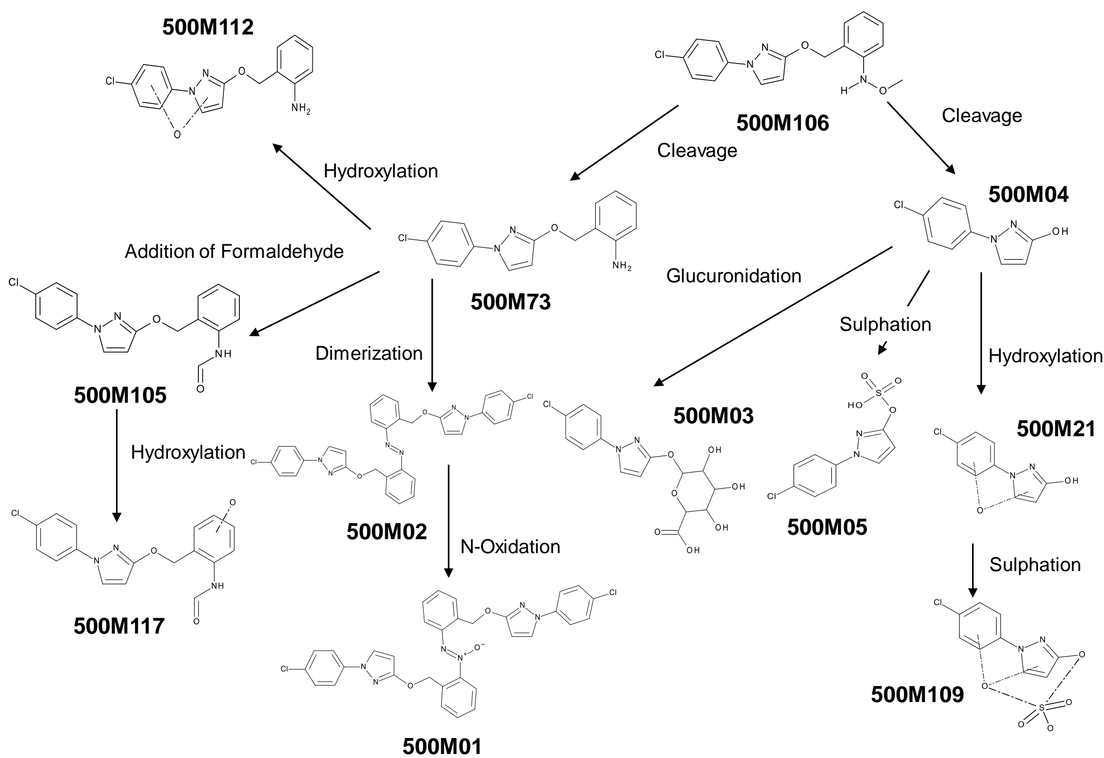
Metabolite identity	Radioactive residues (% of dose)				
	Rat 1 0–48 hours	Rat 2 0–48 hours	Rat 3 0–48 hours	Rat 4 0–48 hours	Rats 1–4 48–168 hours
Characterized by HPLC					
No. of additional HPLC peaks	6	10	7	4	–
% of dose of maximum peak	0.78	0.83	0.78	0.75	–
Total characterized	2.36	4.46	3.51	1.95	–
Total identified and characterized	16.01	15.98	14.28	16.88	1.16

HPLC: high-performance liquid chromatography; RT: retention time

[a] For metabolite 500M109, no unambiguous structure is assigned, but it is given as a generic structure. Several isomers of 500M109 elute in three different peaks (RT 16.0–16.2 minutes, RT 17.6–18.4 minutes and RT 20.2–20.5 minutes) and even co-elute with metabolite 500M05 and two matrix adducts of metabolite 500M05 (RT 20.2–20.5 minutes), where the metabolite ratio could not be determined. Hence, the sum of peaks RT 16.0–16.2 minutes and RT 17.6–18.4 minutes is given additionally.

Source: Fabian & Landsiedel (2016)

Fig. 4. Proposed metabolic pathway of 500M106 in male rats



Source: Fabian & Landsiedel (2016)

(f) Summary of biotransformation of pyraclostrobin

On the basis of the studies that were newly submitted to the present Meeting, including the comparative in vitro metabolism study and the investigations on metabolite 500M106, six steps were proposed for the biotransformation of pyraclostrobin in rats (Fig. 5):

1. desmethoxylation of the side-chain;
2. hydroxylation of the chlorophenyl pyrazole ring system;
3. hydroxylation of the tolyl ring system;
4. cleavage of the ether bond, resulting in chlorophenyl pyrazole or anthranilic acid derivatives;
5. desmethylation of the side-chain; and
6. cleavage of the amide bond in the side-chain.

The combination of these reactions with subsequent conjugation results in a large number of metabolites.

Fig. 5. Proposed biotransformation of pyraclostrobin in rats

Source: Annex 1, reference *100*; Birk, Lutz & Doebbe, 2014; Funk, Glaessgen & Kalyon, 2014; Fabian & Landsiedel, 2016; Funk & Bellwon, 2016a,b

An in vitro comparison of metabolic profiles among humans, rats, rabbits and dogs shows the same key degradation steps as were observed under in vivo conditions, and metabolites such as 500M04, 500M73, 500M108, 500M103, 500M104 and 500M88 were common to all test species. In humans and rabbits, cleavage of the amide bond, resulting in the formation of metabolite 500M106, was the major degradation pathway; 500M106 was subsequently metabolized by conjugation with glucuronic acid to form 500M107. Metabolite 500M02, formed by dimerization, was also identified in these two species. However, no unique human metabolite was detected, based on the study procedures for comparison at a level of 5% of the TRR.

In a rat dosed with 500M106, two major biotransformation steps were observed: 1) cleavage of the N–O bond of the phenylamine moiety, followed by (a) hydroxylation, (b) dimerization with subsequent *N*-oxidation or (c) formation of formaldehyde adducts; and 2) cleavage of the ether bridge between the pyrazole and phenylamine moiety, followed by conjugation (sulfation/glucuronidation) or hydroxylation and sulfation. Metabolite 500M02 was also considered to be covered by the investigations on 500M106, as it has been found in considerable amounts. An enzyme, human carboxylesterase, involved in the formation of 500M106 was confirmed to be present in rat serum.

The results indicate a significant biotransformation of pyraclostrobin, leading to metabolites that were also formed with human hepatocytes or human liver microsomes. In conclusion, 500M02,

500M106 and 500M107, identified as the metabolites in humans, were also identified in other species used for toxicological testing (Annex 1, reference *100*; Birk, Lutz & Doebbe, 2014; Funk, Glaessgen & Kalyon, 2014; Fabian & Landsiedel, 2016; Funk & Bellwon, 2016a,b).

2. Toxicological studies

2.1 Short-term studies of toxicity

(a) Exposure by inhalation

Two 4-week inhalation studies in rats were conducted.

In the first study, three groups of Wistar Crl:Wi(Han) rats (10 of each sex) were exposed, through the head and nose inhalation route, to liquid aerosols (1.6–1.9 µm) of pyraclostrobin (batch no. LJ 27822/199/b; purity 98.7%) dissolved in acetone at a nominal concentration of 0, 1, 30 or 300 mg/m^3 (equal to 0, 0.001, 0.03 and 0.3 mg/L) for 6 hours per day, 5 days per week, for 28 days.

Four male and three female rats died at the high concentration of 300 mg/m^3 (0.3 mg/L) between study days 7 and 24. This concentration is close to the acute 4-hour median lethal concentration (LC_{50}) of 0.58 mg/L observed for an aerosol of pyraclostrobin dissolved in acetone (Ma-Hock & Leibold, 2002). Body weight development in high-concentration (300 mg/m^3) males was impaired. This was evident from significantly lower body weights at day 21 (−6.6%) and from significantly lower body weight gains throughout the study when compared with the acetone control group males. Overall body weight gain of high-concentration males was decreased by 43%. The overall average daily feed intake of high-concentration males tended to be slightly lower, which is in line with the observed body weight effects in this group of animals. Haematology revealed increased white blood cell counts and an increase in absolute and relative neutrophil numbers in both sexes at 300 mg/m^3. This was probably the result of inflammatory processes in the respiratory tract (see below). No treatment-related clinical chemistry findings were noted.

Histopathology identified the respiratory tract (i.e. the nasal cavity, larynx and lungs) as well as the duodenum as target organs at 30 and 300 mg/m^3. A mild to moderate destruction of the olfactory epithelium in the nasal cavity was observed. These changes were characterized by (multi)focal atrophy and/or necrosis, (multi)focal reactive inflammation and signs of repair and regeneration as irregular architecture of the epithelium or gland-like structures. These findings are histomorphological correlates of different stages and intensities of the compound-related irritant effect. The changes in the respiratory epithelium were not as severe as those in the olfactory epithelium. There was minimal to moderate (multi)focal hyperplasia. The minimal to slight hyperplasia of the respiratory epithelium of the larynx observed in three high-concentration males was considered to be treatment related. In the lungs of all groups, a minimal to slight perivascular infiltration of inflammatory cells was observed. As the severity was slightly higher in the high-concentration females, this was considered to be treatment related. In addition, the number of histiocytes (severity) in the alveoli was higher in the high-concentration males, whereas the incidence was elevated in females at the intermediate and high concentrations. A treatment-related increase in the incidence and severity of diffuse mucosal hyperplasia in the duodenum was observed.

In conclusion, the low concentration of 1 mg/m^3 was identified as the no-observed-adverse effect concentration (NOAEC) for the first 4-week inhalation study in rats on the basis of histopathological changes in the nasal cavity, lungs and duodenum at 30 mg/m^3 (Gamer et al., 2005).

In the second study, four groups of Wistar rats (10 of each sex per group) were exposed, through the head and nose inhalation route, to liquid aerosols (1.4–2.2 µm) of pyraclostrobin (batch no. COD-001236; purity 99.02%) dissolved in acetone at a nominal concentration of 3, 10 or 30 mg/m^3 for 6 hours per day, 5 days per week, for 28 days. The study included recovery groups of 10 animals of each sex treated either with acetone (vehicle control) or with 30 mg/m^3 for 28 days followed by a 4-week treatment-free recovery period. The animals were examined for evident signs of toxicity and mortality.

Body weight and feed consumption were measured, an ophthalmoscopic examination was conducted, and haematology and blood chemistry parameters were measured. The exsanguinated animals were necropsied and assessed by gross pathology. The main organs were weighed, and all of the organs were examined histopathologically.

No treatment-related mortality or clinical signs of systemic toxicity were observed in any group. Fluctuations of body weight (body weight loss during inhalation exposure, body weight gain during the treatment-free weekends) were observed in all groups, including controls. No treatment-related changes in haematology or blood chemistry parameters were observed in treated groups.

A weight increase of the duodenum at 10 and 30 mg/m^3 was considered to be treatment related. Histopathology did not reveal any indication of systemic toxicity. However, a minimal to slight atrophy/necrosis of the olfactory epithelium in high-concentration males and females indicated local irritant effects, which were reversible within the 4-week recovery period. In some treated animals and one control animal, a small focal area at the base of the epiglottis was covered by flattened epithelium, which differed from the normal cuboidal to columnar laryngeal epithelium. This finding was also observed in two control animals and two treated animals in the recovery group. This minimal and focal change was regarded to be an adaptive, non-adverse response to the inhalation procedure and also occurred in controls.

The NOAEC for systemic toxicity in the second 4-week inhalation study in rats was 3 mg/m^3, based on the increased weight of the duodenum at 10 mg/m^3, whereas the NOAEC for local effects in the nasal cavity was 10 mg/m^3, based on the effects in the upper respiratory tract at 30 mg/m^3 (Ma-Hock et al., 2014).

Both inhalation studies indicated that pyraclostrobin induced direct irritating damage, and consequently inflammatory reaction, to the respiratory tract. Pyraclostrobin absorbed following inhalation exposure induced toxicity in the duodenum similar to that induced by oral treatment.

2.2 Long-term studies of toxicity and carcinogenicity

Additional data were made available on the histopathology of the 2-year toxicity and carcinogenicity studies in rats that had been evaluated by the 2003 Meeting (Mellert et al., 1999a,b), and two 2-year toxicity and carcinogenicity studies not available to the 2003 Meeting (Mellert, 2002b,c) were submitted.

In the previously evaluated carcinogenicity study (Annex 1, reference *100*), pyraclostrobin (purity 97.1%) was administered to groups of 50 male and 50 female Wistar rats at a dietary concentration of 0, 25, 75 or 200 parts per million (ppm) (equal to 0, 1.1, 3.4 and 9.0 mg/kg bw per day for males and 0, 1.5, 4.6 and 12.3 mg/kg bw per day for females, respectively) for 24 months. Impaired body weight development was observed in male and female rats at 200 ppm. Decreases in cumulative body weight gain approached 10% in males and 22% in females, but feed consumption was only slightly reduced, by about 4%, in females during the first 3 months of treatment. Histopathologically, the incidences of liver necrosis and liver adenomas were increased in males, but the incidence of liver carcinomas was unaffected. In the long-term study in rats that was conducted concurrently with this study of carcinogenicity (Mellert et al., 1999a), the incidence of liver adenomas was lower in treated groups than in the controls, liver carcinomas did not occur in a dose-related manner and the incidence of tumours overall was similar in all groups. On this basis, undue weight was not attached to the apparent increase in liver adenomas in males at 200 ppm. Erosion and ulcers in the glandular stomach were increased in males.

The NOAEL for the 24-month carcinogenicity study in rats was 75 ppm (equal to 3.4 mg/kg bw per day), on the basis of reduced body weight gain in both sexes and histopathological lesions in

liver and stomach in males at 200 ppm (equal to 9.2 mg/kg bw per day). There was no indication of a carcinogenic potential in rats (Mellert et al., 1999b).

Additional histopathological analysis of tissues and organs from the above carcinogenicity study was submitted to the present Meeting. The additional analysis focused on all male animals of the low- and mid-dose groups. The pathological investigations to determine histiocytic sarcomas revealed no malignant systemic tumours related to pyraclostrobin treatment in any group. The detailed histopathological evaluation of mid-dose males identified a low incidence of additional neoplastic findings that did not affect the overall tumour incidence (Mellert, 2002a).

The first carcinogenicity study that had not previously been evaluated by JMPR was conducted as an additional study to the carcinogenicity study by Mellert et al. (1999b). Pyraclostrobin (batch no. J.-Nr. 27882/191/c; purity 97.1%) was administered to groups of 50 male and 50 female Wistar rats at a dietary concentration of 0 or 400 ppm (equivalent to 20 mg/kg bw per day). As the maximum tolerated dose (MTD) was obtained at 200 ppm in the main study, this study was terminated after 399 study days without any further examinations.

The low incidence of mortality (one 400 ppm male and two and one females at 0 and 400 ppm, respectively) was not indicative of a treatment-related effect. No relevant clinical signs were observed. Body weight development was impaired in dosed males, as indicated by statistically significantly lower body weights from study day 7 onwards (11% decrease compared with controls). Body weight development of females was also impaired; however, body weights were statistically significantly lower from study day 70 onwards only (7% decrease compared with controls). Cumulative body weight gain was statistically significantly lower throughout the major part of the study for males and females (15% decrease in males and 12% decrease in females compared with controls). Consistently lower feed consumption was observed for males throughout the study and for females during major parts of the study.

Dietary administration of pyraclostrobin to rats at a concentration of 400 ppm for 399 days resulted in an impairment of body weight development in males and females. Treatment did not affect the survival of rats, and no relevant clinical signs were observed. The study was not designed to identify a NOAEL (Mellert, 2002b).

In the second carcinogenicity study that had not previously been evaluated by JMPR, pyraclostrobin (batch no. J.-Nr. 27882/191/c; purity 97.1%) was administered to groups of 50 female Wistar rats at a dietary concentration of 0 or 600 ppm (equivalent to 30 mg/kg bw per day). As the MTD was obtained at 200 ppm in the main study, the study was terminated after 426 study days without any further examinations.

No mortality or relevant clinical signs were observed. Body weight development was impaired in dosed females, as indicated by statistically significantly lower body weights from study day 35 onwards (12% decrease compared with controls). Cumulative body weight gain was statistically significantly lower throughout the major part of the study for dosed females (25% decrease compared with controls). Feed consumption by treated females was statistically significantly lower almost throughout the entire study (10% decrease compared with controls).

Dietary administration of pyraclostrobin to female rats at a concentration of 600 ppm for 426 days resulted in lower feed consumption and an impairment in body weight development. Treatment did not affect survival or elicit relevant clinical signs. The study was not designed to identify a NOAEL (Mellert, 2002c).

2.3 Reproductive and developmental toxicity

(a) Developmental toxicity

Rabbits

In the previously evaluated developmental toxicity study in rabbits (Schilling, Hellwig & Hildebrand, 1999; Annex 1, reference *100*), a NOAEL for maternal toxicity was not identified owing to a marked decrease in body weight gain and feed consumption at all doses. The pattern of the observations indicated that the decreased body weight gain and feed consumption were likely to have been caused by local gastrointestinal tract effects related to high concentrations of pyraclostrobin or to taste disturbance resulting from regurgitation or leakage of the gavaging solution. Consequently, the Meeting concluded that these observations did not reflect systemic toxicity caused by pyraclostrobin and were not used to establish the ARfD. The Meeting also concluded that the nutritional status of the does was likely to have been compromised by the marked transient reduction in feed intake.

It is for these reasons that the 2003 JMPR concluded that further information on the relationship between local irritation of the gastrointestinal tract and reduced body weight gains in pregnant rabbits and the effect of maternal nutritional deficit on fetal resorptions might allow the ARfD to be refined.

2.4 Special studies

(a) Immunotoxicity

Three immunotoxicity studies in mice were submitted.

In the first study, the ability of pyraclostrobin (batch no. COD-001236; purity 99.02%) to affect the natural killer cell–mediated immune response was investigated in 10 female B6C3F1 mice fed pyraclostrobin at a dietary concentration of 0, 50, 200 or 750 ppm (equal to 0, 13, 50 and 165 mg/kg bw per day, respectively) for 28 days. Additionally, 10 female mice were administered anti-Asialo-GM1 (positive control substance) via a single intravenous injection (0.2 mL/animal) on study day 27, the day prior to scheduled termination and necropsy. The spleen, thymus, lymph nodes (mandibular, mesenteric) and Peyer's patches were collected and examined histopathologically. Individual spleens were placed into individual tubes containing Earle's Balanced Salt Solution (EBSS) with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at 15 mmol/L and supplemented with gentamicin as a bacteriostat. After being weighed, the spleen samples were placed on crushed ice and shipped to ImmunoTox®, Inc. for natural killer cell analysis. The ability of natural killer cells (effector cells) to detect and destroy tumour cells was investigated ex vivo.

Impaired body weight development, decreased body weight gain and lower feed consumption were noted at the high dose. On day 28, body weight decreased by 12.7% and body weight gain decreased by 78.4%, compared with controls. Lower feed consumption was detected from day 0 to day 21. Treatment-related decreases in absolute and relative spleen and thymus weights were noted at the high dose. No histopathological evaluation of the thymus was performed. However, based on the substantial effect on body weight, a stress-related reduction in thymus weights can be assumed. The only treatment-related necropsy finding was a smaller thymus in two 750 ppm females. There was no test substance–related effect on natural killer cell activity at any dose. However, in line with the lower spleen weights, a lower number of spleen cells was noted at the high dose. The significantly higher (25%) spleen cell numbers at the low dose were not test substance related due to lack of an effect in the 200 ppm group. For the positive control (anti-Asialo-GM1), a statistically significant decrease in the natural killer cell activity was observed at effector target ratios of 50:1 and greater (Table 5).

The NOAEL for systemic toxicity was 200 ppm (equal to 50 mg/kg bw per day), based on reduced body weight gain and feed consumption and reduced thymus and spleen weights at 750 ppm (equal to 165 mg/kg bw per day). The NOAEL for immunotoxicity was 750 ppm (equal to 165 mg/kg bw per day), the highest dose tested (Smiraldo, 2012a).

Table 5. Summary of a 28-day natural killer cell immunotoxicity study in female mice

Finding	Pyraclostrobin				Anti-Asialo-GM1
	0 ppm	50 ppm	200 ppm	750 ppm	
Organ weight					
Absolute spleen weight (mg)	60.6	70.4	57.4	33.1**	–
Relative spleen weight (%)	0.27	0.32	0.26	0.17**	–
Absolute thymus weight (mg)	49.1	50.9	46.7	25.0**	–
Relative thymus weight (%)	0.22	0.23	0.21	0.13**	–
Gross pathology					
Thymus small	0/10	0/10	0/10	2/10	0/10
Natural killer activity					
Effector target ratio 200:1	6.2 ± 0.5	5.3 ± 0.4	6.0 ± 0.5	6.3 ± 1.1	1.1± 0.6**
Effector target ratio 100:1	2.0 ± 0.2	1.9 ± 0.3	1.8 ± 0.3	1.4 ± 0.5	0.1 ± 0.4**
Effector target ratio 50:1	1.2 ± 0.3	0.9 ± 0.3	1.2 ± 0.3	0.6 ± 0.3	0.1 ± 0.3*
Effector target ratio 25:1	1.1 ± 0.4	0.8 ± 0.4	0.9 ± 0.3	0.6 ± 0.3	0.4 ± 0.3
Effector target ratio 12.5:1	1.1 ± 0.3	1.1 ± 0.3	0.9 ± 0.3	0.8 ± 0.3	0.5 ± 0.3
Effector target ratio 6.25:1	0.8 ± 0.3	0.8 ± 0.1	0.3 ± 0.3	0.7 ± 0.3	0.5 ± 0.2
Number of spleen cells (×10^7)	9.5 ± 0.6	11.9 ± 0.9*	9.1 ± 0.5	5.3 ± 0.4**	10.0 ± 0.4

ppm: parts per million; *: $P \leq 0.05$; **: $P \leq 0.01$ (Dunnett's test for homogenous data, Wilcoxon rank test for non-homogenous data)

[a] Values may not calculate exactly due to rounding of figures.

Source: Smiraldo (2012a)

In the second study, pyraclostrobin (batch no. COD-001236; purity 99.02%) was administered to groups of 10 female mice at a dietary concentration of 0, 50, 200 or 750 ppm (equal to 0, 13, 50 and 202 mg/kg bw per day, respectively) for 28 days. Additionally, 10 female mice were administered cyclophosphamide monohydrate (CPS) (batch no. 079K1569; purity 100.5%), the positive control substance, via intraperitoneal injection once daily during study days 24–27 at a dose of 50 mg/kg bw per day and a dosing volume of 10 mL/kg bw. On study day 24, all animals were immunized via an intravenous tail vein injection with 0.2 mL of 7.5 × 10^7 sheep red blood cells (sRBCs) in EBSS with HEPES (except for a single animal in the 750 ppm group that was immunized via intraperitoneal injection with 0.4 mL of 7.5 × 10^7 sRBCs in EBSS with HEPES). Mortality, body weight and feed consumption were determined. The exsanguinated animals were necropsied. Spleen, thymus, lymph nodes (mandibular, mesenteric) and Peyer's patches were collected and – except for spleen – placed in 10% neutral-buffered formalin for potential further histopathology. Individual spleens were placed into individual tubes containing EBSS with HEPES at 15 mmol/L and supplemented with gentamicin as a bacteriostat. After being weighed, the spleen samples were placed on crushed ice and shipped to ImmunoTox®, Inc. for the conduct of the splenic antibody-forming cell (AFC) assay.

Treatment-related clinical signs were restricted to the high dose (750 ppm) and consisted of decreased defecation, faeces smaller than normal and yellow material around the urogenital area. No mortality was observed in this study. Statistically significant and treatment-related effects on body weight development were observed only at the high dose. A body weight loss was observed (−5% compared with day 0) during the first week of treatment, and body weight was lower by 11.0% compared with controls on day 28. Statistically significantly lower feed consumption was observed at 200 and 750 ppm for interval days 0–7 (−16% and −25%, respectively, compared with controls). There were no macroscopic findings noted at the scheduled necropsy. Treatment-related and statistically significant decreases in terminal body weight and absolute and relative thymus and spleen weights were

observed in the high-dose animals (Table 6). The lower spleen weights correlated with the lower number of spleen cells. No histopathological evaluation of the thymus was performed. However, based on the substantial effect on body weight, a stress-related reduction of thymus weights can be assumed.

Table 6. Summary of spleen AFC responses to the T cell–dependent antigen, sRBCs, in female mice treated for 28 days with pyraclostrobin

	Pyraclostrobin				CPS
Parameter	**0 ppm**	**50 ppm**	**200 ppm**	**750 ppm**	**50 mg/kg bw**
Organ weights					
Absolute spleen weight (g)	92.4	85.2	82.6	55.4*	46.3*
Relative spleen weight (%)	0.41	0.39	0.37	0.27**	0.21*
Absolute thymus weight (g)	41.3	42.1	39.9	25.5*	13.1*
Relative thymus weight (%)	0.18	0.19	0.18	0.12*	0.06*
AFC responses					
Spleen cells (× 10^7)	14.03	12.32	12.12	7.71**	5.07**
IgM AFC/10^6 spleen cells	2 939	1 559**	1 523*	1 210*	0**
IgM AFC/spleen (× 10^3)	419	189**	184**	93**	0**

AFC: antibody-forming cell; bw: body weight; CPS: cyclophosphamide monohydrate; IgM: immunoglobulin M; ppm: parts per million; *: $P \leq 0.05$; **: $P \leq 0.01$ (Dunnett's test)
Source: Smiraldo (2012b)

Test substance–related statistically significantly lower spleen cell numbers (−45%) were noted in the 750 ppm dose group compared with the vehicle controls. As expected, a statistically significant decrease in the spleen cell number (−64%) was observed in the positive control group (CPS). An apparent, statistically significant suppression of the humoral immune response was observed in all treated groups when evaluated as specific activity (AFC/10^6 spleen cells) or total spleen cell activity (AFC/spleen). However, the overall functional responses of the vehicle control and the pyraclostrobin-exposed groups were enhanced compared with historical control data from vehicle control animals of the same sex and strain. In the vehicle control animals from the pyraclostrobin study, the specific activity was more than twice the mean of 1206 AFC/10^6 spleen cells observed in the historical control data. Similarly, the mean responses of all pyraclostrobin-exposed animals ranged from 1210 to 1559 AFC/10^6 spleen cells, whereas historical control values averaged 1206 AFC/10^6 spleen cells (historical control minimum/maximum of study means: 1116/1499 AFC/10^6 spleen cells) – that is, the response of the pyraclostrobin-treated animals was within or slightly above the historical control range (only values below the historical control range would indicate an immunotoxic effect). The reason for the increased AFC activity in controls could not be determined. Thus, the results of this assay cannot be conclusively interpreted. The positive control group displayed statistically significant decreases in specific activity (−100%) and total spleen activity (−100%), compared with the vehicle control group (Table 6).

In conclusion, test substance–related lower spleen cell numbers were noted in the 750 ppm group (−45%). An apparent suppression of the humoral component of the immune system was noted in all test substance–treated groups when evaluated using the concurrent control of this AFC assay. However, the functional response of the vehicle control was substantially above the historical control mean, and the pyraclostrobin-exposed groups were within or close to the historical control range of the same sex and strain of mice. Therefore, the results of the humoral immune response of this study are not conclusively interpretable (Smiraldo, 2012b).

In the third study, the immunotoxic potential of pyraclostrobin (batch no. COD-001236; purity 99.02%) in 10 female B6C3F1 mice was analysed using dietary concentrations of 0, 50, 200 and 750

ppm (equal to 0, 14, 55 and 191 mg/kg bw per day, respectively) for 28 days. This was the second assay to assess the immunoglobulin M (IgM)–mediated immune response, as the first assay was not interpretable regarding immunotoxicity due to a higher activity of the vehicle control, which exceeded the historical control range by a factor of 2. Additionally, 10 female mice were administered CPS, the positive control substance, via intraperitoneal injection once daily during study days 24–27 at a dose of 50 mg/kg bw per day and a dosing volume of 10 mL/kg bw.

No clinical signs indicative of toxicity were observed throughout the study. Statistically significant and treatment-related effects on body weight development were observed only at the high dose. On day 28, body weight was decreased by 9.8%, and body weight gain was decreased by 90.9%, compared with controls. Test substance–related lower feed consumption was observed in the 750 ppm group throughout the study (−10.5% to −18.5%, compared with the vehicle controls). There were no test substance–related macroscopic findings noted at the scheduled necropsy. Treatment-related and statistically significant decreases in terminal body weight and absolute and relative thymus and spleen weights were observed in the 750 ppm group animals (Table 7). The lower spleen weights correlated with the lower number of spleen cells. No histopathological evaluation of the thymus was performed. However, based on the substantial effect on body weight, a stress-related reduction of thymus weights can be assumed.

Table 7. Summary of a second AFC immunotoxicity study in female mice treated for 28 days with pyraclostrobin

	Pyraclostrobin				**CPS**
Parameter	**0 ppm**	**50 ppm**	**200 ppm**	**750 ppm**	**50 mg/kg bw**
Organ weights					
Absolute spleen weight (g)	94.1	89.4	87.5	67.5*	44.7**
Relative spleen weight (%)	0.42	0.40	0.41	0.33**	0.21**
Absolute thymus weight (g)	45.0	46.0	43.6	29.1**	15.6**
Relative thymus weight (%)	0.20	0.21	0.20	0.14*	0.07**
AFC responses					
Spleen cells ($\times 10^7$)	15.01	12.52*	12.54	9.01**	3.85**
IgM AFC/10^6 spleen cells	990	1 003	1 001	1 459**	0**
IgM AFC/spleen ($\times 10^3$)	148	125	122	129	0**

AFC: antibody-forming cell; bw: body weight; CPS: cyclophosphamide monohydrate; IgM: immunoglobulin M; ppm: parts per million; *: $P \leq 0.05$; **: $P \leq 0.01$ (Dunnett's test); the positive control was compared with the vehicle control using Student's *t*-test

Source: Smiraldo (2012c)

A treatment-related lower number of spleen cells (−40%) was noted at the high dose (750 ppm). The statistically significantly lower spleen cell number at the low dose (50 ppm; −17%) was not considered treatment related, because the values at 50 and 200 ppm were the same and statistical significance was most likely due to the decreased variability within the group. In contrast to the lower spleen cell number, the number of AFCs per spleen was not affected by treatment. Accordingly, at the high dose, a statistically significantly higher (47%) specific activity (AFC/10^6 spleen cells) was noted, compared with the vehicle control group. There were no effects on the absolute and specific activities at the intermediate and low doses. In the positive control group, the total absence of AFCs resulted in a 100% decrease in total and specific AFC activities (Table 7).

The study director reported the no-observed-effect level (NOEL) for immune effects to be 200 ppm (equal to 55 mg/kg bw per day), based on decreased spleen and thymus weights and a lower number of spleen cells at 750 ppm (equal to 191 mg/kg bw per day) (Smiraldo, 2012c). However, the lack of

effects on immunoglobulin A (IgA) and the substantial effect on body weight suggest that the effects on organ weights and on the number of spleen cells are related to the lower body weight, rather than a direct effect on the immune system. In addition, no effect indicating immunosuppression was observed in any study with this compound.

The NOAEL for systemic toxicity was 200 ppm (equal to 55 mg/kg bw per day), based on reduced body weight gain and feed consumption, decreased thymus and spleen weights, and low numbers of spleen cells at 750 ppm (equal to 191 mg/kg bw per day). The NOAEL for immunotoxicity was 750 ppm (equal to 191 mg/kg bw per day), the highest dose tested (Smiraldo, 2012c).

(b) Phototoxicity

Pyraclostrobin (batch no. COD-001236; purity 99.02%) was tested for its ability to induce phototoxic effects in Balb/c 3T3 cells in vitro. The photo-cytotoxicity was estimated by means of the neutral red uptake (NRU) method. Three experiments were carried out with and without irradiation with an ultraviolet A (UVA) source. Vehicle and positive controls were included in each experiment. The first experiment failed to fulfil the acceptance criteria, and the results of the second experiment were confirmed in a third experiment. The latter two experiments fulfilled the acceptance criteria.

Based on an initial range-finding phototoxicity test for the determination of the experimental concentrations, the following concentrations were tested with and without UVA irradiation: 0, 0.5, 1.0, 2.2, 4.6, 10.0, 21.5, 46.4 and 100.0 µg/mL. Precipitation was seen at the top concentration of 100 µg/mL with and without irradiation. In the absence and the presence of UVA irradiation, cytotoxicity was noted, and median effective concentration (EC_{50}) values were calculated.

Based on the results of this study, the test substance was predicted to have no phototoxic potential, as indicated by photo-irritancy factor (PIF) values of 1.6 and 0.9 for the second and third experiments, respectively. The threshold for a negative response is less than or equal to 2. The positive control chlorpromazine led to the expected increased cytotoxicity with UVA irradiation, as indicated by PIF values of 29.8 and 40.8.

Under the experimental conditions of this study, pyraclostrobin is not considered to be a phototoxic substance in the in vitro 3T3 NRU phototoxicity test using Balb/c 3T3 cells (Cetto & Landsiedel, 2012, 2014).

(c) Effects on iron levels in blood serum and urine of rats

A study was conducted to determine the level of iron in serum and urine after oral administration of pyraclostrobin (batch no. CP029053; purity 99.0%) to groups of 10 male and 10 female Wistar rats at a dietary concentration of 0, 50, 500 or 1500 ppm (equal to 0, 3.8, 33.9 and 73.9 mg/kg bw per day for males and 0, 4.1, 37.4 and 78.3 mg/kg bw per day for females, respectively) over a period of 14 days. Clinical signs, body weight and feed consumption were determined. Iron and transferrin levels in blood (day 14) were determined for all animals. For determination of iron in urine, individual animals were transferred to metabolism cages (withdrawal of feed and water), and urine was collected overnight.

No abnormalities were detected in any animal. No animals died during the study. Feed consumption and body weight development were impaired in both sexes at and above 500 ppm. At the high dose, an overall body weight loss was observed.

Serum iron concentrations were dose- and time-dependently decreased in mid- and high-dose animals by up to 50% when compared with the controls. Serum transferrin levels and urinary iron excretion were not affected by treatment. The slight decrease in transferrin concentration in the serum of the high-dose males on day 7 was not consistent over time and was regarded to be incidental and not toxicologically relevant. No treatment-related effects were noted at 50 ppm (Table 8).

Table 8. Summary of effects on iron levels in blood serum and urine of rats administered pyraclostrobin in the diet for 14 days

	0 ppm	50 ppm	500 ppm	1 500 ppm
Males				
Blood chemistry				
Serum iron (µmol/L)				
Day 7	47.01	44.84	36.61	34.85*
Day 14	54.54	46.71	37.72**	27.41**
Serum transferrin (g/L)				
Day 7	5.50	5.34	5.45	4.74**
Day 14	6.38	5.80	6.07	5.85
Urine analysis				
Iron (nmol/L[a])				
Day 15	3.97	3.27	2.69	3.60
Females				
Blood chemistry				
Serum iron (µmol/L)				
Day 7	59.07	60.09	46.48**	57.49
Day 14	53.61	58.31	45.55*	41.97**
Serum transferrin (g/L)				
Day 7	5.40	5.23	5.62	4.92
Day 14	5.86	5.68	6.13	5.63
Urine analysis				
Iron (nmol/L[a])				
Day 15	2.61	2.44	2.78	2.89

ppm: parts per million; *: $P \leq 0.05$; **: $P \leq 0.01$ (Kruskal Wallis plus Wilcoxon test)
[a] The units for iron concentrations in urine are not clear from the original report.
Source: Mellert et al. (2003a)

This mechanistic study indicated that pyraclostrobin-induced anaemia was related to lower absorption of iron from the gut, resulting in lower serum iron levels (Mellert et al., 2003a).

(d) Effects on oxidative stress in liver

Pyraclostrobin (batch no. CP029053; purity 99.0%) was administered to groups of 10 male Wistar rats at a dietary concentration of 0, 75 or 200 ppm (equal to 0, 5.1 and 13.4 mg/kg bw per day for 14 days and 0, 5.3 and 13.6 mg/kg bw per day for 28 days, respectively) for 14 or 28 days. Feed consumption and body weights were determined weekly. The animals were examined for signs of toxicity or mortality at least once a day. Potential induction of oxidative stress by pyraclostrobin in liver was determined by measuring lipid peroxidation.

No animals died during the study. No abnormal clinical signs were detected. Lipid peroxidation in liver was decreased markedly after 14 days in animals receiving 200 ppm (416, 346.7 and 236.2 nmol malondialdehyde [MDA] equivalents per gram liver at 0, 75 and 200 ppm, respectively). After 28 days, lipid peroxidation was still decreased in both treated groups, although to a smaller extent (539.5, 467.3

and 460.1 nmol MDA equivalents per gram liver at 0, 75 and 200 ppm, respectively). The reduction of thiobarbituric acid–reactive material is not indicative of an induction of oxidative stress in the liver and is not considered to represent an adverse effect (Mellert, Beimborn & Van Ravenzwaay, 2003).

(e) In vitro haemolytic potential

In order to clarify the possible haemolytic potential of pyraclostrobin, the effect on haemolysis was investigated in vitro using 0.001%, 0.01% and 0.1% weight per volume (w/v) pyraclostrobin (batch no. CP029053; purity 99.0%) solutions. The assay is based on the integrity of the erythrocyte membrane after incubation with the test compound. Haemoglobin crosses the cell membrane into the test solution after damage of the membrane. The concentration of free haemoglobin can then be determined and correlated directly with the damage caused to the erythrocyte membrane by the test compound. To demonstrate any haemolytic effect, photographs of the erythrocyte suspension and the supernatant were taken.

Pyraclostrobin did not cause haemolysis in the in vitro haemolysis test even at relatively high concentrations (0.1% w/v) and after stirring the erythrocyte suspension for 2 hours (Deckardt & Van Ravenzwaay, 2003).

(f) Combination study of pyraclostrobin with vitamin B_{12}

Pyraclostrobin (batch no. LJ-Nr.27882/199/b; purity 98.7%) was administered to groups of 12 male Wistar rats at a dietary concentration of 0 or 1500 ppm (equivalent to 150 mg/kg bw per day) over a period of 4 weeks. Animals received additionally 0.1 mL vitamin B_{12} subcutaneously each day. Feed consumption and body weights were determined weekly. The animals were examined for clinical signs of toxicity or mortality at least once a day. Haematological examinations and serum iron determinations were carried out on days 7, 14, 21 and 27. At necropsy, pH was determined in forestomach and glandular stomach.

The administration of pyraclostrobin resulted in decreased feed consumption and body weight. The decreases were similar in the groups with and without vitamin B_{12} administration. Haematology examinations revealed an iron deficiency anaemia after administration of pyraclostrobin. The administration of vitamin B_{12} did not affect pyraclostrobin-induced changes in red blood cell parameters or serum iron level. The pH measurement in forestomach and glandular stomach did not reveal any substance-related changes. The absolute and relative weights of the duodenum were increased in the groups receiving pyraclostrobin either with or without vitamin B_{12} administration. There was basically no difference between the two groups. No effect was seen in the group receiving vitamin B_{12} only.

In conclusion, the simultaneous administration of excessive vitamin B_{12} and pyraclostrobin did not inhibit pyraclostrobin-induced anaemia, serum iron deficiency or duodenal weight increase. The pH values in the stomach did not show any treatment-related effects. This study showed evidence that serum iron deficiency is the causative agent for anaemia and duodenal thickening (Mellert, 2003a; Mellert et al., 2003b).

(g) Combination study of dimoxystrobin with iron

Dimoxystrobin, which is a strobilurin fungicide similar to pyraclostrobin, was administered to groups of 10 male Wistar rats at a dietary concentration of 0 or 4500 ppm (equal to 0, 206.6 and 171.2 mg/kg bw per day for controls, 4500 ppm or 4500 ppm plus iron complex group, respectively) over a period of 14 days and to groups of 10 female Wistar rats at a dietary concentration of 0, 500 or 4500 ppm (equal to 0, 37.7, 17.7, 191.3 and 84.7 mg/kg bw per day for controls, 500 ppm, 500 ppm plus iron complex, 4500 ppm and 4500 ppm plus iron complex, respectively) over a period of 7 days. Simultaneously, additional groups received an iron complex (Myofer® 100) intramuscularly (males once daily at a dose of 100 mg/kg bw on study days 0, 7, 11 and 13, and females twice daily at a dose of 50 mg/kg bw from day 2 to day 6). Feed consumption and body weights were determined weekly.

The animals were examined for signs of toxicity or mortality at least once a day. On several days, the following serum parameters were determined: iron, transferrin (males only) and unsaturated iron binding capacity (males only). At necropsy, duodenal weights were determined.

Iron levels in the serum were increased in all groups receiving the iron complex (with and without dimoxystrobin) and reduced in the groups receiving dimoxystrobin only. Feed consumption was statistically significantly decreased in all treated males and in females treated with 4500 ppm dimoxystrobin and/or iron complex. Both the administration of 4500 ppm dimoxystrobin and the administration of the iron complex caused a significant impairment of body weight. In the groups receiving 4500 ppm dimoxystrobin and iron complex, the effect was additive. Duodenal weights were statistically significantly increased in the 500 ppm (females) and 4500 ppm (both sexes) groups. Treatment with 500 ppm dimoxystrobin and iron complex as well as 4500 ppm dimoxystrobin and iron complex led to a lower increase in duodenal weights in all treatment groups. The increase was not statistically significant in females of the 4500 ppm plus iron complex treatment group when compared with controls. Moreover, the increase was not dose dependent. Although the duodenal weights did not completely reach the control values, these data indicate that iron administered to animals treated with dimoxystrobin had an inhibitory effect on the increase in duodenal weights, in the sense of preventing effects on the duodenum that are caused by a reduction in serum iron levels following treatment with dimoxystrobin.

In conclusion, this study indicates that the administration of iron complex can reduce the dimoxystrobin-induced increase in duodenal weights. Dimoxystrobin-induced iron deficiency might therefore be a causative factor for increased duodenal weights (Mellert, 2003b; Mellert et al., 2002; Mellert & Kaufmann, 2004).

(h) Effect of dimoxystrobin on iron absorption and transport in the duodenum

In a non-GLP-compliant study investigating the effect of dimoxystrobin on the mucosal uptake and transfer of iron into the carcass after oral administration, groups of five female Wistar rats were treated with control diet or diet containing 4500 ppm dimoxystrobin (equivalent to 450 mg/kg bw per day) for 24, 96 or 168 hours. The everted duodenums of these rats, removed under anaesthesia, were incubated for 5 minutes in buffer containing $^{59}Fe^{2+}$-ascorbate, and Fe^{2+} uptake was measured by counting radioactivity in the duodenal segments and by autoradiography. Iron transfer across the serosal membrane (i.e. iron transfer into the body) was determined with tied-off duodenal segments in anaesthetized rats that were exposed to 4500 ppm dimoxystrobin in the feed for 24 or 96 hours. $^{59}Fe(nitrilotriacetate)_2$ was injected into duodenal segments. After varying time intervals (10, 20 and 40 minutes), the animals were terminated, and the duodenal segments were removed, washed and weighed. Radioactivity in the duodenal segments and the remaining carcass, representing mucosal retention and mucosal transfer, respectively, was determined by gamma counting. The sum of the mucosal retention and mucosal transfer represents the total mucosal uptake.

Mucosal iron uptake showed a statistically significant reduction after treatment with dimoxystrobin for 96 and 168 hours. This was confirmed by autoradiography performed on animals that had been treated for 168 hours compared with those on control diet. Mucosal iron uptake was slightly and statistically non-significantly reduced after treatment of rats with dimoxystrobin for 24 hours. Iron transfer across the serosal membrane (i.e. iron transfer into the body) was significantly reduced after a 96-hour treatment with dimoxystrobin, with the reduction of iron transfer being proportional to the reduction in uptake. After 24 hours of treatment, no significant effect was found.

In summary, the results of this study clearly demonstrate that repeated treatment of rats with dimoxystrobin considerably reduces both uptake of iron by the duodenum mucosa and transfer of iron into the body (Srai, 2003).

2.5 *Toxicity of metabolites*

New acute, short-term toxicity and genotoxicity studies for seven metabolites of pyraclostrobin were submitted.

(a) 500M04 (pyrazolon)

Toxicity data submitted on metabolite 500M04 included studies on acute oral toxicity in rats, irritation to eye and skin in rabbits, hypersensitization in guinea-pigs and short-term oral toxicity in rats (summarized in Table 9). In vitro and in vivo genotoxicity studies with this metabolite are described in section (c) on genotoxicity below.

Table 9. Summary of acute toxicity, irritation, sensitization and short-term toxicity of 500M04

Route (method)	Species/strain	Purity (%); batch no.	Result	Reference
Oral (gavage)	Wistar rats	100.5; 27967/95	LD_{50} > 2 000 mg/kg bw in both sexes	Kuehlem (1997a)
Skin irritation	New Zealand white rabbits	100.5; 27967/95	Negative	Kuehlem (1997b)
Eye irritation	New Zealand white rabbits	100.5; 27967/95	Weakly positive	Kuehlem (1997c)
Dermal sensitization (maximization test)	Dunkin-Hartley guinea-pigs	100.5; 27967/95	Not sensitizing	Kuehlem & Hellwig (1997)
Ninety-day oral toxicity	Wistar rats	99.6; L84-174	NOAEL = 100 mg/kg bw for males, 300 mg/kg bw for females	Buesen et al. (2013)

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

Acute oral toxicity

500M04 was investigated in a study in which a group of three male and three female Wistar rats were administered the metabolite by gavage at a dose of 200 or 2000 mg/kg bw. The test material was administered in 0.5% tylose CB 30000 at a dosing volume of 10 mL/kg bw. The animals were starved overnight prior to dosing. Mortality and signs of reaction to treatment were recorded during a subsequent 14-day observation period. The surviving animals were killed on the following day. All animals were subjected to necropsy.

No deaths occurred after oral administration of 200 or 2000 mg/kg bw. At a dose of 2000 mg/kg bw, male rats showed an impaired or poor general state, dyspnoea, apathy and staggering up to 1 day after administration, whereas female rats did not show any symptoms. Likewise, no symptoms were noted for males or females at the dose of 200 mg/kg bw. Normal body weight gain was observed during the 14 days of the observation period with the exception of one 200 mg/kg bw female, which displayed a body weight loss during the second week of observation. No remarkable findings were observed at necropsy. The LD_{50} was greater than 2000 mg/kg bw (Kuehlem, 1997a).

Skin irritation in rabbits

The potential of 500M04 to irritate the skin of rabbits was assessed by semi-occluded application of 0.5 g of the test material to the closely clipped dorsa of three New Zealand white rabbits for 4 hours. Dermal reactions were assessed 1, 24, 48 and 72 hours after removal of the dressings.

No erythema or oedema was observed in any rabbit after 24–72 hours. 500M04 did not show a skin irritation potential under the test conditions chosen (Kuehlem, 1997b).

Eye irritation in rabbits

The potential of 500M04 to cause damage to the conjunctiva, iris or cornea was assessed in three New Zealand white rabbits that were subjected to a single ocular instillation of 0.1 mL of 500M04. Ocular reactions were examined 1, 24, 48 and 72 hours after treatment.

No ocular reactions on the cornea or iris were observed. Slight to moderate conjunctival redness and chemosis were observed in all animals at the 1-hour reading point. Discharge was observed only in one animal. At the 72-hour reading point, all effects were fully reversed.

Based on the findings of this study, 500M04 is slightly irritating to the eye under the test conditions chosen (Kuehlem, 1997c).

Sensitization in guinea-pigs

For the determination of the potential sensitizing properties of 500M04, a maximization test based on the method of Magnusson and Kligman was conducted using a control group and a treated group of five and 10 female Pirbright white Dunkin Hartley guinea-pigs, respectively. The test substance concentrations for the main test were selected based on the results of the pretests and the results of the first challenge. The intradermal induction was performed with a 5% test substance preparation in 1% aqueous tylose CB 30000 solution or in Freund's complete adjuvant/0.9% aqueous sodium chloride solution (1:1). The epicutaneous induction was conducted with a 25% test substance preparation in 1% aqueous tylose CB 30000 solution. Two challenges were performed 14 and 21 days after percutaneous induction.

After the first challenge with a 10% test substance preparation, very slight to well-defined skin reactions were observed in three test group animals. The second challenge with a 10% substance preparation did not cause any skin reactions.

Based on the results of this study and applying the evaluation criteria, it was concluded that 500M04 does not have a sensitizing effect on the skin of the guinea-pig in the maximization test under the test conditions chosen (Kuehlem & Hellwig, 1997).

Short-term oral toxicity in rats

Three groups of 10 male and 10 female Wistar rats received 500M04 (batch no. L84-174; purity 99.6%) at a target dose of 100, 300 or 1000 mg/kg bw per day (mean intakes 103, 302 and 1017 mg/kg bw per day for males and 106, 316 and 1066 mg/kg bw per day for females, respectively) for 3 months by the dietary route. A further group of 10 male and 10 female rats received the basal control diet as a contemporaneous control. Mortality, clinical signs, body weight, feed consumption and ophthalmological parameters were measured. Water consumption was monitored from day 49 onwards. A functional observational battery (FOB) was performed on all animals. Haematology and blood chemistry parameters were measured. All animals were terminated, necropsied and assessed by gross pathology. The organs were sampled, weighed and examined histopathologically.

No effects on clinical signs, mortality, body weight, feed consumption, ophthalmology or FOB test parameters were detected. Daily drinking-water consumption was consistently increased in both sexes at the high dose (1000 mg/kg bw per day) throughout the observation period, with males more severely affected than females (Table 10). The increased water consumption is also evident if the average water consumption between days 49 and 91 is calculated. The effect on water consumption in mid- and low-dose animals was neither consistent over time nor dose dependent, and the difference in the overall mean water consumption relative to the controls was marginal. Thus, only the effect at 1000 mg/kg bw per day was considered to be treatment related.

Table 10. Summary of findings in short-term toxicity study in rats administered 500M04

	Males				Females			
Finding	0 mg/kg bw per day	100 mg/kg bw per day	300 mg/kg bw per day	1 000 mg/kg bw per day	0 mg/kg bw per day	100 mg/kg bw per day	300 mg/kg bw per day	1 000 mg/kg bw per day
Water consumption								
g/animal[a]	22.5	23.0	23.4	31.5	20.5	21.3	20.5	24.5
% relative to control[a]	–	2.2	4.1	39.7	–	4.2	−0.1	19.5
Haematology								
RBCs (10^{12}/L)	8.82	8.80	8.66	8.02**	7.53	7.41	7.58	7.54
HGB (mmol/L)	9.1	9.1	9.1	8.8*	8.4	8.3	8.5	8.4
MCV (fL)	49.7	50.1	50.4	53.5**	52.4	52.6	53.2	53.1
MCH (fmol)	1.04	1.04	1.05	1.10*	1.12	1.12	1.12	1.12
Ret (%)	2.0	1.9	2.2	2.9*	2.6	2.9	2.9	2.6
Clinical chemistry								
Total protein (g/L)	62.99	62.03	61.77	59.01**	64.11	64.98	65.03	66.65
Triglycerides (mmol/L)	1.16	1.18	1.44	1.86**	0.64	0.49	0.58	0.97
Urine analysis								
Urine volume (mL)	3.2	3.9	3.5	6.5**	2.4	2.7	3.0	3.9**
Specific gravity (g/L)	1 060	1 055	1 062	1 040**	1 071	1 062	1 051	1 045**
Crystal[b]	2	2	3*	3**	2	2	2	2
Histopathology: Kidney[c]								
Eosinophic material, renal pelvis	0/10	0/10	1/10 (2.0)	6/10 (2.8)	0/10	0/10	0/10	1/10 (2.0)
Hyperplasia, urothelial	0/10	0/10	1/10 (1.0)	7/10 (1.4)	0/10	0/10	0/10	3/10 (2.0)
Mineralization, medulla	2/10 (1.0)	4/10 (1.0)	0/10	7/10 (1.0)	8/10 (1.3)	9/10 (1.3)	10/10 (1.6)	10/10 (2.3)
Nephropathy chronic	4/10 (1.0)	7/10 (1.0)	5/10 (1.0)	9/10 (1.3)	6/10 (1.0)	1/10 (1.0)	1/10 (1.0)	9/10 (1.3)
Ulceration, papillary	0/10	0/10	0/10	1/10 (2.0)	0/10	0/10	0/10	0/10
Histopathology: Spleen[c]								
Haematopoiesis, extramedullary	6/10 (1.0)	10/10 (1.4)	10/10 (1.5)	10/10 (2.0)	8/10 (1.2)	–	–	9/10 (1.2)

bw: body weight; HGB: haemoglobin; MCH: mean cell haemoglobin; MCV: mean corpuscular volume; RBC: red blood cells; Ret: reticulocytes; *: $P \leq 0.05$; **: $P \leq 0.01$ (Kruskal-Wallis and Wilcoxon-tests, two-sided)

[a] Values were calculated based on mean individual daily consumption. Values may not calculate exactly due to rounding of mean values; no statistics were performed because $n = 2$.

[b] Semiquantitative parameter: Grade 0 = none, Grade 1 = few, Grade 2 = many, Grade 3 = masses.

[c] Mean severity grading given in parentheses. Histopathological findings were graded minimal (Grade 1), slight (Grade 2), moderate (Grade 3), marked (Grade 4) and massive/severe (Grade 5). The mean severity is the sum of the gradings divided by the incidence.

Source: Buesen et al. (2013)

Treatment-related haematological changes were restricted to high-dose males and consisted of a slight regenerative normochromic-macrocytic anaemia, indicated by decreased erythrocyte counts and haemoglobin values and increased mean corpuscular volume, mean cell haemoglobin and relative reticulocyte counts. Treatment-related clinical chemistry findings were restricted to high-dose males and consisted of decreased total protein and globulin values as well as increased triglyceride levels. Urine analysis revealed increased mean urine volumes, which were accompanied by lower specific gravity of the urine in both sexes at 1000 mg/kg bw per day. This is consistent with the higher water consumption noted at this dose. In addition, more crystals of unknown origin were found in the urine sediment of males at and above 300 mg/kg bw per day. The aforementioned findings were considered to be treatment related (Table 10).

No statistically significant absolute or relative organ weight differences were observed. There were no treatment-related gross necropsy findings. Treatment-related histopathological findings were observed in the kidneys of mid-dose males and high-dose males and females as well as in the spleen of high-dose males.

In the kidney, the eosinophilic material was localized in the lumen of the renal pelvis in males at 300 and 1000 mg/kg bw per day and in females at 1000 mg/kg bw per day. The urothelial hyperplasia most probably represented a reactive response to the presence of the eosinophilic material in the renal pelvis in males at 300 and 1000 mg/kg bw per day and in females at 1000 mg/kg bw per day. The increased mineralization was seen in the medulla at 1000 mg/kg bw per day in both sexes. Chronic progressive nephropathy showed a slightly increased incidence and severity in both sexes at the high dose. Papillary ulceration was detected at 1000 mg/kg bw per day in males. Increased severity of extramedullary haematopoiesis in the spleen was detected in high-dose males (Table 10).

The NOAEL was 100 mg/kg bw per day, based on renal lesions and urine analysis findings in males at 300 mg/kg bw per day (Buesen et al., 2013).

(b) 500M106

Short-term oral toxicity in rats

500M106 (batch no. L87-218; purity 97.7%) was administered to groups of 10 male and 10 female Wistar rats by gavage at a target dose of 0, 100, 300 or 1000 mg/kg bw per day for 28 days. The test item was administered as a suspension in 0.5% aqueous carboxymethylcellulose. Mortality, body weight, feed consumption, water consumption, ophthalmological examination, a FOB and urine analysis were conducted. Haematology and clinical chemistry parameters were examined. All animals were terminated, necropsied and assessed by gross pathology. The organs were sampled, weighed and examined histopathologically.

No test substance–related effects on clinical signs, mortality, ophthalmoscopy, body weight, feed consumption, FOB test parameters, motor activity or clinical chemistry parameters were observed.

Treatment-related and adverse haematological changes were restricted to high-dose males and females and consisted of a slight, regenerative normochromic-normocytic anaemia, indicated by decreased erythrocyte counts, haemoglobin and haematocrit in males and increased absolute reticulocyte counts in both sexes. Platelet counts were significantly higher in all treated male groups. However, the values were within the range observed in historical control animals of this strain and age (683–973 $\times 10^9$/L). A statistically significant increase in the number of reticulocytes was noted in both sexes at the low dose. Again, the numbers were within the historical control range (males: 102.1–184.6 $\times 10^9$/L; females: 102.2–189.8 $\times 10^9$/L) and not dose related (Table 11).

Changes in absolute and relative organ weights were noted in high-dose males and consisted of increased liver and duodenum weights (Table 11). There were no treatment-related gross necropsy findings.

Table 11. Summary of 4-week oral toxicity study of 500M106 in rats

	Males				Females			
Finding	**0 mg/kg bw per day**	**100 mg/kg bw per day**	**300 mg/kg bw per day**	**1 000 mg/kg bw per day**	**0 mg/kg bw per day**	**100 mg/kg bw per day**	**300 mg/kg bw per day**	**1 000 mg/kg bw per day**
Haematology								
RBC (10^{12}/L)	7.86	7.67	7.88	7.35**	7.15	7.04	6.88	6.9
HGB (mmol/L)	8.8	8.8	8.8	8.4**	8.2	8.2	8.0	8.3
HCT (%)	41.7	41.5	41.4	39.4**	37.5	37.3	36.8	36.8
Platelets (10^9/L)[a]	712	772*	775*	817**	756	676	711	733
Reticulocytes (10^9/L)[b]	141.2	172.4**	151.7	248.1**	146.1	173.7*	191.5	211.7**
Organ weights								
Absolute duodenum weight (g)	0.477	0.508	0.485	0.532*	0.442	0.432	0.425	0.456
Relative duodenum weight (%)	0.176	0.185	0.184	0.200*	0.256	0.245	0.241	0.263
Absolute liver weight (g)	7.32	7.57	7.23	8.00*	4.70	4.68	4.83	4.95
Relative liver weight (%)	2.69	2.75	2.73	3.00**	2.72	2.65	2.74	2.84*
Absolute spleen weight (g)	0.583	0.531	0.549	0.578	0.462	0.373	0.407	0.419
Relative spleen weight (%)	0.214	0.193	0.207	0.217	0.266	0.211	0.231	0.240
Histopathology: Liver[c]								
Hypertrophy, centrilobular	0/10	0/10	0/10	4/10 (1.0)	0/10	0/10	0/10	0/10
Histopathology: Spleen[c]								
Haematopoiesis, extramedullary	2/10 (1.0)	2/10 (1.0)	3/10 (1.0)	8/10 (1.9)	1/10 (1.0)	0/10	3/10 (1.0)	6/10 (1.5)

bw: body weight; HCT: haematocrit; HGB: haemoglobin; RBC: red blood cells; *: $P \leq 0.05$; **: $P \leq 0.01$ (Kruskal-Wallis plus Wilcoxon test, two-sided)

[a] Historical control data for platelets: males: 683–973 × 10^9/L.

[b] Historical control data for reticulocytes: males: 102.1–184.6 × 10^9/L; females: 102.2–189.8 × 10^9/L.

[c] Mean severity grading given in parentheses. Histopathological findings were graded minimal (Grade 1), slight (Grade 2), moderate (Grade 3), marked (Grade 4) and massive/severe (Grade 5). The mean severity is the sum of the gradings divided by the incidence.

Source: Buesen et al. (2017)

Treatment-related histopathological findings were restricted to the high dose and noted in the liver of high-dose males and the spleen of high-dose males and females. In the liver of high-dose males, a minimal centrilobular hepatocellular hypertrophy was noted, which correlated with the increased liver weights. In the spleen, an increased incidence and severity of extramedullary haematopoiesis was noted,

which correlated with the slight anaemia observed at this dose. There was no histopathological correlate to the slightly increased absolute and relative duodenum weights (Table 11).

The NOAEL for 500M106 in rats was 300 mg/kg bw per day, on the basis of the treatment-related effects on red blood cell parameters (regenerative anaemia in both sexes), duodenum (increased weight in males), liver (increased weight and hepatocellular hypertrophy in males) and spleen (extramedullary haematopoiesis) at 1000 mg/kg bw per day (Buesen et al., 2017).

(c) Genotoxicity

Several metabolites of pyraclostrobin were evaluated for potential genotoxicity in in vitro tests for mutagenicity in bacterial and mammalian cells, for chromosome damage (clastogenicity) and for unscheduled DNA synthesis. The results of these studies are summarized in Table 12.

Table 12. Summary of in vitro and in vivo genotoxicity studies with metabolites of pyraclostrobin

Metabolite/study	Test object	Concentration	Purity (%), lot/batch no.	Results	Reference
500M04 (pyrazolon)					
In vitro					
Reverse mutation test	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537; *Escherichia coli* WP2*uvr*A	10, 33, 100, 333, 1 000, 2 600 and 5 200 µg/plate ±S9	97.6, L83-44-2	Negative	Woitkowiak (2012)
Forward mutation test	Chinese hamster ovary cells (*Hprt* locus)	Experiment 1: 21.9, 43.8, 87.5, 175 and 350 µg/mL +S9; 21.9, 43.8, 87.5 and 175 µg/mL −S9 Experiment 2: 31.3, 62.5, 125 and 250 µg/mL +S9; 62.5, 125, 250, 500 and 1 000 µg/mL −S9	99.6, L84-174	Negative	Schulz & Landsiedel (2012a)
Chromosomal aberrations	Chinese hamster ovary cells (V79 cells)	62.5, 125 and 250 µg/mL ±S9; 100, 150, 200 and 250 µg/mL +S9	97.6, L83-44-2	Positive with S9	Schulz & Landsiedel (2012b)
In vivo					
Micronucleus test	Bone marrow cells from NMRI mice (five males)	500, 1 000 and 2 000 mg/kg bw (once by gavage, analysed 24 hours after treatment) 2 000 mg/kg bw (once by gavage, analysed 48 hours after treatment)	99.6, L84-174	Negative	Fabian & Landsiedel (2013); Schulz & Landsiedel (2013a)

Metabolite/study	Test object	Concentration	Purity (%), lot/batch no.	Results	Reference
500M24					
In vitro					
Reverse mutation test	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A	33, 100, 333, 1 000, 2 500 and 5 000 µg/plate ±S9	98.8, L82-125	Negative	Woitkowiak (2014)
Forward mutation test	L5178Y mouse lymphoma cells (*Tk*$^{+/-}$ locus)	287.5, 575, 1 150 and 2 300 µg/mL ±S9; 431.3, 862.5, 1 725 and 2 300 µg/mL +S9; 143.8, 287.5, 575, 1 150 and 2 300 µg/mL −S9	98.8, L82-125	Negative	Schulz & Landsiedel (2014a)
Chromosomal aberrations	Chinese hamster ovary cells (V79 cells)	575, 1 150 and 2 300 µg/mL ±S9; 1 150, 1 725 and 2 300 µg/mL −S9	98.8, L82-125	Positive without S9	Schulz & Landsiedel (2014b)
In vivo					
Micronucleus test	Bone marrow cells from NMRI mice (five males)	500, 1 000 and 2 000 mg/kg bw (once by gavage, analysed 24 hours after treatment) 2 000 mg/kg bw (once by gavage, analysed 48 hours after treatment)	98.8, L82-125	Negative	Dony (2014); Schulz & Landsiedel (2016a)
500M49					
In vitro					
Reverse mutation test	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A	33, 100, 333, 1 000, 2 500 and 5 000 µg/plate ±S9	100.0, L82-115	Negative	Woitkowiak (2013a)
Forward mutation test	L5178Y mouse lymphoma cells (*Tk*$^{+/-}$ locus)	125, 250, 500, 1 000 and 2 000 µg/mL ±S9; 375, 750 and 1 500 µg/mL +S9; 62.5 µg/mL −S9	100.0, L82-115	Negative	Schulz & Landsiedel (2014c)
Chromosomal aberrations	Chinese hamster ovary cells (V79 cells)	250, 500, 1 000 and 2 000 µg/mL ±S9	100.0, L82-115	Negative	Schulz & Landsiedel (2014d)

Metabolite/study	Test object	Concentration	Purity (%), lot/batch no.	Results	Reference
500M51					
In vitro					
Reverse mutation test	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A	10, 33, 100, 333, 1 000, 2 600 and 5 200 µg/plate ±S9	97.9, L82-117	Negative	Woitkowiak (2013b)
Forward mutation test	L5178Y mouse lymphoma cells ($Tk^{+/-}$ locus)	250, 500, 1 000 and 2 000 µg/mL ±S9; 375, 750, 1 500 and 2 000 µg/mL +S9; 7.8, 15.6, 31.3, 62.5, 125, 250, 500, 1 000 and 2 000 µg/mL −S9	97.9, L82-117	Negative	Schulz & Landsiedel (2014e)
Chromosomal aberrations	Chinese hamster ovary cells (V79 cells)	500, 1 000 and 2 000 µg/mL +S9; 125, 250, 500, 1 000 and 2 000 µg/mL −S9	97.9, L82-117	Negative	Schulz & Landsiedel (2014f)
500M76					
In vitro					
Reverse mutation test	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A	22, 110, 550, 2 750 and 5 500 µg/plate ±S9	94.5, 01586-236	Negative	Engelhardt & Hoffmann (2000)
Forward mutation test	Chinese hamster ovary cell (*Hprt* locus)	62.5, 125, 250, 500, 750 and 1 000 µg/mL +S9; 12.5, 25, 50, 100, 200 and 400 µg/mL −S9 9.38, 18.75, 37.5, 75, 150 and 300 µg/mL −S9	94.5, 01586-236	Negative	Engelhardt & Leibold (2003)
Chromosomal aberrations	Chinese hamster ovary cells (V79 cells)	250, 500 and 750 µg/mL +S9; 125, 250 and 500 µg/mL −S9; 700, 750 and 800 µg/mL +S9; 500, 550 and 600 µg/mL −S9	94.5, 01586-236	Positive with and without S9	Schulz & Landsiedel (2013b, 2014g)
In vivo					
Micronucleus test	Bone marrow cells from NMRI mice (five males)	125, 250 and 500 mg/kg bw (once by gavage, analysed 24 hours after treatment) 500 mg/kg bw (once by gavage,	99.0, L83-122	Negative	Schulz & Landsiedel (2012c)

Metabolite/study	Test object	Concentration	Purity (%), lot/batch no.	Results	Reference
		analysed 48 hours after treatment)			
500M02					
In vitro					
Reverse mutation test	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A	4, 20, 100, 500, 2 500 and 5 000 µg/plate ±S9	99.9, 01185-022	Negative	Engelhardt & Hoffmann (1999)
		33, 100, 333, 1 000, 2 500 and 5 000 µg/plate ±S9	98.3, L85-192	Negative	Woitkowiak (2016)
Forward mutation test	L5178Y mouse lymphoma cells (*Tk*$^{+/-}$ locus)	4.69, 9.38, 18.75, 37.5, 75 and 150 µg/mL ±S9; 1.56, 3.13, 6.25, 12.5, 25, 50 and 100 µg/mL ±S9	98.3, L85-192	Negative	Schulz & Landsiedel (2016b)
Micronucleus test	Human lymphocytes	4.3, 6.6, 7.6, 9.9, 13.2 and 14.8 µg/mL +S9; 4.3, 7.6, 13.2, 22.2, 33.3 and 100 µg/mL −S9	98.3, L84-192	Negative	Chang (2016a)
500M106					
In vitro					
Reverse mutation test	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A	3.3, 10, 33, 100, 333, 1 000, 2 600 and 5 200 µg/plate ±S9	97.6, L87-58	Negative	Woitkowiak & Landsiedel (2016)
Forward mutation test	L5178Y mouse lymphoma cells (*Tk*$^{+/-}$ locus)	1.56, 3.13, 6.25, 12.5, 25, 50 and 100 µg/mL ±S9; 4.69, 9.38, 18.75, 37.5, 50, 75 and 100 µg/mL +S9	97.6, L87-58	Positive with S9	Schulz & Landsiedel (2016c)
Micronucleus test	Human lymphocytes	1.9, 2.6, 3.3, 3.5, 4.6, 5.8, 6.1, 8.0, 10.7, 14.0, 18.7, 32.7 and 100 µg/mL ±S9	97.6, L87-58	Negative	Chang (2016b)
In vivo					
Micronucleus test	Bone marrow cells from NMRI mice (5 males)	500, 1 000 and 2 000 mg/kg bw (once by gavage, analysed 24 hours after treatment)	97.6, L87-58	Negative	Dony (2016)
		2 000 mg/kg bw (once by gavage, analysed 48 hours after treatment)			

Metabolite/study	Test object	Concentration	Purity (%), lot/batch no.	Results	Reference
Muta™Mouse gene mutation assay	Male CD2-LacZ80/HazfBR mice (Muta™Mouse)	100, 300 and 1 000 mg/kg bw (gavage for 28 consecutive days)	97.6, L87-58	Negative	Ueda (2017)

bw: body weight; *Hprt*: hypoxanthine–guanine phosphoribosyltransferase; S9: 9000 × *g* supernatant fraction from rat liver homogenate; *Tk*: thymidine kinase

3. Observations in humans

Manufacturing plant personnel are monitored by regular medical examinations. There are no specific parameters available for monitoring the effects of pyraclostrobin. The surveillance programme includes a general physical examination, including neurological status, red and white blood cell counts and liver enzymes. Adverse health effects suspected to be related to pyraclostrobin exposure have not been observed (BASF, 2017).

Information on 33 persons exposed to pyraclostrobin was published in the Morbidity and Mortality Weekly Report series of the United States Centers for Disease Control and Prevention (Gergely & Calvert, 2008). In all but one incident, the exposure was to spray drift from aerial application. The most severe incident pertained to 27 workers who were exposed to off-target drift from a nearby field while removing tassels from corn plants (to prevent autopollination and enable hybridization). Some workers reported feeling wet droplets on their skin and seeing mist coming from the aircraft. All workers received skin decontamination on-site by a hazardous materials team before being transported to an emergency department for observation until their symptoms resolved.

The most common symptom was upper respiratory tract pain or irritation (26 patients), followed by chest pain (20 patients). Three patients had nausea, and one patient each had pruritis, skin redness, eye pain, weakness, headache, dizziness and chest pain. According to United States National Institute for Occupational Safety and Health classifications, low-severity illness or injury includes "illnesses manifested by skin, eye, or upper respiratory irritation". These illnesses might also include fever, headache, fatigue or dizziness. Typically, the illness or injury resolves without treatment, and time lost from work or normal activities is less than 3 days.

The other incidents pertained to single cases with off-target drift of pyraclostrobin from nearby aerial applications. The individuals were exposed by riding a motorcycle near a field or by spray drifting to their home yard. Symptoms reported were headache, eye pain partially associated with conjunctivitis and dizziness. The last case was from a crop-duster pilot who – when his plane crashed during take-off – was exposed to spilling of the liquid fungicide.

Comments

Biochemical aspects

Based on in vivo studies and in vitro incubations with rat serum or plasma, six biotransformation reactions were observed: 1) desmethoxylation of the side-chain, 2) hydroxylation of the chlorophenyl pyrazole ring system, 3) hydroxylation of the tolyl ring system, 4) cleavage of the ether bond, resulting in chlorophenyl pyrazole or anthranilic acid derivatives, 5) desmethylation of the side-chain and 6) cleavage of the amide bond in the side-chain. The combination of these reactions with subsequent conjugation resulted in a large number of metabolites (Birk, Lutz & Doebbe, 2014).

An in vitro comparison of metabolic profiles among rats, rabbits, dogs and humans shows the same key degradation steps in all species. The metabolites 500M04, 500M73, 500M108, 500M103, 500M104 and 500M88 were common to all test species. 500M02, 500M106 and 500M107, identified as the metabolites in humans, were also identified in other species used for toxicological testing (Funk, Glaessgen & Kalyon, 2014; Fabian & Landsiedel, 2016; Funk & Bellwon, 2016a,b).

Toxicological data

Additional data were made available on the histopathology of the 2-year toxicity and carcinogenicity studies in rats that had been evaluated by the 2003 Meeting (Mellert et al., 1999a,b), and two supplementary carcinogenicity studies not available to the 2003 Meeting (Mellert, 2002b,c) were submitted. The two new studies were conducted at concentrations (400 and 600 ppm, respectively) higher than those used in the first study. As these concentrations were above the MTD because of toxicity resulting in early termination, these studies provided no additional information relevant to the risk assessment.

Three new immunotoxicity studies were submitted, one of which was uninterpretable due to the inconsistent results in negative and positive controls.

In one immunotoxicity study, female mice were administered pyraclostrobin in the diet at 0, 50, 200 or 750 ppm (equal to 0, 13, 50 and 165 mg/kg bw per day, respectively) for 28 days. The NOAEL for immunotoxicity was 750 ppm (equal to 165 mg/kg bw per day), the highest dose tested. The NOAEL for systemic toxicity was 200 ppm (equal to 50 mg/kg bw per day), based on reduced body weight gain and feed consumption and reduced thymus and spleen weights at 750 ppm (equal to 165 mg/kg bw per day). The reduced spleen and thymus weights were considered to be secondary to the extreme reductions in body weight gain in mice receiving 750 ppm (Smiraldo, 2012a).

Another immunotoxicity study was conducted in female mice administered pyraclostrobin in the diet at 0, 50, 200 or 750 ppm (equal to 0, 14, 55 and 191 mg/kg bw per day, respectively) for 28 days. The NOAEL for immunotoxicity was 750 ppm (equal to 191 mg/kg bw per day), the highest dose tested. The NOAEL for systemic toxicity was 200 ppm (equal to 55 mg/kg bw per day), based on reduced body weight gain and feed consumption, decreased thymus and spleen weights, and low numbers of spleen cells at 750 ppm (equal to 191 mg/kg bw per day). The reduced spleen and thymus weights were considered secondary to the extreme reductions in body weight gain in mice receiving 750 ppm (Smiraldo, 2012c).

The Meeting concluded that pyraclostrobin is not immunotoxic.

A study of phototoxicity in vitro indicated that pyraclostrobin was not phototoxic (Cetto & Landsiedel, 2012, 2014).

Two repeated-dose inhalation studies in rats were submitted. In the first study, rats were exposed, through the head and nose inhalation route, to an aerosol of pyraclostrobin at 0, 1, 30 or 300 mg/m^3 for 6 hours per day, 5 days per week, for 4 weeks (Gamer et al., 2005). In the second study, rats were exposed, through the head and nose inhalation route, to an aerosol of pyraclostrobin at 0, 3, 10 or 30 mg/m^3 for 6 hours per day, 5 days per week, for 4 weeks (Ma-Hock et al., 2014). In both studies, there were local irritant effects leading to inflammation of the nasal tract at 30 mg/m^3. In addition, mucosal hyperplasia in the duodenum was observed at 30 mg/m^3 in the first study (NOAEC of 1 mg/m^3) and not seen in the second study at any tested concentration.

Additional studies indicated that the mucosal hyperplasia observed in mice, rats and dogs following repeated dietary exposure could be induced by reduced uptake of iron in the duodenum, resulting in lower serum iron levels, but not by local irritation (Mellert et al., 2003a,b; Srai, 2003). It is unlikely that this mode of action would be applicable to effects seen after a single dose (Appendix 3).

Toxicological data on metabolites and/or degradates

500M04

500M04 (pyrazolon) is a metabolite in rats, rabbits and humans. Its acute oral LD_{50} is greater than 2000 mg/kg bw (Kuehlem, 1997a). 500M04 was not irritating to the skin of rabbits (Kuehlem, 1997b), slightly irritating to the eye of rabbits (Kuehlem, 1997c) and not sensitizing in guinea-pigs (Kuehlem & Hellwig, 1997).

500M04 was tested for genotoxicity in an adequate range of in vitro and in vivo assays. It gave negative results for gene mutation (Schulz & Landsiedel, 2012a; Woitkowiak, 2012) and a positive response in an in vitro chromosomal aberration assay (Schulz & Landsiedel, 2012b), but it was negative in an in vivo micronucleus test (Schulz & Landsiedel, 2013a).

In a 3-month toxicity study in which rats were administered 500M04 by the dietary route 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 kidney effects at 300 mg/kg bw per day (Buesen et al., 2013). The Meeting noted that effects on the kidney were also identified with the parent compound in a short-term toxicity study in rats evaluated by the 2003 JMPR, but at higher doses (Annex 1, reference *100*). Other changes noted for the parent compound (e.g. reductions in body weight and feed consumption, effects on clinical chemistry parameters, liver hypertrophy and mucosal hypertrophy in the duodenum) were not observed for 500M04 in the 3-month toxicity study.

The Meeting concluded that the toxicity of 500M04 was lower than that of pyraclostrobin.

500M106

500M106 is a metabolite in rats, rabbits and humans. In a 28-day study in which rats were administered 500M106 by gavage at a dose of 0, 100, 300 or 1000 mg/kg bw per day, the NOAEL was 300 mg/kg bw per day, based on effects on the duodenum, liver, spleen and haematological system at 1000 mg/kg bw per day (Buesen et al., 2017). These effects were also observed for pyraclostrobin, but at much higher doses (reviewed by 2003 JMPR; Annex 1, reference *100*).

500M106 was tested for genotoxicity in an adequate range of in vitro and in vivo assays. Negative results were seen in an Ames test (Woitkowiak & Landsiedel, 2016) and an in vitro micronucleus assay (Chang, 2016b). In a forward mutation test, 500M106 was positive with S9 (Schulz & Landsiedel, 2016c). In two genotoxicity studies in vivo (micronucleus assay in mice and Muta™Mouse transgenic mouse model), 500M106 showed no genotoxicity (Dony, 2016; Ueda, 2017).

The Meeting concluded that the toxicity of 500M106 was similar to or lower than that of pyraclostrobin.

500M02

500M02 was tested for genotoxicity in an adequate range of in vitro assays. No evidence of genotoxicity was found (Engelhardt & Hoffmann, 1999; Chang, 2016a; Schulz & Landsiedel, 2016b; Woitkowiak, 2016).

The subchronic toxicity of 500M02 was considered to be tested in the 28-day rat study with 500M106 (Buesen et al., 2017), as 500M02 is metabolically formed from 500M106 to a substantial extent (~11.5% of the dose).

The Meeting concluded that, on the basis of its formation from 500M106 and the absence of genotoxicity, the toxicity of 500M02 was similar to or lower than that of pyraclostrobin.

500M24

500M24 was tested for genotoxicity in an adequate range of in vitro and in vivo assays. It gave negative results for gene mutation (Schulz & Landsiedel, 2014a; Woitkowiak, 2014) and a positive response in an in vitro chromosomal aberration assay (Schulz & Landsiedel, 2014b), but it was negative in an in vivo micronucleus test (Dony, 2014; Schulz et al., 2016a).

500M49

500M49 was tested for genotoxicity in an adequate range of in vitro assays. No evidence of genotoxicity was found (Woitkowiak, 2013a; Schulz & Landsiedel, 2014c,d).

500M51

500M51 was tested for genotoxicity in an adequate range of in vitro assays (Woitkowiak, 2013b; Schulz & Landsiedel, 2014e,f). No evidence of genotoxicity was found.

500M76

500M76 was tested for genotoxicity in an adequate range of in vitro and in vivo assays, producing mainly negative results (Engelhardt & Hoffmann, 2000; Engelhardt & Leibold, 2003; Schulz & Landsiedel, 2012c). It gave a positive response in an in vitro chromosomal aberration assay (Schulz & Landsiedel, 2013b, 2014b), but it was negative in an in vivo micronucleus test (Schulz & Landsiedel, 2012c).

500M07 (plant metabolite)

No toxicological information on 500M07 was submitted to the present Meeting; however, 500M07 is a rat metabolite found in serum and is formed early in the metabolic pathway.

Human data

Information on 33 cases of accidental exposure to pyraclostrobin was submitted (Gergely & Calvert, 2008). In almost all incidents, the exposure was to spray drift from aerial application. The most severe incident involved 27 subjects. Skin, eye and upper respiratory irritation were frequently reported.

Toxicological evaluation

The Meeting concluded that no revision of the ADI established by the 2003 Meeting was necessary.

The Meeting established a new ARfD of 0.7 mg/kg bw, based on the overall NOAEL of 5.8 mg/kg bw per day in 90-day and 1-year dog feeding studies (evaluated by the 2003 Meeting). A safety factor of 8 (2.5 for interspecies toxicodynamic differences, 3.2 for interindividual toxicodynamic differences) was applied. The previous ARfD was withdrawn.

Vomiting and diarrhoea seen during the first week of dosing of dogs with feed at 11 mg/kg bw per day (Annex 1, reference *100*) were identified as the critical effects. These critical effects are considered to be secondary to a direct, local effect of pyraclostrobin on the gastrointestinal tract, which is local concentration related and independent of absorption and metabolism (Appendix 2). Therefore, the default 100-fold safety factor was modified based on the scheme outlined by the International Programme on Chemical Safety (IPCS) on chemical-specific adjustment factors (IPCS, 2005) by removing the interindividual and interspecies toxicokinetic factors of 3.2 and 4, respectively.

The Meeting concluded that the effects secondary to local irritation following gavage dosing with pyraclostrobin were not relevant to human dietary risk assessment, and therefore the basis for the previously established ARfD was no longer applicable, as this was a gavage study in rabbits.

Acute reference dose (ARfD)

0.7 mg/kg bw

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

Absorption, distribution, excretion and metabolism in mammals	
Metabolism	Desmethoxylation of the side-chain
	Hydroxylation of the chlorophenyl pyrazole ring system
	Hydroxylation of the tolyl ring system
	Cleavage of the ether bond, resulting in chlorophenyl pyrazole or anthranilic acid derivatives
	Desmethylation of the side-chain
	Cleavage of the amide bond in the side-chain
Toxicologically significant compounds in animals and plants	Pyraclostrobin
Other toxicological studies	
Immunotoxicity	No immunotoxicity
Phototoxicity	No phototoxicity
Studies on toxicologically relevant metabolites	
500M04 (pyrazolon)	Oral LD_{50} > 2 000 mg/kg bw (rats)
	3-month oral toxicity study NOAEL 100 mg/kg bw per day (rats)
	No evidence of genotoxicity in vivo
500M24	No evidence of genotoxicity in vivo
500M49	No evidence of genotoxicity in vitro
500M51	No evidence of genotoxicity in vitro
500M76	No evidence of genotoxicity in vivo
500M02	No evidence of genotoxicity in vitro
500M106	Four-week oral toxicity study NOAEL 300 mg/kg bw per day (rats)
	No evidence of genotoxicity in vivo
Human data	
	Skin, eye or upper respiratory irritation

Summary

	Value	**Study**	**Safety factor**
ARfD	0.7 mg/kg bw	Ninety-day and 1-year feeding studies in dogs	8

References

BASF (2017). Pyraclostrobin (BAS 500 F). “Joint Meeting on Pesticide Residues” (JMPR). Toxicology: monograph. Unpublished report. BASF SE, Ludwigshafen/Rhein and Limburgerhof, Germany. BASF document ID 2017/1211. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Birk B, Lutz T, Doebbe A (2014). Further investigations of metabolites in rat plasma samples after dosing with ^{14}C-pyraclostrobin. Unpublished report. BASF SE, Limburgerhof, Germany. BASF document ID 2014/1315930. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Buesen R, Strauss V, Moreno MCR, Becker M, Van Ravenzwaay B (2013). Reg. No. 298327 (metabolite of BAS 500 F, pyraclostrobin) – repeated dose 90-day oral toxicity study in Wistar rats – administration via the diet. Unpublished report. BASF SE, Limburgerhof, Germany. BASF document ID 2013/1042164. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Buesen R, Strauss V, Moreno MCR, Becker M, Van Ravenzwaay B (2017). Reg. No. 399379 (metabolite of BAS 500 F, pyraclostrobin) – repeated-dose 28-day oral toxicity study in Wistar rats – administration by gavage. Unpublished report. BASF SE, Limburgerhof, Germany. BASF document ID 2017/1288407. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Cetto V, Landsiedel R (2012). BAS 500 F (pyraclostrobin) – in vitro 3T3 NRU phototoxicity test. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2012/1189936. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Cetto V, Landsiedel R (2014). Amendment no. 1 – BAS 500 F (pyraclostrobin) – in vitro 3T3 NRU phototoxicity test. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2014/1092412. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Chang S (2016a). Reg. No. 369315 (metabolite of BAS 500 F, pyraclostrobin): micronucleus test in human lymphocytes in vitro. Unpublished report. Envigo CRS GmbH, Rossdorf, Germany. BASF document ID 2016/1135652. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Chang S (2016b). Reg. No. 399379 (metabolite of BAS 500 F, pyraclostrobin) – micronucleus test in human lymphocytes in vitro. Unpublished report. Envigo CRS GmbH, Rossdorf, Germany. BASF document ID 2016/1109533. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Deckardt K, Van Ravenzwaay B (2003). In vitro hemolysis test with BAS 500 F (screening). Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2003/1013685. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Dony E (2014). Reg. No. 5916421 (metabolite of BAS 500 F, pyraclostrobin): micronucleus assay in bone marrow cells of the mouse. Unpublished report. Harlan Cytotest Cell Research GmbH, Rossdorf, Germany. BASF document ID 2013/1389659. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Dony E (2016). Reg. No. 399379 (metabolite of BAS 500 F, pyraclostrobin), micronucleus assay in bone marrow cells of the mouse. Unpublished report. Envigo CRS GmbH, Rossdorf, Germany. BASF document ID 2016/1295116. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Engelhardt G, Hoffmann HD (1999). *Salmonella typhimurium*/*Escherichia coli* reverse mutation assay (Ames standard plate test and Prival preincubation test) with Reg. No. 369315. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 1999/10736. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Engelhardt G, Hoffmann HD (2000). *Salmonella typhimurium*/*Escherichia coli* reverse mutation assay (standard plate test and preincubation test) with Reg. No. 413038. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2000/1000005. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Engelhardt G, Leibold E (2003). In vitro gene mutation test with Reg. No. 413038 in CHO cells (*HPRT* locus assay). Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2003/1004384. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Fabian E, Landsiedel R (2013). ^{14}C-Reg. No. 298327 (metabolite of BAS 500 F, pyraclostrobin) – study on the kinetics in mice. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2012/1278425. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Fabian E, Landsiedel R (2016). ^{14}C-Reg. No. 399379 (metabolite of BAS 500 F, pyraclostrobin) – study on kinetics in Wistar rats after oral administration. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2015/1241735. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Funk D, Bellwon P (2016a). Influence of WWL229 on the in-vitro metabolism of ^{14}C-BAS 500 F with human hepatocytes and human liver cytosol. Unpublished report. BASF SE, Limburgerhof, Germany. BASF document ID 2016/1225031. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Funk D, Bellwon P (2016b). Metabolisation of ^{14}C-BAS 500 F in rat serum. Unpublished report. BASF SE, Limburgerhof, Germany. BASF document ID 2016/1225032. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Funk D, Glaessgen WE, Kalyon B (2014). Comparative in-vitro metabolism with ^{14}C-BAS 500 F. Unpublished report. BASF SE, Limburgerhof, Germany. BASF document ID 2014/1001562. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Gamer AO, Deckardt K, Burkhardt S, Van Ravenzwaay B (2005). BAS 500 F – subacute inhalation study in Wistar rats – 20 aerosol exposures during 4 weeks. Unpublished report. BASF SE, Limburgerhof, Germany. BASF document ID 2005/1013950. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Gergely RM, Calvert GM (2008). Acute pesticide poisoning associated with pyraclostrobin fungicide – Iowa, 2007. MMWR Morb Mortal Wkly Rep. 56(51):1343–5 (http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5651a3.htm, accessed 26 September 2018).

IPCS (2005). Chemical-specific adjustment factors for interspecies differences and human variability: guidance document for use of data in dose/concentration–response assessment. Genera: World Health Organization, International Programme on Chemical Safety (Harmonization Project Document No. 2; http://apps.who.int/iris/handle/10665/43294, accessed 26 September 2018).

Kuehlem C (1997a). Study on the acute oral toxicity of pyrazolon in rats. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 1997/10963. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Kuehlem C (1997b). Study on the acute dermal irritation/corrosion of pyrazolon in the rabbit. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 1997/10964. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Kuehlem C (1997c). Study on the acute eye irritation of pyrazolon in the rabbit. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 1997/10965. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Kuehlem C, Hellwig J (1997). Pyrazolon – maximization test in guinea pigs. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 1997/10968. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Ma-Hock L, Leibold E (2002). BAS 500 F – acute inhalation toxicity study in Wistar rats – 4-hour liquid aerosol exposure. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2002/1012053. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Ma-Hock L, Strauss V, Treumann S, Becker M, Van Ravenzwaay B (2014). BAS 500 F (pyraclostrobin) – repeated dose 28-day inhalation toxicity study with recovery period in Wistar rats, aerosol exposure. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2014/1003946. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W (2002a). Amendment no. 1: BAS 500 F – carcinogenicity study in Wistar rats. Administration in the diet for 24 months. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2002/1005114. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W (2002b). Summary of results – BAS 500 F – first supplementary carcinogenicity study in Wistar rats – administration in the diet for 24 months. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2002/1004123. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W (2002c). BAS 500 F – second supplementary carcinogenicity study in female Wistar rats – administration in the diet for 24 months. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2002/1004124. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W (2003a). Amendment no. 1 to the report: BAS 500 F – administration in the diet over 4 weeks with simultaneous subcutaneous administration of vitamin B_{12}. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2003/1014738. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W (2003b). Amendment no. 1 to the report: Screening study BAS 505 F – administration in the diet with simultaneous parenteral administration of iron to Wistar rats. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2003/1009197. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W, Kaufmann W (2004). Amendment no. 2 to the report: Screening study BAS 505 F – administration in the diet with simultaneous parenteral administration of iron to Wistar rats. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2004/1010601. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W, Beimborn DB, Van Ravenzwaay B (2003). BAS 500 F – determination of lipid peroxidation in the liver of male Wistar rats. Administration in the diet for 14 and 28 days. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2003/1009622. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W, Deckardt K, Gembardt C, Pappritz G, Hildebrand B (1999a). BAS 500F – chronic toxicity study in Wistar rats, administration in the diet for 24 months. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 99/11672. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany [cited in Annex 1, reference *100*].

Mellert W, Deckardt K, Gembardt C, Pappritz G, Hildebrand B (1999b). BAS 500F – carcinogenicity study in Wistar rats, administration in the diet for 24 months. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 99/11868. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany [cited in Annex 1, reference *100*].

Mellert W, Deckardt K, Kaufmann W, Leibold E (2002). Screening study BAS 505 F – administration in the diet with simultaneous parenteral administration of iron to Wistar rats. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2002/1013984. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W, Deckardt K, Gembart C, Van Ravenzwaay B (2003a). BAS 500 F – determination of iron in urine and serum of Wistar rats – administration in the diet over 14 days. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2003/1009534. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W, Deckardt K, Kuttler K, Hellwig J (2003b). BAS 500 F – administration in the diet over 4 weeks with simultaneous subcutaneous administration of vitamin B_{12}. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2003/1013463. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Schilling K, Hellwig J, Hildebrand B (1999). BAS 500F – prenatal developmental toxicity study in Himalayan rabbits, oral administration (gavage). Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 1999/11512. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany [cited in Annex 1, reference *100*].

Schulz M, Landsiedel R (2012a). Reg. No. 298327 (metabolite of BAS 500 F, pyraclostrobin) – in vitro gene mutation test in CHO cells (*HPRT* locus assay). Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2012/1272482. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Schulz M, Landsiedel R (2012b). Reg. No. 298327 (metabolite of BAS 500 F, pyraclostrobin) – in vitro chromosome aberration assay in V79 cells. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2012/1185707. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Schulz M, Landsiedel R (2012c). Reg. No. 413038 (metabolite of BAS 500 F, pyraclostrobin) – micronucleus test in bone marrow cells of the mouse. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2012/1220183. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Schulz M, Landsiedel R (2013a). Reg. No. 298327 (metabolite of BAS 500 F, pyraclostrobin) – micronucleus test in bone marrow cells of the mouse. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2013/1026779. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Schulz M, Landsiedel R (2013b). Reg. No. 413038 (metabolite of BAS 500 F, pyraclostrobin) – in vitro chromosome aberration assay in V79 cells. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2012/1044766. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Schulz M, Landsiedel R (2014a). Reg. No. 5916421 (metabolite of BAS 500 F, pyraclostrobin) – in vitro gene mutation test in L5178Y mouse lymphoma cells ($TK^{+/-}$ locus assay, microwell version). Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2013/1298449. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Schulz M, Landsiedel R (2014b). Reg. No. 5916421 (metabolite of BAS 500 F, pyraclostrobin) – in vitro micronucleus assay in V79 cells (cytokinesis block method). Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2013/1363549. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Schulz M, Landsiedel R (2014c). Reg. No. 5916420 (metabolite of BAS 500 F, pyraclostrobin) – in vitro gene mutation test in L5178Y mouse lymphoma cells (*TK*$^{+/-}$ locus assay, microwell version). Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2013/1298447. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Schulz M, Landsiedel R (2014d). Reg. No. 5916420 (metabolite of BAS 500 F, pyraclostrobin) – in vitro micronucleus assay in V79 cells (cytokinesis block method). Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2013/1361921. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Schulz M, Landsiedel R (2014e). Reg. No. 78810 (metabolite of BAS 500 F, pyraclostrobin) – in vitro gene mutation test in L5178Y mouse lymphoma cells (*TK*$^{+/-}$ locus assay, microwell version). Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2013/1298448. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Schulz M, Landsiedel R (2014f). Reg. No. 78810 (metabolite of BAS 500 F, pyraclostrobin) – in vitro micronucleus assay in V79 cells (cytokinesis block method). Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2013/1361922. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Schulz M, Landsiedel R (2014g). Amendment no. 1 – Reg. No. 413038 (metabolite of BAS 500 F, pyraclostrobin) – in vitro chromosome aberration assay in V79 cells. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2014/1145892. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Schulz M, Landsiedel R (2016a). Amendment no. 1: Cytogenetic study in vivo with BAS 500 F in the mouse micronucleus test. Single oral administration. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2016/1309356. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Schulz M, Landsiedel R (2016b). Reg. No. 369315 (metabolite of BAS 500 F, pyraclostrobin) – in vitro gene mutation test in L5178Y mouse lymphoma cells (*TK* locus assay, microwell version). Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2016/1225056. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Schulz M, Landsiedel R (2016c). Reg. No. 399379 (metabolite of BAS 500 F, pyraclostrobin) – in vitro gene mutation test in L5178Y lymphoma cells (*TK*$^{+/-}$ locus assay, microwell version). Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2016/1171390. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Smiraldo PG (2012a). A 28-day oral (dietary) natural killer cell immunotoxicity study of BAS 500 F in female B6C3F1 mice. Unpublished report. WIL Research Laboratories Inc., Ashland, OH, USA. BASF document ID 2011/1035857. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Smiraldo PG (2012b). A 28-day oral (dietary) antibody forming cell immunotoxicity study of BAS 500 F in female B6C3F1 mice. Unpublished report. WIL Research Laboratories Inc., Ashland, OH, USA. BASF document ID 2012/1020986. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Smiraldo PG (2012c). A 28-day oral (dietary) antibody forming cell immunotoxicity study of BAS 500 F in female B6C3F1 mice. Unpublished report. WIL Research Laboratories Inc., Ashland, OH, USA. BASF document ID 2012/1084176. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Srai K (2003). BAS 505 F – study on the effect on the mucosal iron transport in female Wistar rats – administration in the diet. Unpublished report. Royal Free and University College Medical School, London, United Kingdom. BASF document ID 2003/1014047. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Thiaener J, Bellwon P (2016). Metabolism investigation of ^{14}C-Reg. No. 399379 (metabolite of BAS 500F, pyraclostrobin) in faeces and urine of male Wistar rats. Unpublished report. BASF SE, Limburgerhof, Germany. BASF document ID 2015/1198492. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Ueda M (2017). Reg. No. 399379 (metabolite of BAS 500 F, pyraclostrobin): transgenic mice (Muta™Mouse) gene mutation assay. Unpublished report. BSRC – Biosafety Research Center, Iwata Shizuoka, Japan. BASF document ID 2017/1002442. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Woitkowiak C (2012). Reg. No. 298327 (metabolite of BAS 500 F, pyraclostrobin) – *Salmonella typhimurium* / *Escherichia coli* reverse mutation assay. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2012/1220416. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Woitkowiak C (2013a). Reg. No. 5916420 (metabolite of BAS 500 F, pyraclostrobin) – *Salmonella typhimurium* / *Escherichia coli* reverse mutation assay. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2013/1281928. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Woitkowiak C (2013b). Reg. No. 78810 (metabolite of BAS 500 F, pyraclostrobin) – *Salmonella typhimurium* / *Escherichia coli* reverse mutation assay. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2013/1255749. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Woitkowiak C (2014). Reg. No. 5916421 (metabolite of BAS 500 F, pyraclostrobin) – *Salmonella typhimurium*/*Escherichia coli*, reverse mutation test. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2013/1323364. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Woitkowiak C (2016). Reg. No. 369315 (metabolite of BAS 500 F, pyraclostrobin) – *Salmonella typhimurium*/*Escherichia coli* – reverse mutation assay (Prival modification). Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2016/1200488. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Woitkowiak C, Landsiedel R (2016). Reg. No. 399379 (metabolite of BAS 500 F, pyraclostrobin) – *Salmonella typhimurium* / *Escherichia coli* – reverse mutation assay. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2016/1119837. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Appendix 1: List of metabolites of pyraclostrobin

The metabolites of pyraclostrobin are identified in Table A1-1.

Table A1-1. Identification of metabolites of pyraclostrobin in animals and humans

Metabolite code	Reg. no.	Synonyms	CAS no.	Compound found in:	Structure
BAS 500 F (pyraclostrobin)	304428	500M00	175013-18-0		
500M02	369315	BF 500-7	Not assigned	Human, dog, rabbit (in vitro)	
500M03	Not assigned	na	Not assigned	Rat Human, rabbit (in vitro)	
500M04	298327	BF 500-5	76205-19-1	Rat Human, rat, rabbit, dog (in vitro)	
500M05	Not assigned	na	Not assigned	Rat	
500M06	Not assigned	na	Not assigned	Rat (in vivo, plasma) Human, rat, rabbit (in vitro)	
500M07	340266	BF 500-3	512165-96-7	Rat	

Metabolite code	Reg. no.	Synonyms	CAS no.	Compound found in:	Structure
500M08	Not assigned	na	Not assigned	Rat (in vivo, plasma)	
500M13	Not assigned	na	Not assigned	Rat (in vivo, plasma)	
500M15	Not assigned	na	Not assigned	Rat (in vivo, plasma)	
500M18	Not assigned	na	Not assigned	Rat	
500M19	Not assigned	na	Not assigned	Rat	
500M21	Not assigned	na	Not assigned	Rat	
500M22	Not assigned	na	Not assigned	Rat	
500M23	Not assigned	na	Not assigned	Rat	

Metabolite code	Reg. no.	Synonyms	CAS no.	Compound found in:	Structure
500M24	5916421	na	Not assigned	Rat	
500M25	Not assigned	na	Not assigned	Rat	
500M26	Not assigned	na	Not assigned	Rat	
500M29	Not assigned	na	Not assigned	Rat (in vivo, plasma)	
500M30	Not assigned	na	Not assigned	Rat (in vivo, plasma)	
500M31	Not assigned	na	Not assigned	Rat (in vivo, plasma)	
500M32	Not assigned	na	Not assigned	Rat (in vivo, plasma)	
500M33	Not assigned	na	Not assigned	Rat	

Metabolite code	Reg. no.	Synonyms	CAS no.	Compound found in:	Structure
500M34	Not assigned	na	Not assigned	Rat (in vivo, plasma)	
500M35	412040	na	Not assigned	Rat (in vivo, plasma)	
500M37	Not assigned	na	Not assigned	Rat (in vivo, plasma)	
500M38	Not assigned	na	Not assigned	Rat (in vivo, plasma)	
500M39	Not assigned	na	Not assigned	Rat	
500M40	Not assigned	na	Not assigned	Rat	
500M44	Not assigned	na	Not assigned	Rat	
500M45	Not assigned	na	Not assigned	Rat (in vivo, plasma)	
500M46	Not assigned	na	Not assigned	Rat (in vivo, plasma)	

Metabolite code	Reg. no.	Synonyms	CAS no.	Compound found in:	Structure
500M48	Not assigned	na	Not assigned	Rat	
500M51	78810	na	6268-38-8	Rat	
500M52	Not assigned	na	Not assigned	Rat	
500M73	358672	BF 500-4	Not assigned	Human, rat, rabbit, dog (in vitro)	
500M88	322410	BF 500-1	220897-76-7	Human, rat, rabbit, dog (in vitro)	
500M103	Not assigned	na	Not assigned	Human, rat, rabbit (in vitro)	
500M104	Not assigned	na	Not assigned	Rat (in vivo, plasma) Human, rat, rabbit (in vitro)	
500M106	399379	na	Not assigned	Rat (in vivo, plasma) Human, rabbit (in vitro)	

Metabolite code	Reg. no.	Synonyms	CAS no.	Compound found in:	Structure
500M107	Not assigned	na	Not assigned	Rat (in vivo, plasma) Human, rabbit (in vitro)	
500M108	Not assigned	na	Not assigned	Rat (in vivo, plasma) Human, rat, rabbit (in vitro)	

CAS: Chemical Abstracts Service; na: not applicable; Reg. no.: registration number
Source: Funk, Glaessgen & Kalyon (2014)

Appendix 2: Summary of potential direct/secondary local effects induced by pyraclostrobin in toxicity studies

The potential direct or secondary local effects induced by pyraclostrobin in toxicity studies in mice, rats, rabbits and dogs are summarized in Table A2-1.

Table A2-1. Effects induced by pyraclostrobin in toxicity studies

Study (route)	Species	NOAEL for local effect	Local effect	Secondary effect to local effect	Reference
Skin irritation	Rabbits	–	Slightly irritating	–	Annex 1, reference *100*
Eye irritation	Rabbits	–	Slightly irritating	–	Annex 1, reference *100*
Three-month oral toxicity (feeding)	Mice	9.2 mg/kg bw per day	Erosion/ulcer of glandular stomach	–	Annex 1, reference *100*
Three-month oral toxicity (feeding)	Dogs	5.8/6.2 mg/kg bw per day	–	Vomiting and diarrhoea during first 2 weeks	Annex 1, reference *100*
Twelve-month oral toxicity (feeding)	Dogs	5.4 mg/kg bw per day	–	Vomiting and diarrhoea during first week	Annex 1, reference *100*
Four-week inhalation (inhalation, nasal)	Rats	10 mg/m^3 (overall)	Inflammation, necrosis, atrophy, regeneration in of nasal cavity, larynx and/or lung	–	Gamer et al. (2005); Ma-Hock et al. (2014)
Two-year carcinogenicity (feeding)	Rats	3.4 mg/kg bw per day	Erosion/ulcer of stomach	–	Annex 1, reference *100*
Developmental toxicity (gavage)	Rats	10 mg/kg bw per day (maternal)	–	Decreased feed consumption on GDs 6–8 (maternal)	Annex 1, reference *100*
Developmental toxicity (gavage)	Rabbits	3 mg/kg bw per day (maternal) 5 mg/kg bw per day (embryo/fetal)[a]	–	Lower body weight gain immediately after dosing (maternal) Implantation loss/reduced fetal weight (embryo/fetal)	Annex 1, reference *100*

ARfD: acute reference dose; bw: body weight; GD: gestation day; NOAEL: no-observed-adverse-effect level

[a] Point of departure for setting ARfD in 2003.

Appendix 3: Proposed mode of action for the induction of mucosal hyperplasia in the duodenum by strobilurin fungicides, including pyraclostrobin

The hypothesized mode of action (MOA) and relevance to humans for pyraclostrobin-induced mucosal hyperplasia in the duodenum is assessed using the International Programme on Chemical Safety (IPCS) framework for analysing the relevance of a non-cancer MOA for humans (IPCS, 2007).

Data on toxicity of pyraclostrobin, orysastrobin and dimoxystrobin

1. Occurrence of duodenal mucosal hyperplasia in studies on toxicity of pyraclostrobin

In subchronic oral (dietary admixture) studies on the toxicity of pyraclostrobin in mice, rats and dogs, duodenal mucosal thickening and hyperplasia were detected. The results of these studies are summarized in Table A3-1. The duodenal changes observed in the short-term studies were not accompanied by inflammatory or degenerative changes in the small intestines. The dose ranges of the long-term studies in mice and rats were lower than the NOAELs for this effect in the short-term studies, and no such changes or related neoplastic lesions were observed in these long-term studies (Annex 1, reference *100*).

Table A3-1. Histopathology of duodenal lesions in oral toxicity studies with pyraclostrobin[a]

Study/dose/finding	Males						Females					
91-day study in mice												
Dose (mg/kg bw per day)	0	9.2	30.4	119	274	476	0	12.9	40.4	162	374	635
Thickening of mucosa	0/10	0/10	0/10	10/10	10/10	10/10	0/10	0/10	0/10	6/10	10/10	9/10
18-month carcinogenicity study in mice												
Dose (mg/kg bw per day)	0	1.4	4.1	17.2			0	1.6	4.8	20.5	32.8	
Thickening of mucosa	0/10	0/10	0/10	0/10			0/10	0/10	0/10	0/10	0/10	
4-week study in rats												
Dose (mg/kg bw per day)	0	1.8	9.0	42	120		0	2.0	9.6	47	126	
Mucosal hyperplasia	–	–	–	P	P		–	–	–	P	P	
91-day study in rats												
Dose (mg/kg bw per day)	0	4	11	35	69	106	0	4	13	41	80	119
Mucosal hyperplasia	2/10	1/10	1/10	4/10	5/10	10/10	2/10	1/10	2/10	1/10	1/10	10/10
24-month carcinogenicity study in rats												
Dose (mg/kg bw per day)	0	1.2	3.4	9.2			0	1.5	4.6	12.6		
Mucosal hyperplasia	–	–	–	–			–	–	–	–		

Study/dose/finding	Males					Females				
3-month study in dogs										
Dose (mg/kg bw per day)	0	2.8	5.8	12.9		0	3.0	6.2	13.6	
Mucosal hypertrophy	0/5	0/5	0/5	2/5		0/5	0/5	0/5	1/5	
12-month study in dogs										
Dose (mg/kg bw per day)	0	2.7	5.4	10.8		0	2.7	5.4	11.2	
Mucosal hypertrophy	0/5	0/5	0/5	0/5		0/5	0/5	0/5	0/5	

–: no lesions; bw: body weight; P: present; the incidence was increased, but detailed information was not submitted for the 2018 JMPR

[a] Yellow cells show the increased incidence of duodenal lesions induced by pyraclostrobin.

Source: Annex 1, reference *100*

2. Relationship of iron deficiency anaemia with duodenal mucosal changes induced by pyraclostrobin treatment

Duodenal hyperplastic changes in rats and mice were accompanied by the typical iron deficiency hypochromic microcytic anaemia (decreased levels of haemoglobin, mean corpuscular volume, mean cell haemoglobin and/or mean cell haemoglobin concentration), although less clearly so at the lowest-observed-adverse-effect level (LOAEL), where anaemia was barely detectable (Annex 1, reference *100*) (Table A3-2). In fact, in the 90-day toxicity study in dogs, no finding indicating anaemia was observed at the doses at which mild duodenal changes were observed in 1–2/5 animals.

Serum iron concentrations and transferrin levels and urinary iron excretion were not examined in any standard toxicity studies in mice, rats or dogs.

Table A3-2. Anaemia and duodenal changes in oral toxicity studies with pyraclostrobin[a]

	Males						Females					
91-day study in mice												
Dose (mg/kg bw per day)	0	9.2	30.4	119	274	476	0	12.9	40.4	162	374	635
Haemoglobin (mmol/L)	11.8	11.6	11.6	11.4	11.4	10.6***	11.4	11.5	11.2	11.0	10.9**	10.4**
Haematocrit (L/L)	0.57	0.56	0.55	0.54*	0.54*	0.52*	0.52	0.53	0.52	0.52	0.51	0.50
MCV (10^{-15} L)	48.3	48.0	47.3	47.0*	46.7*	42.6*	46.4	46.7	46.3	46.8	46.0	42.6
MCH (10^{-15} mol/L)	0.99	0.99	1.00	1.00	0.98	0.87**	1.02	1.01	1.00	1.00	0.98*	0.90**
MCHC (mmol/L)	20.6	20.7	21.1	21.1	21.0	20.5	22.0	21.6	21.7	21.3***	21.2***	21.0***
Duodenal hyperplastic change	–	–	–	+	+	+	–	–	–	+	+	+

	Males						Females					
4-week study in rats												
Dose (mg/kg bw per day)	0	1.8	9.0	42	120		0	2.0	9.6	47	126	
RBC	–	–	–	–	–		–	–	–	↓	↓	
Haemoglobin	–	–	–	–	↓		–	–	–	↓	↓	
Duodenal hyperplastic change	–	–	–	+	+		–	–	–	+	+	
91-day study in rats												
Dose (mg/kg bw per day)	0	4	11	35	69	106	0	4	13	41	80	119
Erythrocytes (10^{12}/L)	8.5	8.5	8.8	8.6	8.4	8.2	8.0	7.9	8.0	7.7	7.4**	7.1**
Haemoglobin (mmol/L)	9.7	9.5	9.8	9.7	9.5	9.4	9.2	9.3	9.3	9.3	8.7*	8.6**
Haematocrit (L/L)	0.43	0.43	0.44	0.44	0.44	0.43	0.41	0.41	0.42	0.42	0.40	0.39
MCV (10^{-15} L)	50.6	50.1	50.2	51.3	52.3*	52.9*	51.5	52.0	52.1	53.9***	53.8***	54.8***
MCH (10^{-15} mol/L)	1.14	1.12	1.12	1.13	1.14	1.15	1.16	1.18	1.17	1.20**	1.19*	1.21**
MCHC (mmol/L)	22.5	22.3	22.3	21.9**	21.8**	21.7***	22.6	22.6	22.4	22.4	22.2***	22.2***
Duodenal hyperplastic change	–	–	–	+	+	+	–	–	–	–	–	+
3-month study in dogs												
Dose (mg/kg bw per day)	0	2.8	5.8	12.9			0	3.0	6.2	13.6		
Haematology	–	–	–	–			–	–	–	–		
Duodenal hyperplastic change	–	–	–	+			–	–	–	+		
12-month study in dogs												
Dose (mg/kg bw per day)	0	2.7	5.4	10.8			0	2.7	5.4	11.2		
Haematology	–	–	–	–			–	–	–	–		

–: no lesions; +: present; ↓: decrease (detailed information was not submitted for the 2018 JMPR); bw: body weight; MCH: mean cell haemoglobin; MCHC: mean cell haemoglobin concentration; MCV: mean corpuscular volume; RBC: red blood cells; *: $P \leq 0.05$; **: $P \leq 0.02$; ***: $P \leq 0.002$ (Kruskal-Wallis plus Mann-Whitney *U*-test)

[a] Yellow cells show the effects induced by pyraclostrobin.

Source: Annex 1, reference *100*

Although pyraclostrobin is a weak irritant, no mucosal damage to the duodenum, such as inflammation, erosion or ulcer, was observed in any of the toxicity studies in mice, rats and dogs. Therefore, the duodenal hyperplasia observed in toxicity studies is not considered a regenerative change

in response to mucosal damage, as is seen with a variety of cytotoxic and highly irritating chemical substances (Meek et al., 2003).

3. Effects on iron levels in serum and urine in rats

In a study conducted to determine the levels of iron in serum and urine after oral administration of pyraclostrobin to Wistar rats for 14 days in the diet at 0, 50, 500 or 1500 ppm (equal to 0, 3.8, 33.9 and 73.9 mg/kg bw per day for males and 0, 4.1, 37.4 and 78.3 mg/kg bw per day for females, respectively), serum iron concentrations were dose- and time-dependently decreased in both sexes at 500 and 1500 ppm, with up to a 50% decrease in males, and serum transferrin levels were decreased in males and females at 1500 ppm (Table A3-3). Iron levels in the urine were not affected by the treatment. This study suggested that pyraclostrobin-induced anaemia was related to lower absorption of iron from the gut, resulting in lower iron levels in serum (Mellert et al., 2003).

Table A3-3. Summary of changes in iron or transferrin in the serum by pyraclostrobin in rats

	Males				**Females**			
	0 ppm	**50 ppm**	**500 ppm**	**1 500 ppm**	**0 ppm**	**50 ppm**	**500 ppm**	**1 500 ppm**
Serum iron (µmol/L)								
Day 7	47.01	44.84	36.61	34.85*	59.07	60.09	46.48**	57.49
Day 14	54.54	46.71	37.72**	27.41**	53.61	58.31	45.55*	41.97**
Serum transferrin (g/L)								
Day 7	5.50	5.34	5.45	4.74**	5.40	5.23	5.62	4.92
Day 14	6.38	5.80	6.07	5.85	5.86	5.68	6.13	5.63

bw: body weight; *: $P \leq 0.05$; **: $P \leq 0.01$
Source: Mellert et al. (2003)

In a study in which orysastrobin, a strobilurin fungicide similar to pyraclostrobin (Fig. A3-1), was administered to male Wistar rats in feed at a concentration of 0, 10, 100 or 2500 ppm (equal to 0, 0.7, 7.4 and 143 mg/kg bw per day, respectively) for 14 days, decreases in serum iron and transferrin levels and an increase in the weight of the duodenum were observed in the rats at 2500 ppm. No further details were provided (FSCJ, 2005).

4. Mucosal proliferative lesions in duodenum in rats treated with orysastrobin

Data on other strobilurin fungicides were added to the present analysis to provide support for the findings on pyraclostrobin.

It has been reported that orysastrobin, another strobilurin fungicide, induces duodenal changes similar to those induced by pyraclostrobin (FSCJ, 2005; Van Ravenzwaay et al., 2007). The mucosal hyperplasia was observed in both sexes of mice, rats and dogs (Table A3-4). In rats exposed to the carcinogenic dose of 2500 ppm orysastrobin, increased epithelial cell proliferation was detected in the duodenal mucosa after 4 weeks, and decreased serum iron levels, increased serum transferrin levels and unsaturated iron capacity were seen after 1 week. Most of these changes are reversible; however, some hyperplastic lesions progressed to tumours in the duodenum of rats and mice. The carcinogenic effect in the duodenum was presumably caused by an attempt to increase iron absorption from the gastrointestinal tract in order to compensate for the markedly decreased serum iron levels. The reversible mechanism of action with a clear threshold dose and the absence of mutagenic potential in vivo were reported (Van Ravenzwaay et al., 2007).

Fig. A3-1. Chemical structures of pyraclostrobin, orysastrobin and dimoxystrobin

Pyraclostrobin

Dimoxystrobin

Orysastrobin

Table A3-4. Histopathology of duodenal lesions in oral toxicity studies of orysastrobin

Species	Duration	Dietary concentrations (ppm)	NOAEL for duodenal lesions (ppm)	Findings
Rats	90 days	0, 300, 1 000, 3 000, 5 000	<300	Thickening of duodenal mucosa
	24 months	0, 100, 500, 2 500	100	Thickening of duodenal mucosa
			500	Carcinoma and/or adenoma in duodenum
Mice	18 months	0, 100, 500, 2 000	500	Thickening of duodenal mucosa
			100	Focal hyperplasia in pylorus (at the transition of glandular stomach to duodenum)
			500	Adenocarcinoma in duodenum
Rat (mechanistic study)	4 weeks	0, 10, 100, 2 500	100	Increased epithelial cell proliferation activity (BrdU immunohistochemistry)
	2 weeks	0, 10, 100, 2 500	100	Decreased serum iron and increased serum transferrin and unsaturated iron capacity

BrdU: 5-bromo-2′-deoxyuridine; NOAEL: no-observed-adverse-effect level; ppm: parts per million

Source: Modified Tables 2 and 3 in Van Ravenzwaay et al. (2007)

Orysastrobin increased the incidences of tumours in the duodenum in rats and mice at the highest dose, suggesting a carcinogenic potential by long-term and high-dose exposure to strobilurin fungicides that induce duodenal hyperplasia. Duodenal hyperplasias following treatment with pyraclostrobin were observed in short-term studies, but no increases in hyperplasia or tumours were observed in long-term studies in rats and mice. The highest doses (200 ppm in rats; 180 ppm in mice) in the carcinogenicity studies were lower than those in the short-term studies in rats and mice. The doses, however, were considered to be acceptable for the evaluation of carcinogenicity, because long-term treatment with pyraclostrobin at 360 ppm in mice seriously depressed their body weights, indicating that the dose exceeded the MTD. Although strobilurin fungicides have the potential to promote the formation of duodenal tumours from mucosal hyperplasia due to continuous stimulation by negative feedback in response to the low iron level in the serum in rodents (FSCJ, 2005), long-term treatment with pyraclostrobin is not concluded to be carcinogenic in the duodenum.

5. Cell proliferation activity

Cell proliferation activity in the duodenum in mice and rats was studied after administration of orysastrobin (see Fig. A3-1 above), a strobilurin fungicide similar to pyraclostrobin.

In a 4-week study in which male C57BL/6J Rj mice were fed orysastrobin at a concentration of 0, 10, 100 or 2000 ppm (equal to 0, 1.9, 20.9 and 437 mg/kg bw per day, respectively), cell proliferation activity in the duodenum was increased at 2000 ppm. The increase showed a recovery after the diet was withdrawn for 2 weeks. No further details were provided (FSCJ, 2005).

In a 4-week study in which male Wistar rats were fed orysastrobin at a concentration of 0, 10, 100 or 2500 ppm (equal to 0, 0.6, 6.1 and 148 mg/kg bw per day, respectively), cell proliferation activity in the duodenum was increased at 2500 ppm. The increase showed a recovery after the diet was withdrawn for 2 weeks. No further details were provided (FSCJ, 2005).

No such studies have been conducted with pyraclostrobin.

6. Effect of dimoxystrobin on iron absorption and transport in the duodenum

In a study investigating the effect of dimoxystrobin, a compound similar to pyraclostrobin, on the mucosal uptake and transfer of iron into the carcass after oral administration in rats treated with dimoxystrobin at 0 or 4500 ppm (equivalent to 450 mg/kg bw per day) in the diet for 24, 96 or 168 hours, the everted duodenums were incubated to measure Fe^{2+} uptake in the duodenal segments. Iron transfer across the serosal membrane (i.e. iron transfer into the body) was measured using tied-off duodenal segments in rats exposed to 4500 ppm dimoxystrobin in the feed for 24 or 96 hours (Srai, 2003).

Mucosal iron uptake showed a statistically significant reduction after treatment with dimoxystrobin for 96 and 168 hours. The autoradiographic data confirmed the reduction of iron uptake. The data show the decrease in density of silver grains along the villus length, suggesting the reduction in uptake by the treatment.

Iron transfer across the serosal membrane (i.e. iron transfer into the body) was significantly reduced after a 96-hour treatment with dimoxystrobin, with the reduction of iron transfer being proportional to the reduction in uptake. After 24 hours of treatment, no significant effect was found. The results are summarized in Table A3-5.

In summary, the results clearly demonstrated that repeated treatment of rats with dimoxystrobin considerably reduced both uptake of iron by the duodenal mucosa and transfer of iron into the body (Srai, 2003).

Table A3-5. Iron transfer across the serosal membranes in the duodenum of dimoxystrobin-treated rats

Incubation times with iron	Treatment period with dimoxystrobin	Group	Mucosal retention[a]	Mucosal transfer[a]	Total mucosal uptake[a]
10 minutes	24 hours	Control	16.6 ± 3.7[b]	29 ± 10.2	45.6 ± 12.3
		Treated	17.8 ± 5.1	15.3 ± 5*	33.1 ± 8.9
	96 hours	Control	41.1 ± 18.5	10.6 ± 3.8	51.7 ± 21
		Treated	13.4 ± 9.5*	4.6 ± 2.1*	18 ± 9.4*
20 minutes	24 hours	Control	8.7 ± 3.5	12.6 ± 7.9	21.3 ± 10.4
		Treated	10.3 ± 2.2	13.3 ± 3.6	23.6 ± 5.6
	96 hours	Control	34.5 ± 9.7	37.7 ± 5.3	72.2 ± 11.7
		Treated	12.4 ± 2.4*	5.7 ± 2**	18.1 ± 2.6**
40 minutes	24 hours	Control	42.8 ± 10.5	33.6 ± 11.1	76.3 ± 13.8
		Treated	38.4 ± 4.9	20.9 ± 10.4	59.2 ± 13.3
	96 hours	Control	27.1 ± 10.8	38.9 ± 13.6	66.1 ± 15.8
		Treated	36.2 ± 17.7	17.5 ± 8.7*	53.7 ± 24.9

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

[a] Radioactivity of ^{59}Fe as measured by gamma counting in the intestinal tissue or in the carcass was referred to as mucosal retention or mucosal transfer, respectively. The sum of mucosal retention and mucosal transfer represents total mucosal uptake.

[b] Mean ± standard deviation.

Source: Srai (2003)

7. Combination study of dimoxystrobin with trivalent iron

Dimoxystrobin was administered to groups of 10 male Wistar rats at a dietary concentration of 0 or 4500 ppm (equal to 0, 206.6 and 171.2 mg/kg bw per day for controls, 4500 ppm or 4500 ppm plus iron complex group, respectively) over a period of 14 days and to groups of 10 female Wistar rats at a dietary concentration of 0, 500 or 4500 ppm (equal to 0, 37.7, 17.7, 191.3 and 84.7 mg/kg bw per day for controls, 500 ppm, 500 ppm plus iron complex, 4500 ppm and 4500 ppm plus iron complex, respectively) over a period of 7 days. Simultaneously, additional groups received an iron complex (Myofer® 100) intramuscularly (males once daily at a dose of 100 mg/kg bw on study days 0, 7, 11 and 13, and females twice daily at a dose of 50 mg/kg bw from day 2 to day 6).

Iron levels in the serum were increased in all groups receiving the iron complex (with and without dimoxystrobin) and reduced in the groups receiving dimoxystrobin only. Duodenal weights were statistically significantly increased in the 500 ppm (females) and 4500 ppm (both sexes) groups. In the iron complex groups at 500 and 4500 ppm (both sexes), the increase in duodenal weights was lower than in the corresponding dimoxystrobin groups. The increases in duodenal weights in the iron complex groups were not statistically significant in females compared with controls. Although the duodenal weights did not completely reach the control values, these data indicate that iron administered to animals treated with dimoxystrobin had an inhibitory effect on the increase in duodenal weights, in the sense of preventing effects on the duodenum that are caused by a reduction in serum iron levels following treatment with dimoxystrobin.

In immunohistochemical staining with proliferative cell nuclear antigen (PCNA), the number of positive cells was increased by approximately 200% at 4500 ppm with or without the iron complex in males. In females, the number of positive cells was increased by approximately 150% at 500 ppm with or without the iron complex and at 4500 ppm without the iron complex. The number of positive

cells at 4500 ppm with the iron complex was reduced and similar to that in the control group with the iron complex.

In conclusion, the administration of an iron complex can reduce the dimoxystrobin-induced increase in duodenal weights. Dimoxystrobin-induced iron deficiency might therefore be a causative factor for increased duodenal weights (Mellert et al., 2002; Mellert, 2003; Mellert & Kaufmann, 2004).

Postulated MOA for pyraclostrobin-induced mucosal hyperplasia of the duodenum in rodents and dogs

The data for pyraclostrobin support an MOA involving the following key events:

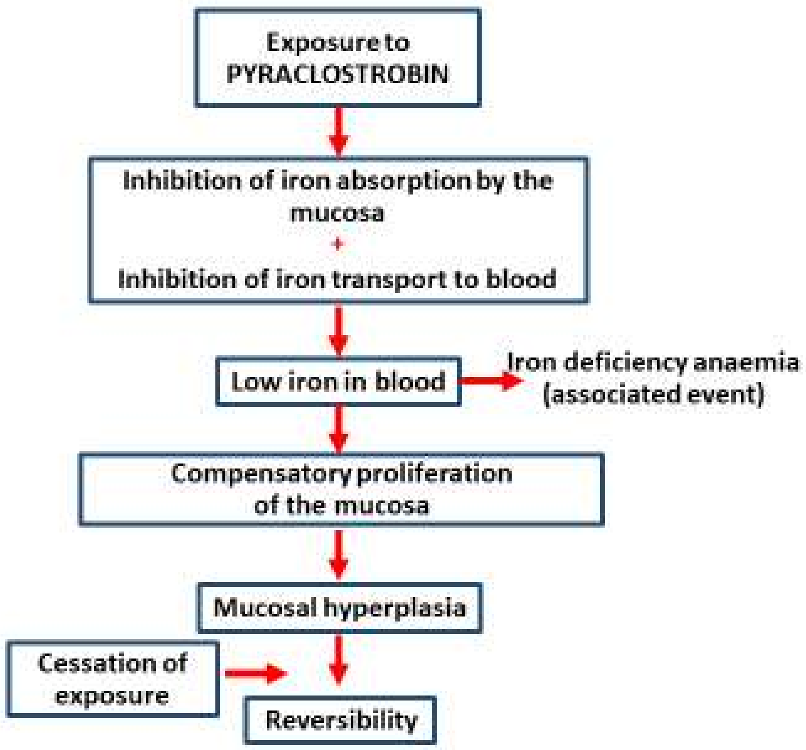
- inhibition of iron absorption from mucosa and iron transport to the circulatory system in the duodenum;
- iron deficiency in blood;
- increased cell proliferation activity in the mucosa of the duodenum as a compensatory response to the iron-deficient status in blood;
- reversible mucosal hyperplasia in the duodenum upon cessation of exposure;

and the following associated event:

- iron deficiency anaemia.

A diagrammatic representation of the postulated MOA is given in Fig. A3-2.

Fig. A3-2. Proposed MOA for the induction of mucosal hyperplasia in the duodenum by pyraclostrobin



Dose–response relationship and concordance

Key event: inhibition of iron absorption and transfer

In the report on inhibition of iron absorption and transfer, there was no description of a dose–response relationship because the study was conducted using a single-dose treatment with dimoxystrobin, a compound similar to pyraclostrobin (Srai, 2003).

Key event: Serum iron levels (see Table A3-3)

In rats treated with pyraclostrobin at 0, 50, 500 or 1500 ppm for 14 days in the diet, the NOAEL for the decrease in iron level in the serum was 50 ppm (equal to 3.8 mg/kg bw per day), with a LOAEL of 500 ppm (equal to 33.9 mg/kg bw per day) (Mellert et al., 2003).

Key event: Mucosal hyperplasia (see Table A3-1)

Mucosal hyperplasia following exposure to pyraclostrobin showed clear dose dependency in mice, rats and dogs. The NOAEL for mucosal hyperplasia in mice was 30.4 mg/kg bw per day, with a LOAEL of 119 mg/kg bw per day, in a 91-day oral toxicity study. The NOAEL for mucosal hyperplasia in rats was 11 mg/kg bw per day, with a LOAEL of 35 mg/kg bw per day, in a 90-day oral toxicity study. The NOAEL for mucosal hyperplasia in dogs was 5.8 mg/kg bw per day, with a LOAEL of 12.9 mg/kg bw per day, in a 90-day study. Long-term studies conducted in mice and rats were conducted at lower doses than the NOAELs for the 90-day studies, and no mucosal hyperplasia was observed in either study (Annex 1, reference *100*).

Key event: Cell proliferation activity

No data on cell proliferation activity in animals treated with pyraclostrobin were obtained.

In male mice treated with orysastrobin, a strobilurin fungicide similar to pyraclostrobin, at a dietary concentration of 0, 10, 100 or 2000 ppm (equal to 0, 1.9, 20.9 and 437 mg/kg bw per day, respectively) for 4 weeks, the NOAEL for cell proliferation activity in the duodenum was 100 ppm, with a LOAEL of 2000 ppm. The increase showed a recovery after the diet was withdrawn for 2 weeks (FSCJ, 2005).

In male rats treated with orysastrobin at a dietary concentration of 0, 10, 100 or 2500 ppm (equal to 0, 0.6, 6.1 and 148 mg/kg bw per day, respectively) for 4 weeks, the NOAEL for cell proliferation activity in the duodenum was 100 ppm, with a LOAEL of 2500 ppm. The increase showed a recovery after the diet was withdrawn for 2 weeks (FSCJ, 2005).

Associated event: Anaemia (see Table A3-2)

In a 3-month study in mice, the NOAEL for haematological effects was 30.4 mg/kg bw per day, with a LOAEL of 119 mg/kg bw per day. In a 4-week study in rats, the NOAEL for haematological effects was 9.6 mg/kg bw per day, with a LOAEL of 47 mg/kg bw per day. In a 3-month study in rats, the NOAEL for haematological effects was 11 mg/kg bw per day, with a LOAEL of 35 mg/kg bw per day. No haematological effects were observed in dogs (Annex 1, reference *100*).

The dose concordance of key and associated events from studies with pyraclostrobin or other strobilurin fungicides in mice, rats and dogs is summarized in Table A3-6.

Table A3-6. Dose concordance of key and associated events in the MOA for the induction of mucosal hyperplasia in the duodenum in mice, rats and dogs by pyraclostrobin

	NOAEL (mg/kg bw per day)	LOAEL (mg/kg bw per day)
Key events		
Decreased serum iron level		
Rat 14-day	3.8	33.9
Mucosal hyperplasia		
Mouse 3-month	30.4	119
Rat 4-week	9.0	42
Rat 3-month	11	35
Dog 3-month	5.8	12.9
Associated event		
Anaemia		
Mouse 3-month	30.4	119
Rat 4-week	9.6	47
Rat 3-month	11	35
Dog 3-month	12.9	–[a]

bw: body weight; LOAEL: lowest-observed-adverse-effect level; NOAEL: no-observed-adverse-effect level

[a] No findings indicating anaemia.

Temporal relationship

The temporal relationships among each key or associated event and pyraclostrobin treatment are summarized in Table A3-7.

Table A3-7. Temporal relationships of key and associated events for the induction of mucosal hyperplasia of the duodenum by pyraclostrobin or other strobilurin fungicides in mice, rats and dogs

	Within 96 hours	7 days	14 days	4 weeks	3 months
Key event: inhibition of iron absorption or transport					
Dimoxystrobin[a]					
Inhibition of absorption/transfer of iron in the duodenum	YES				
Key event: low iron in blood					
Pyraclostrobin					
Decreased iron and transferrin levels in serum in rats		YES	YES		
Orysastrobin					
Decreased serum iron level in rats			YES		
Key events: mucosal hyperplasia and cell proliferation					
Pyraclostrobin					
Mucosal hyperplasia					
Mouse					YES

	Within 96 hours	7 days	14 days	4 weeks	3 months
Rat				YES	YES
Dog					YES
Orysastrobin					
Cell proliferation activity in the duodenum					
Mice				YES	
Rats				YES	
Associated event					
Pyraclostrobin					
Anaemia					
Mouse					YES
Rat				YES	YES
Dog					

[a] See structure in Fig. A3-1 above.

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

The key and associated events occurred (Table A3-6) at increasing doses, in the order shown in the proposed MOA (Fig. A3-2). In fact, the dose–response relationships of the key events assessed for pyraclostrobin were consistent with the proposed sequence in the MOA.

The key events (Table A3-7) also indicated a clear, well-matched temporal relationship with the duodenum response in each step of the proposed MOA (Fig. A3-2), because the inhibitions of the absorption and transfer of iron in the duodenum, the initial event, occurred very quickly (within 96 hours), followed by the low iron levels in the serum after 7 days, the increased cell proliferation activity in the duodenum after 4 weeks and the mucosal hyperplasia in the duodenum after 4 weeks or 3 months. Most MOA studies were conducted using rats. However, from the available data, it can be concluded that there was no remarkable temporal difference in the events leading to mucosal hyperplasia in mice, rats or dogs.

Although the data on inhibition of the absorption and transfer of iron were obtained from studies using dimoxystrobin (Srai, 2003) only, given the similar toxicological profiles of the strobilurins, including the decrease in serum iron (Mellert et al., 2002) and mucosal cell proliferation following exposure to orysastrobin (Mellert & Kaufmann, 2004), they can be applied, at least qualitatively, to pyraclostrobin.

Biological plausibility

1. Occurrence of duodenal mucosal lesions in iron deficiency in rats

A study in which rats were treated with an iron-deficient diet has shown that this treatment results in duodenal changes similar to those observed with pyraclostrobin. Administration of an iron-deficient diet to Wistar rats resulted within 14 days in reduced serum iron concentrations, hypochromic microcytic anaemia, and an increase in duodenal epithelial cell proliferation (Cunha et al., 2008). After 5 weeks of iron deficiency, hypochromic microcytic anaemia and a clear increase in duodenal weight but no pronounced effects on cell proliferation were observed. Increased duodenal weights corresponded to significant increases in mucosal area, indicating a diffuse, simple mucosal hyperplasia. The sequence of events following iron depletion thus appears to be: 1) reduced serum iron levels, 2) induction of hypochromic microcytic anaemia, 3) increased duodenal epithelial cell proliferation and 4) increased duodenal weight (increased mucosal area). Iron deficiency anaemia was reversible after a

2-week recovery period, while duodenal weights were still increased. Intramuscular iron supplementation in animals fed with iron-deficient diet resulted in the maintenance of body iron levels at normal values, and neither anaemia nor increased duodenal cell proliferation was detected after 14 days. Thus, increased duodenal mucosal hyperplasia was shown to be secondary to depletion of body iron and consequent anaemia and possibly reflects an attempt to increase iron absorption to counteract iron deficiency.

2. Effect of dimoxystrobin on iron absorption and transport in the duodenum

In a study investigating the effect of dimoxystrobin, a compound similar to pyraclostrobin, on the mucosal uptake and transfer of iron into the carcass after oral administration in rats treated with dimoxystrobin at 0 or 4500 ppm (equivalent to 450 mg/kg bw per day) in the diet for 24, 96 or 168 hours, the everted duodenums were incubated to measure Fe^{2+} uptake in the duodenal segments. Iron transfer across the serosal membrane (i.e. iron transfer into the body) was measured using tied-off duodenal segments in rats exposed to 4500 ppm dimoxystrobin in the feed for 24 or 96 hours (Srai, 2003).

Mucosal iron uptake showed a statistically significant reduction after treatment with dimoxystrobin for 96 and 168 hours. The autoradiographic data confirmed the reduction of iron uptake. The data show the decrease in density of silver grains along the villus length, suggesting the reduction in uptake by the treatment.

Iron transfer across the serosal membrane (i.e. iron transfer into the body) was significantly reduced after a 96-hour treatment with dimoxystrobin, with the reduction of iron transfer being proportional to the reduction in uptake.

In summary, the results clearly demonstrated that repeated treatment of rats with dimoxystrobin considerably reduced both uptake of iron by the duodenal mucosa and transfer of iron into the body (Srai, 2003).

On the basis of the chemical similarity of dimoxystrobin to pyraclostrobin, inhibition of both the absorption and the transfer of iron in the duodenum likely occurred as a result of pyraclostrobin treatment.

Alternative MOA hypotheses

1. Mucosal hyperplasia as a consequence of cytotoxicity and regenerative proliferation

High concentrations of hexavalent chromium (Cr(VI)), captan and folpet induced duodenal tumours in mice (Thompson et al., 2017) as a consequence of cytotoxicity-induced regenerative proliferation.

When B6C3F1 mice were exposed to 180 ppm Cr(VI) in their drinking-water, 12 000 ppm captan in their feed or 16 000 ppm folpet in their feed for 28 days, villous enterocyte hypertrophy and mild crypt epithelial hyperplasia were observed in all exposed mice.

Folpet is not genotoxic in vivo and causes duodenal glandular tumours in mice, but not in rats. Folpet reacts with thiol groups and is rapidly hydrolysed at pH 7. Both reactions produce thiophosgene, which reacts with thiols and other functional groups. At sufficiently high, prolonged dietary doses, folpet irritates the mouse duodenum, resulting in cytotoxicity, with consequent regenerative proliferation and ultimately tumour development (Gordon, Cohen & Singh, 2012). Based on MOA analysis and assessment of human relevance, folpet, like captan, another trichloromethylthio-related fungicide with similar toxic and carcinogenic effects, is not likely to be a human carcinogen at doses that do not cause cytotoxicity and regenerative proliferation (Cohen et al., 2010).

The sequence of events involving cytotoxicity and regenerative proliferation is a common MOA for tumorigenesis by non–DNA-reactive chemicals (Meek et al., 2003).

This MOA should be excluded for pyroclostrobin because of the following:

- This MOA appears applicable to mice only, whereas duodenal hyperplasia induced by strobilurin fungicides was found in several species.
- Common histopathological findings in this MOA are inflammation or degenerative change, which have not been observed with strobilurin fungicides.

2. Brunner's gland hyperplasia induced by vascular endothelial growth factor

Vascular endothelial growth factor receptor tyrosine kinase inhibitors are reported to cause reversible mucosal hyperplasia (adenosis) in the duodenum of rats (Inomata et al., 2014). At 4 weeks, there was degeneration and necrosis of Brunner's gland epithelium accompanied by neutrophil infiltration around the affected glands. The main function of Brunner's gland is to protect the duodenal mucosa from the acidic gastric contents and abrasion of the intestinal mucosa by the passage of rough ingesta. At 13 weeks, the inflammation was more extensive and followed by reactive hyperplasia of the duodenal epithelium. Similar changes were not present in similar time-course studies in dogs and monkeys, suggesting that this is a rodent- or species-specific change (Inomata et al., 2014).

This MOA should be excluded because the target is Brunner's gland and because of the morphological findings (adenosis, which is observed in the muscular layer to the serosa).

3. Direct damage to DNA in the duodenal epithelium

Pyraclostrobin is not genotoxic in vitro or in vivo. Although no data were provided on DNA damage to the duodenum by the treatment, there is no reason to conclude that pyraclostrobin causes direct damage to DNA in the duodenal epithelium. Some genotoxic carcinogens, such as methylnitronitrosoguanidine, are known to induce tumours in various areas of the gastrointestinal tract in experimental animals, but there are no reports on induction of tumours in the duodenum only.

This MOA should be excluded because pyraclostrobin does not exhibit genotoxicity. There is no evidence of tumour induction in the duodenum only by genotoxic carcinogens.

Uncertainty, inconsistency or data gaps

The data for the present analysis were obtained not only from studies on pyraclostrobin, but also from studies on similar strobilurin fungicides – specifically, dimoxystrobin and orysastrobin. Several key events were evaluated based on the studies on these other strobilurin fungicides. Therefore, there are uncertainties or data gaps related to the lack of studies on pyraclostrobin on the basis of which to evaluate some of the key events. Although the level of inhibitory activity of iron absorption and transfer varied depending on the compound, the comparison of data on duodenal hyperplasia induced by several strobilurin fungicides contributes to the consistency of this MOA.

Human relevance of the proposed MOA

1. Is the weight of evidence sufficient to establish the MOA in animals?

Mucosal hyperplasia in the duodenum was consistently observed in mice, rats and dogs, with dose responsiveness (Table A3-8). Whereas serum iron levels or absorption and transfer of iron in the duodenum were not measured in mice or dogs, morphological and physiological features related to iron absorption in the duodenum are considered common among mice, rats, dogs and humans based on current knowledge. No evidence of inflammatory reaction or degeneration of the duodenal mucosa was obtained in any species. Overall, cell proliferation and consequently mucosal hyperplasia will occur as a compensatory response to lower iron levels in the serum due to inhibition of the uptake of iron in those species.

Table A3-8. Comparison of key and associated events for pyraclostrobin in animal species

Key/associated events	Mice	Rats	Dogs	Humans	Comments
Key events					
Inhibition of absorption and transfer of iron	NE	Yes	NE	Likely	Evidence from dimoxystrobin, a strobilurin fungicide similar to pyraclostrobin
Decrease in serum iron level	Likely	Yes	Likely	Likely	Suggestive based on haematological profiles of anaemia
Cell proliferation	Yes	Yes	NE	Likely	Evidence from orysastrobin, a strobilurin fungicide similar to pyraclostrobin
Mucosal hyperplasia in the duodenum	Yes	Yes	Yes	Likely	
Associated event					
Iron deficiency anaemia in response to low serum iron levels	Yes	Yes	Yes	Yes	Serum iron levels were not measured in mice or dogs

NE: not examined

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

Pyraclostrobin is a non-genotoxic substance, and no carcinogenicity was observed in rats or mice. Hyperplasia of the duodenal mucosa due to pyraclostrobin exposure was observed in all animal species examined (mice, rats and dogs). It was revealed from various studies in rats that iron deficiency occurs as a result of pyraclostrobin exposure, and mucosal hyperplasia occurs to enlarge the surface area for the absorption of iron in the feed from the duodenum. In general, hyperplasia is a change that shows recoverability when the cause is eliminated (cessation of exposure).

In humans, iron deficiency anaemia is one of the most common diseases worldwide. Iron deficiency anaemia attributable to nutritional deficiency or blood loss remains the most common, treatable anaemia in the world (Brugnara, 2003; Muñoz, Villar & Garcia-Erce, 2009). The most common cause of iron deficiency anaemia is blood loss from lesions in the gastrointestinal tract (Liu & Kaffes, 2012). Once the cause for the underlying blood loss or dietary deficiency is identified, the finding of anaemia with microcytic hypochromic erythrocytes in conjunction with abnormal serum biochemical indices (low iron, low transferrin saturation, low ferritin) usually leads to the administration of oral iron supplements, with improvement of anaemia in the vast majority of cases.

Intestinal structure and function in 11 children with iron deficiency anaemia were reported (Ercan et al., 1991). In six cases, there were histological abnormalities of small intestinal mucosa in varying degrees, consisting of villous damage, increased activity in the crypts, increased lymphoplasmocytic infiltration and changes in the surface epithelium. Ultrastructurally, microvilli lesions, mitochondrial changes and an increase in lysosomes were observed. These changes were due to impairment of cell metabolism of small intestinal epithelia. However, mucosal hyperplasia is not detected in human patients with iron deficiency anaemia.

The structure and function of the human duodenum are similar to those of mice, rats and dogs (Treuting, Valasek & Dintzis, 2012). After chronic exposure to strobin compounds, a similar duodenal mucosal hyperplastic lesion may be observed if iron deficiency anaemia occurs in humans.

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

There are no quantitative data comparing the kinetics or dynamics of the inhibition of iron uptake in the duodenum among humans and experimental animals. More information is necessary to enable a quantitative comparison.

Conclusion

The MOA is considered to be an interspecies MOA, because there are no qualitative differences between mucosal hyperplasias and their effective doses among rodents and dogs. Major MOA studies on key events were conducted in rats; however, anatomical and functional similarities in the duodenum among these species result in similar effects on iron absorption and transport in the duodenum by the treatment. Clear evidence of a relationship between mucosal hyperplasia and iron deficiency anaemia, which is a common type of anemia in humans, has not been provided, and there was no related report on occupational workers. In contrast, the effects on iron absorption and transport in the duodenum observed in rodents and dogs are considered to be common to humans due to the anatomical and functional similarities in the duodenum.

In conclusion, a similar MOA is plausible in humans following long-term and high-dose exposure to strobilurin fungicides.

References for Appendix 3

Brugnara C (2003). Iron deficiency and erythropoiesis: new diagnostic approaches. Clin Chem. 49:1573–8.

Cohen SM, Gordon EB, Singh P, Arce GT, Nyska A (2010). Carcinogenic mode of action of folpet in mice and evaluation of its relevance to humans. Crit Rev Toxicol. 40:531–45.

Cunha GC, Van Ravenzwaay B, Mellert W, Kaufmann W (2008). Iron deficiency causes duodenum mucosal hyperplasia in male Wistar rats. Toxicol Lett. 177:156–67.

Ercan O, Ulukutlu L, Ozbay G, Arda O (1991). Intestinal effects of iron deficiency anemia in children. Turk J Pediatr. 33:85–98.

FSCJ (2005). Orysastrobin. Food Safety Commission of Japan (https://www.fsc.go.jp/fsciis/evaluationDocument/show/kya20081030045, accessed 26 September 2018) (in Japanese).

Gordon E, Cohen SM, Singh P (2012). Folpet-induced short term cytotoxic and proliferative changes in the mouse duodenum. Toxicol Mech Methods. 22:54–9.

Inomata A, Nakano-Ito K, Fujikawa Y, Sonoda J, Hayakawa K, Ohta E et al. (2014). Brunner's gland lesions in rats induced by a vascular endothelial growth factor receptor inhibitor. Toxicol Pathol. 42:1267–74.

IPCS (2007). Part 1: IPCS framework for analysing the relevance of a cancer mode of action for humans and case-studies. Part 2: IPCS framework for analysing the relevance of a non-cancer mode of action for humans. Geneva, Switzerland: World Health Organization, International Programme on Chemical Safety (Harmonization Project Document No. 4; http://www.inchem.org/documents/harmproj/harmproj/harmproj4.pdf, accessed 26 September 2018).

Liu K, Kaffes AJ (2012). Iron deficiency anemia: a review of diagnosis, investigation and management. Eur J Gastroenterol Hepatol. 24:109–16.

Meek ME, Bucher JR, Cohen SM, Dellarco V, Hill RN, Lehman-McKeeman LD et al. (2003). A framework for human relevance analysis of information on carcinogenic modes of action. Crit Rev Toxicol. 33:591–653.

Mellert W (2003). Amendment no. 1 to the report: Screening study BAS 505 F – administration in the diet with simultaneous parenteral administration of iron to Wistar rats. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2003/1009197. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W, Kaufmann W (2004). Amendment no. 2 to the report: Screening study BAS 505 F – administration in the diet with simultaneous parenteral administration of iron to Wistar rats. Unpublished report. BASF AG,

Ludwigshafen/Rhein, Germany. BASF document ID 2004/1010601. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W, Deckardt K, Kaufmann W, Leibold E (2002). Screening study BAS 505 F – administration in the diet with simultaneous parenteral administration of iron to Wistar rats. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2002/1013984. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Mellert W, Deckardt K, Gembart C, Van Ravenzwaay B (2003). BAS 500 F – determination of iron in urine and serum of Wistar rats – administration in the diet over 14 days. Unpublished report. BASF AG, Ludwigshafen/Rhein, Germany. BASF document ID 2003/1009534. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Muñoz M, Villar I, Garcia-Erce JA (2009). An update on iron physiology. World J Gastroenterol. 15:4617–26.

Srai K (2003). BAS 505 F – study on the effect on the mucosal iron transport in female Wistar rats – administration in the diet. Unpublished report. Royal Free and University College Medical School, London, United Kingdom. BASF document ID 2003/1014047. Submitted to WHO by BASF AG, Ludwigshafen/Rhein, Germany.

Thompson CM, Wolf JC, McCoy A, Suh M, Proctor DM, Kirman CR et al. (2017). Comparison of toxicity and recovery in the duodenum of B6C3F1 mice following treatment with intestinal carcinogens captan, folpet, and hexavalent chromium. Toxicol Pathol. 45:1091–1101.

Treuting PM, Valasek MA, Dintzis SM (2012). Upper gastrointestinal tract. In: Treuting PM, Dintzis SM, editors. Comparative anatomy and histology. A mouse and human atlas. London, United Kingdom: Academic Press; 155–75.

Van Ravenzwaay B, Akiyama M, Landsiedel R, Kieczka H, Cunha G, Schneider S et al. (2007). Toxicological overview of a novel strobilurin fungicide, orysastrobin. J Pestic Sci. 32:270–7.

PYRIOFENONE

First draft prepared by
April Neal-Kluever,[1] Angelo Moretto[2] and Carl Cerniglia[3]

[1] Center for Food Safety and Applied Nutrition, Food and Drug Administration, College Park, Maryland, United States of America (USA)
[2] Department of Biomedical and Clinical Sciences, University of Milan, and International Centre for Pesticides and Health Risk Prevention, ASST Fatebenefratelli Sacco, Milan, Italy
[3] Division of Microbiology, National Center for Toxicological Research, Food and Drug Administration, Jefferson, Arkansas, USA

Explanation....651
Evaluation for acceptable intake....652
1. Biochemical aspects....652
1.1 Absorption, distribution and excretion....652
(a) Single-dose and repeated-dose absorption, distribution and excretion of ^{14}C-(phenyl)-pyriofenone....653
(b) Enterohepatic circulation of ^{14}C-(phenyl)-pyriofenone....658
(c) Plasma and whole blood kinetics of ^{14}C-(phenyl)-pyriofenone....659
(d) Single-dose absorption, distribution and excretion of ^{14}C-(pyridyl)-pyriofenone....660
(e) Plasma and whole blood kinetics of ^{14}C-(pyridyl)-pyriofenone....663
(f) Accumulation ratios....663
1.2 Biotransformation....664
2. Toxicological studies....665
2.1 Acute toxicity....665
(a) Lethal doses....665
(b) Dermal irritation....665
(c) Ocular irritation....665
(d) Dermal sensitization....666
2.2 Short-term studies of toxicity....667
(a) Oral administration....667
(b) Dermal application....675
2.3 Long-term studies of toxicity and carcinogenicity....676
2.4 Genotoxicity....690
2.5 Reproductive and developmental toxicity....691
(a) Multigeneration studies....691
(b) Developmental toxicity....695
2.6 Special studies....698
(a) Neurotoxicity....698
(b) Immunotoxicity....699
(c) Mechanistic studies....700
(d) Studies on metabolites....701
(e) Microbiological effects....702
3. Observations in humans....702
Comments....702
Toxicological evaluation....705
References....708

Explanation

Pyriofenone (Fig. 1) is the International Organization for Standardization–proposed common name for (5-chloro-2-methoxy-4-methyl-3-pyridyl)(4,5,6-trimethoxy-*o*-tolyl) methanone, with the Chemical Abstracts Service number 688046-61-9.

Pyriofenone is an aryl phenyl ketone fungicide used to control powdery mildew on grapevines.

Fig. 1. Chemical structure of pyriofenone

Source: Knight (2009)

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

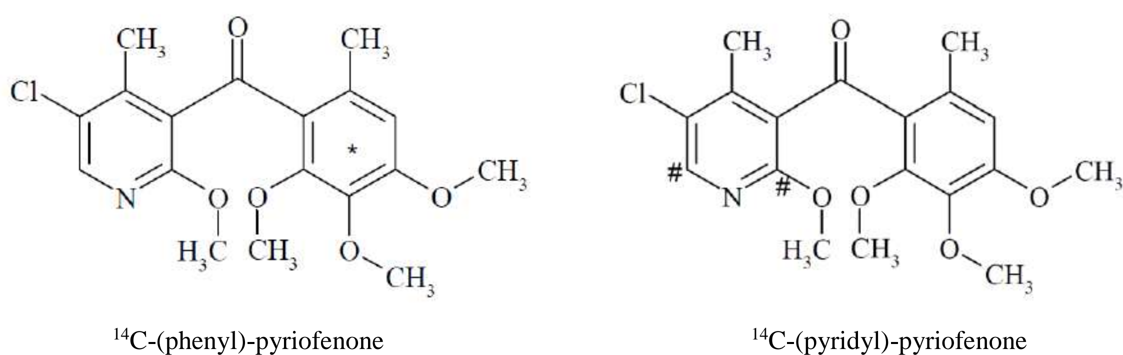
All critical studies contained statements of compliance with good laboratory practice (GLP) and were conducted in accordance with relevant national or international test guidelines, unless otherwise specified. No additional information from a literature search was identified that complemented the toxicological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

Evaluation for acceptable intake

1. Biochemical aspects

The positions of the pyriofenone radiolabels used in the absorption, distribution, metabolism and excretion (ADME) studies described below are shown in Fig. 2.

Fig. 2. Positions of pyriofenone radiolabels in ADME studies



^{14}C-(phenyl)-pyriofenone ^{14}C-(pyridyl)-pyriofenone

* denotes the position of the radiolabel (uniform) in the phenyl ring and # denotes the position of the radiolabel at positions 2 and 6 of the pyridyl ring
Source: Knight (2009)

1.1 Absorption, distribution and excretion

In a toxicokinetics study (Knight, 2009), Fischer rats (four of each sex per dose) received ^{14}C-(phenyl)-pyriofenone (batch no. EPPS-06-045-73-18 [CP-3098]; specific activity 6.22 MBq/mg; radiochemical purity >97%) or ^{14}C-(pyridyl)-pyriofenone (batch no. CP-3097; specific activity 4.75 MBq/mg; radiochemical purity >97%) in a single oral dose of 5 or 200 mg/kg body weight (bw). Urine was collected at 0–6 hours, at 6–24 hours and at 4-hour intervals up to 120 hours post-dosing. Faeces and cage washes were collected at 24-hour intervals up to 120 hours post-dosing. Blood samples were taken by cardiac puncture immediately prior to termination. Animals were terminated by cervical dislocation 120 hours after dosing, and selected tissues and organs were analysed for radioactivity using

liquid scintillation counting. Parent compound and metabolites were identified using high-performance liquid chromatography with radioactivity detection and thin-layer chromatography.

In addition to the above single-dose studies with both radiolabels, investigations included the following:

- *Repeated-dose excretion and distribution:* Four rats of each sex received oral doses of ^{14}C-(phenyl)-pyriofenone at 5 mg/kg bw per day for 14 days. The other radiolabelled compound, ^{14}C-(pyridyl)-pyriofenone, was not tested in repeated-dose experiments. Urine and faeces were collected for 24 hours after the first and seventh daily doses. Cage washes were collected at the end of each excreta collection period. Following the final dose, samples of excreta, tissues, organs, carcasses and cage washes were collected and assessed as described for the single-dose studies.
- *Biliary excretion:* Five rats of each sex per dose were used to assess the role of biliary excretion in pyriofenone metabolism. A flexible cannula was inserted into the common bile duct of each animal, and the other end of the cannula was inserted into the duodenum. The animals were permitted a recovery period of at least 3 days, during which bile flow through the cannula was checked daily. On the day of dosing, the rats received a single oral administration of ^{14}C-(phenyl)-pyriofenone or ^{14}C-(pyridyl)-pyriofenone at 5 or 200 mg/kg bw. Bile was collected from each rat at 0–3, 3–6, 6–9, 9–12, 12–24 and 24–48 hours after dosing. Urine and faeces were collected at 24-hour intervals up to 48 hours post-dosing. The rats were terminated at 48 hours, and the gastrointestinal tract (including contents) and liver were assessed for radioactivity, along with the remaining carcass. The interiors of the cages were rinsed after termination, and the washings were assessed for radioactivity.
- *Enterohepatic circulation:* Cannulated rats (five of each sex) were administered ^{14}C-(phenyl)-pyriofenone or ^{14}C-(pyridyl)-pyriofenone in a single oral dose of 5 or 200 mg/kg bw, and bile was collected at 0–9 hours post-dosing. A portion of this bile pool (0.5 mL) was administered to recipient animals via duodenal cannulas, and samples were collected as described above under biliary excretion.
- *Single-dose plasma and whole blood kinetics:* Twelve rats of each sex per dose received ^{14}C-(phenyl)-pyriofenone or ^{14}C-(pyridyl)-pyriofenone in a single oral dose of 5 or 200 mg/kg bw. Rats in each group were further divided into three subgroups (four of each sex). Blood samples were collected before dosing and at 0.25, 0.5, 1, 2, 3, 4, 6, 12, 24, 72, 96 and 120 hours after dosing.
- *Repeated-dose plasma and whole blood kinetics:* Twelve rats of each sex per dose received ^{14}C-(phenyl)-pyriofenone at an oral dose of 5 mg/kg bw per day for 14 days. Rats in each group were further divided into three subgroups (four of each sex). Blood samples were collected before dosing and at 0.25, 0.5, 1, 2, 3, 4, 6, 12, 24, 72, 96 and 120 hours after dosing.
- *Tissue distribution experiments:* Nine rats of each sex per dose received ^{14}C-(phenyl)-pyriofenone or ^{14}C-(pyridyl)-pyriofenone in a single oral dose of 5 or 200 mg/kg bw. Groups of six animals (three of each sex) were terminated at times determined by the plasma kinetics.

(a) Single-dose and repeated-dose absorption, distribution and excretion of ^{14}C-(phenyl)-pyriofenone

In bile duct–cannulated rats administered ^{14}C-(phenyl)-pyriofenone at a dose of 5 mg/kg bw, radioactivity was rapidly excreted into bile (65–73% of the dose) and urine (2.8–13% of the dose) 48 hours after dosing (Table 1). In intact rats, radioactivity in faeces accounted for 82–89% of the dose 120 hours after dosing, whereas radioactivity in urine accounted for 11–17% of the dose. No significant

radioactivity was detected in the carcass 120 hours after dosing (Table 2). These data suggest that pyriofenone is rapidly taken up and initially excreted into bile, with final excretion occurring predominately via faeces and minor excretion occurring via urine. No sex-specific differences were observed.

Table 1. Excretion of radioactivity 0–48 hours after administration of single oral doses of ^{14}C-(phenyl)- or ^{14}C-(pyridyl)-pyriofenone to bile duct–cannulated rats

	% of dose							
	^{14}C-(phenyl) label				^{14}C-(pyridyl) label			
	5 mg/kg bw		200 mg/kg bw		5 mg/kg bw		200 mg/kg bw	
	Males	Females	Males	Females	Males	Females	Males	Females
Bile	73	65	33	42	74	81	41	49
Urine	2.8	13	1.8	4.6	7.5	7.6	2.2	3.4
Cage wash	0.15	0.34	0.05	0.14	0.09	0.13	0.05	0.13
Faeces	23	15	59	51	14	6.3	54	45
Liver	0.10	0.04	0.07	0.05	0.10	0.04	0.09	0.04
GI tract + contents	0.13	0.11	1.63	0.92	0.02	0.06	0.39	0.21
Carcass	0.05	0.24	1.72	0.80	0.17	0.16	0.32	0.85
Total	99.5	92.9	97.0	99.3	95.6	95.2	98.2	98.1
Absorption[a]	76.2	77.9	36.1	47.2	81.7	88.8	43.8	53.0

bw: body weight; GI: gastrointestinal

[a] Sum of means for bile, urine, liver and residual carcass.

Source: Knight (2009)

Table 2. Excretion of radioactivity 0–120 hours after administration of single oral doses of ^{14}C-(phenyl)- or ^{14}C-(pyridyl)-pyriofenone to rats

	% of dose							
	^{14}C-(phenyl) label				^{14}C-(pyridyl) label			
	5 mg/kg bw		200 mg/kg bw		5 mg/kg bw		200 mg/kg bw	
	Males	Females	Males	Females	Males	Females	Males	Females
Urine	11	17	6.1	8.1	20	14	8.3	9.1
Cage wash	0.52	1.6	0.45	0.58	2.3	2.2	0.50	0.98
Faeces	89	82	91	85	73	78	89	89
Carcass	0.15	0.09	0.12	0.11	0.20	0.03	0.13	0.11
Total	100	101	97.6	94.6	94.5	94.1	97.6	98.9

bw: body weight

Source: Knight (2009)

The highest concentrations of radioactivity in tissues 120 hours after administration of ^{14}C-(phenyl)-pyriofenone at 5 mg/kg bw were found in liver, kidney and blood cells (Table 3). Tissue:plasma ratios were highest for the liver and kidney in both sexes. Intermediate ratios were

calculated for whole blood in both sexes and for carcass, fat, bone and gastrointestinal tract plus contents in female animals. Tissue:plasma ratios were less than 1 for all other tissues (Table 4).

In rats administered ^{14}C-(phenyl)-pyriofenone at 200 mg/kg bw, excretion followed a similar pattern as was seen with 5 mg/kg bw. However, at the high dose, a higher proportion of radioactivity was found in faeces (51–59% of the dose) than in bile (33–42% of the dose) in bile duct–cannulated rats 48 hours after dosing. The majority of radioactivity was eliminated in faeces 120 hours after dosing (85–91% of the dose) in intact rats. Overall recoveries of radioactivity (excluding tissues) were 97.6% and 94.6% of the dose for males and females, respectively (Tables 1 and 2). The data suggest that absorption at the high dose became saturated and that a lower proportion of the dose was absorbed into bile compared with the low dose. No sex-specific differences were noted.

Similar to animals dosed with 5 mg/kg bw, the highest radioactivity levels in tissues after exposure to 200 mg/kg bw were in liver, followed by kidney, blood cells, whole blood and fat (females) (Table 3). Tissue:plasma ratios were highest for the fat (females), liver and kidney (both sexes) and gastrointestinal tract (females). Intermediate ratios were calculated for whole blood (both sexes), heart (females), lungs (females), spleen (both sexes), ovaries and uterus (females) and residual carcass (females). Tissue:plasma ratios were less than 1 for all other tissues (Table 4).

Table 3. Mean concentrations of radioactivity in tissues 120 hours after administration of single or repeated oral doses of ^{14}C-(phenyl)-pyriofenone to rats

	Mean concentrations of radioactivity (µg equivalents/g)					
	1 × 5 mg/kg bw		1 × 200 mg/kg bw		14 × 5 mg/kg bw	
	Males	Females	Males	Females	Males	Females
Sample	**Mean ± sd**	**Mean ± sd**	**Mean ± sd**	**Mean ± sd**	**Mean ± sd**	**Mean ± sd**
Plasma	0.026 ± 0.002	0.006 ± 0.001	0.585 ± 0.096	0.230 ± 0.033	0.131 ± 0.011	0.022 ± 0.003
Whole blood	0.042 ± 0.003	0.006 ± 0.004	1.364 ± 0.137	0.431 ± 0.041	0.411 ± 0.060	0.067 ± 0.004
Blood cells	0.068 ± 0.006	0.010 ± 0.006	2.500 ± 0.450	0.810 ± 0.066	0.819 ± 0.172	0.146 ± 0.010
Brain	nd	nd	nd	nd	0.026 ± 0.003	nd
Heart	0.015 ± 0.002	nd	0.473 ± 0.131	0.245 ± 0.036	0.124 ± 0.033	0.029 ± 0.003
Kidney	0.065 ± 0.006	0.024 ± 0.003	1.925 ± 0.171	1.630 ± 0.176	0.486 ± 0.061	0.208 ± 0.028
Liver	0.163 ± 0.016	0.041 ± 0.006	4.346 ± 0.480	1.703 ± 0.479	0.892 ± 0.027	0.184 ± 0.015
Lungs	0.012 ± 0.001	nd	0.504 ± 0.025	0.248 ± 0.031	0.147 ± 0.021	0.047 ± 0.004
Spleen	0.018 ± 0.003	nd	0.639 ± 0.089	0.301 ± 0.015	0.250 ± 0.024	0.065 ± 0.008
Adrenal glands	nd	nd	nd	nd	0.096 ± 0.007	0.027 ± 0.020
Pituitary gland	nd	nd	nd	nd	nd	nd
Thyroid	nd	nd	nd	nd	0.256 ± 0.052	nd
Epididymis	0.006 ± 0.001	–	0.226 ± 0.021	–	0.046 ± 0.003	–
Testes	0.002 ± 0.003	–	0.041 ± 0.081	–	0.037 ± 0.003	–
Ovaries	–	nd	–	0.315 ± 0.364	–	0.009 ± 0.019
Uterus	–	nd	–	0.394 ± 0.046	–	0.016 ± 0.002
Bone	0.007 ± 0.008	0.004 ± 0.007	0.129 ± 0.257	nd	0.064 ± 0.007	0.032 ± 0.022

Sample	Mean concentrations of radioactivity (µg equivalents/g)					
	1 × 5 mg/kg bw		1 × 200 mg/kg bw		14 × 5 mg/kg bw	
	Males	Females	Males	Females	Males	Females
	Mean ± sd	Mean ± sd	Mean ± sd	Mean ± sd	Mean ± sd	Mean ± sd
Bone marrow	nd	nd	nd	nd	0.081 ± 0.022	nd
Fat (abdominal)	nd	0.002 ± 0.004	nd	2.936 ± 0.760	0.032 ± 0.008	0.005 ± 0.011
Muscle (skeletal)	0.001 ± 0.003	nd	0.055 ± 0.110	nd	0.043 ± 0.003	nd
GI tract + contents	0.020 ± 0.005	0.014 ± 0.004	0.510 ± 0.061	1.331 ± 0.471	0.076 ± 0.041	0.029 ± 0.004
Residual carcass	0.009 ± 0.002	0.006 ± 0.004	0.304 ± 0.051	0.268 ± 0.348	0.065 ± 0.005	0.036 ± 0.011
% of dose recovered in total tissues[a]	0.31 ± 0.05	0.07 ± 0.02	0.21 ± 0.03	0.24 ± 0.02	2.25 ± 0.08	0.40 ± 0.05

bw: body weight; GI: gastrointestinal; nd: not detected; sd: standard deviation

[a] Excluding plasma and carcass. Recoveries calculated assuming the following % body weight values:

Whole blood	7%
Bone	5.46%
Bone marrow	0.35%
Fat (abdominal)	7.1%
Muscle (skeletal)	45.5%

Source: Knight (2009)

Table 4. Tissue:plasma ratios of radioactivity in tissues 120 hours after administration of single oral doses of ^{14}C-(phenyl)-pyriofenone to rats

Sample	Tissue:plasma ratios of radioactivity			
	5 mg/kg bw		200 mg/kg bw	
	Males	Females	Males	Females
	Mean ± sd	Mean ± sd	Mean ± sd	Mean ± sd
Plasma	1	1	1	1
Whole blood	1.62 ± 0.022	1.46 ± 0.246	2.36 ± 0.211	1.89 ± 0.144
Brain	nd	nd	nd	nd
Heart	0.581 ± 0.120	nd	0.817 ± 0.216	1.09 ± 0.241
Kidney	2.51 ± 0.087	4.21 ± 0.605	3.33 ± 0.286	7.13 ± 0.435
Liver	6.24 ± 0.306	7.16 ± 1.08	7.49 ± 0.559	7.32 ± 0.940
Lungs	0.466 ± 0.032	nd	0.876 ± 0.112	1.09 ± 0.102
Spleen	0.697 ± 0.083	nd	1.10 ± 0.108	1.33 ± 0.185
Adrenal glands	nd	nd	nd	nd
Pituitary gland	nd	nd	nd	nd
Thyroid	nd	nd	nd	nd
Epididymis	0.24 ± 0.008	–	0.393 ± 0.056	–
Testes	0.223 ± na	–	0.230 ± na	–

Sample	Tissue:plasma ratios of radioactivity											
	5 mg/kg bw						200 mg/kg bw					
	Males			Females			Males			Females		
	Mean	±	sd	Mean	±	sd	Mean	±	sd	Mean	±	sd
Ovaries		–			nd			–		2.67	±	0.566
Uterus		–			nd			–		1.75	±	0.384
Bone	0.515	±	0.016	2.21	±	na	0.729	±	na		nd	
Bone marrow		nd			nd			nd			nd	
Fat (abdominal)		nd		1.51	±	na		nd		13.3	±	4.77
Muscle (skeletal)	0.205	±	na		nd		0.374	±	na		nd	
GI tract + contents	0.761	±	0.155	2.44	±	0.827	0.900	±	0.230	5.71	±	1.59
Residual carcass	0.326	±	0.043	1.42	±	0.290	0.532	±	0.133	2.59	±	1.37

bw: body weight; GI: gastrointestinal; na: not applicable; nd: not detected; sd: standard deviation
Source: Knight (2009)

In rats administered ^{14}C-(phenyl)-pyriofenone at 5 mg/kg bw per day for 14 days, radioactivity in faeces accounted for the majority of excreted radioactivity, with 99–103% of the daily dose found in faeces 120 hours after the final daily dose (Table 5).

Table 5 . Mean excretion and retention of radioactivity after administration of up to 14 consecutive daily oral doses of ^{14}C-(phenyl)-pyriofenone at 5 mg/kg bw to rats

Sample	Time point	Mean excretion (% of dose)[a]					
		Males			Females		
		Mean	±	sd	Mean	±	sd
Urine	Day 1 + 24 h	6.44	±	0.32	8.03	±	3.07
Cage wash	Day 1 + 24 h	0.37	±	0.09	0.71	±	0.09
Faeces	Day 1 + 24 h	65.24	±	2.91	58.31	±	13.78
Total		72.04	±	2.99	67.04	±	11.47
Urine	Day 7 + 24 h	9.61	±	1.16	8.86	±	2.89
Cage wash	Day 7 + 24 h	0.53	±	0.19	0.68	±	0.50
Faeces	Day 7 + 24 h	88.86	±	9.06	89.67	±	6.01
Total		98.99	±	9.62	99.20	±	3.00
Urine	Day 14 + 6 h	3.47	±	0.45	2.02	±	0.39
	Day 14 + 24 h	6.21	±	0.93	7.93	±	1.39
	Day 14 + 48 h	1.53	±	1.03	2.40	±	0.72
	Day 14 + 72 h	0.50	±	0.37	0.54	±	0.18
	Day 14 + 96 h	0.19	±	0.09	0.21	±	0.04
	Day 14 + 120 h	0.14	±	0.05	0.10	±	0.01

Sample	Time point	Mean excretion (% of dose)[a]					
		Males			Females		
		Mean	±	sd	Mean	±	sd
Total urine		12.04	±	2.21	13.18	±	2.36
Cage wash	Day 14 + 120 h	0.88	±	0.37	1.59	±	0.25
Faeces	Day 14 + 24 h	84.62	±	3.89	84.92	±	5.87
	Day 14 + 48 h	14.50	±	11.59	12.02	±	3.83
	Day 14 + 72 h	2.69	±	2.85	1.31	±	0.36
	Day 14 + 96 h	0.85	±	0.73	0.34	±	0.11
	Day 14 + 120 h	0.41	±	0.22	0.17	±	0.02
Total faeces		103.08	±	11.85	98.75	±	3.01
Residual carcass	Day 14 + 120 h	1.05	±	0.09	0.57	±	0.18
Overall total		117.04	±	14.35	114.09	±	4.18

bw: body weight; sd: standard deviation

[a] Results are expressed as % of the dose based on the dose received by an animal on a single day (day 1, 7 or 14), not the cumulative dose received during that period.

Source: Knight (2009)

Similar to the situation following administration of a single dose of 5 mg/kg bw, radioactivity remaining in the carcass after 14 daily doses of ^{14}C-(phenyl)-pyriofenone at 5 mg/kg bw accounted for 0.57–1.1% of the daily dose (Table 5), and concentrations of radioactivity in tissues were relatively low (Table 3). The highest concentrations in tissues occurred in liver, kidney, whole blood and blood cells (Table 5). Generally, tissue concentrations were higher in males than in females and, except for red blood cells, declined over time. Concentrations of radioactivity in blood cells increased with time for both sexes.

The extent of absorption was estimated by summing the values for bile, urine, liver and carcass after administration of ^{14}C-(phenyl)-pyriofenone to bile duct–cannulated rats. On this basis, it was estimated that the extent of absorption of a low dose (5 mg/kg bw) of ^{14}C-(phenyl)-pyriofenone was 76% of the dose for males and 78% of the dose for females. For the high dose (200 mg/kg bw) of ^{14}C-(phenyl)-pyriofenone, the extent of absorption was 36% of the dose for males and 47% of the dose for females (Table 1).

(b) Enterohepatic circulation of ^{14}C-(phenyl)-pyriofenone

The bile was retained from some of the cannulated rats given ^{14}C-(phenyl)-pyriofenone at a dose of 5 mg/kg bw. This bile was subsequently administered into the duodenum of cannulated recipient animals to determine the extent of enterohepatic circulation. Forty-eight hours after single intraduodenal doses of bile, 66% of the dose was excreted in the bile of recipient male rats. Most of the radioactivity, 65% of the dose, was excreted in bile 24 hours after dosing. Urinary excretion 48 hours after dosing accounted for 11% of the dose, whereas faecal excretion accounted for 20% of the dose. No radioactivity was detected in the carcass at 48 hours. Overall recovery was 96% of the dose. The extent of reabsorption was estimated by summing the values for bile, urine, liver and carcass. On this basis, it was estimated that the extent of reabsorption was 76% of the dose.

(c) Plasma and whole blood kinetics of ^{14}C-(phenyl)-pyriofenone

Following administration of a single oral dose of ^{14}C-(phenyl)-pyriofenone at 5 mg/kg bw, peak mean plasma and whole blood concentrations of radioactivity were reached at 12 hours (Table 6). At 200 mg/kg bw, peak mean plasma concentrations of radioactivity were reached at 6 hours (males) and 12 hours (females), whereas peak mean whole blood concentrations of radioactivity were reached at 6 hours (males) and 2 hours (females). After 14 daily oral doses at 5 mg/kg bw, peak mean plasma and whole blood concentrations of radioactivity were reached at 2 hours (males) and 12 hours (females) (Table 7).

Table 6. Pharmacokinetic parameters derived from mean radioactivity concentrations in plasma and whole blood after administration of single oral doses of ^{14}C-(phenyl)- or ^{14}C-(pyridyl)-pyriofenone to rats

	^{14}C-(phenyl) label				^{14}C-(pyridyl) label			
	5 mg/kg bw		**200 mg/kg bw**		**5 mg/kg bw**		**200 mg/kg bw**	
	Males	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**
Plasma								
C_{max} (µg/g)	0.60	0.58	13	6.2	0.88	0.66	15	7.4
T_{max} (h)	12	12	6	12	4	12	6	24
AUC_{120} (µg·h/g)	26	17	460	230	33	16	620	330
$t_{½}$ (h)	26	17[a]	24	13	46[a]	13[a]	30	20
AR[b]	1.5	1.0	1.4	0.78	2.8	0.77	1.8	1.2
Whole blood								
C_{max} (µg/g)	0.37	0.34	9.4	4.4	0.53	0.40	9.8	5.2
T_{max} (h)	12	12	6	2	12	12	6	24
AUC_{120} (µg·h/g)	19	11	430	170	25	9.9	530	230
$t_{½}$ (h)	36	18[a]	58[a]	18	30	13	54[a]	22
AR[b]	2.2	1.1	3.5	1.1	1.8	0.80	3.2	1.3

AR: accumulation ratio; AUC_{120}: area under the concentration–time curve at 120 hours; bw: body weight; C_{max}: maximum concentration; $t_{½}$: terminal half-life; T_{max}: time at which the maximum concentration is reached

[a] Data did not meet all the acceptance criteria, and therefore this value should be treated cautiously.

[b] Calculated as AR = 1.44*($t_{½}$/dosing interval).

Source: Knight (2009)

Table 7. Summary of pharmacokinetic parameters in rats after administration of 14 consecutive oral daily doses of ^{14}C-(phenyl)-pyriofenone at 5 mg/kg bw

	^{14}C-(phenyl) label, 14 × 5 mg/kg bw	
	Males	**Females**
Plasma		
C_{max} (µg/g)	1.2	0.77
T_{max} (h)	2	12
AUC_{120} (µg·h/g)	54	18
$t_{½}$ (h)	37	26

	^{14}C-(phenyl) label, 14 × 5 mg/kg bw	
	Males	**Females**
AR[b]	2.2	1.6
Whole blood		
C_{max} (µg/g)	1.2	0.55
T_{max} (h)	2	12
AUC_{120} (µg·h/g)	74	20
$t_{½}$ (h)	102[a]	64[a]
AR[b]	6.1	3.8

AR: accumulation ratio; AUC_{120}: area under the concentration–time curve at 120 hours; bw: body weight; C_{max}: maximum concentration; $t_{½}$: terminal half-life; T_{max}: time at which the maximum concentration is reached

[a] Data did not meet all the acceptance criteria, and therefore this value should be treated cautiously.

[b] Calculated as AR = 1.44*($t_{½}$/dosing interval).

Source: Knight (2009)

The extent of exposure, reflected by the area under the concentration–time curve at 120 hours (AUC_{120}), was greater in males than in females, particularly at 200 mg/kg bw. The terminal half-life ($t_{½}$) was longer in males than in females and longer in whole blood than in plasma. Both the maximum concentration (C_{max}) and the extent of exposure (AUC_{120}) increased with increasing dose; however, this increase was less than proportional, indicating non-linear, dose-dependent kinetics.

Following single oral doses, the whole blood:plasma ratio (based on AUC_{120}) was less than 1, was higher in males than in females and increased with increasing dose. After repeated oral doses, the whole blood:plasma ratio was greater than 1 and indicated accumulation of radioactivity in red blood cells during repeated dose administration.

(d) Single-dose absorption, distribution and excretion of ^{14}C-(pyridyl)-pyriofenone

In bile duct–cannulated rats administered ^{14}C-(pyridyl)-pyriofenone at 5 mg/kg bw, radioactivity was rapidly excreted into bile (74–81% of the dose) and urine (7.5–7.6% of the dose) (Table 1). Radioactivity in faeces accounted for 73–78% of the dose, whereas radioactivity in urine accounted for 14–20% of the dose, 120 hours after dosing in intact rats (Table 2). Similar to ^{14}C-(phenyl)-pyriofenone, the data suggest that ^{14}C-(pyridyl)-pyriofenone is rapidly taken up and initially excreted into bile, with final excretion occurring predominately via faeces, with minor excretion occurring via urine. No sex-specific differences were noted.

Radioactivity retained in the tissues accounted for less than 1% of the dose in both male and female rats. The highest concentrations were measured in the liver in both sexes, then kidney (both sexes), plasma (males), whole blood (males) and blood cells (males). Females consistently had lower levels of radioactivity in tissues than males, with the highest value observed in liver (0.046 µg equivalents/g). In males, concentrations of radioactivity in all tissues other than plasma, whole blood, blood cells, kidney and liver were less than or equal to 0.05 µg equivalents/g or below the limit of detection (Table 8). Tissue:plasma ratios were highest for the liver (both sexes), kidney (both sexes), gastrointestinal tract including contents (females) and fat (females). Intermediate ratios were calculated for whole blood in both sexes. Tissue:plasma ratios were less than 1 for all other tissues (Table 9).

In bile duct–cannulated rats administered ^{14}C-(pyridyl)-pyriofenone at 200 mg/kg bw, excretion followed a similar pattern as with 5 mg/kg bw, except that most of the radioactivity was recovered in faeces (45–54% of the dose) rather than bile (41–49% of the dose) 48 hours after dosing (Table 1). The majority of radioactivity was eliminated in faeces (89% of the dose) 120 hours after dosing in intact rats (Table 2). Overall recoveries of radioactivity in intact animals (excluding tissues) 120 hours after dosing were 98% and 99% of the dose for males and females, respectively (Table 2).

Table 8. Mean concentrations of radioactivity in tissues 120 hours after administration of single oral doses of ^{14}C-(pyridyl)-pyriofenone to rats

Sample	Mean concentrations of radioactivity (µg equivalents/g)			
	1 × 5 mg/kg bw		1 × 200 mg/kg bw	
	Males	Females	Males	Females
	Mean ± sd	Mean ± sd	Mean ± sd	Mean ± sd
Plasma	0.055 ± 0.005	0.003 ± 0.002	0.877 ± 0.112	0.464 ± 0.228
Whole blood	0.084 ± 0.013	0.007 ± 0.001	3.593 ± 1.092	0.954 ± 0.421
Blood cells	0.127 ± 0.019	0.016 ± 0.003	8.016 ± 2.664	1.855 ± 0.914
Brain	0.005 ± 0.001	nd	0.045 ± 0.091	nd
Heart	0.021 ± 0.003	nd	0.587 ± 0.058	0.207 ± 0.140
Kidney	0.118 ± 0.015	0.023 ± 0.003	3.039 ± 0.422	2.937 ± 0.956
Liver	0.356 ± 0.049	0.046 ± 0.010	6.124 ± 0.889	2.857 ± 1.146
Lungs	0.027 ± 0.009	nd	0.658 ± 0.087	0.357 ± 0.093
Spleen	0.026 ± 0.005	nd	0.823 ± 0.108	0.392 ± 0.068
Adrenal glands	0.017 ± 0.012	nd	nd	nd
Pituitary gland	nd	nd	nd	nd
Thyroid	nd	nd	nd	nd
Epididymis	0.011 ± 0.001	–	0.340 ± 0.102	–
Testes	0.008 ± 0.001	–	0.210 ± 0.024	–
Ovaries	–	nd	–	0.219 ± 0.439
Uterus	–	nd	–	0.224 ± 0.162
Bone	nd	nd	nd	nd
Bone marrow	0.005 ± 0.010	nd	nd	nd
Fat (abdominal)	nd	0.011 ± 0.003	0.499 ± 0.144	6.439 ± 0.846
Muscle (skeletal)	0.007 ± 0.001	nd	0.202 ± 0.013	nd
GI tract + contents	0.046 ± 0.001	0.028 ± 0.009	1.166 ± 0.447	6.354 ± 4.107
Residual carcass	0.013 ± 0.003	0.002 ± 0.004	0.346 ± 0.058	0.289 ± 0.336
% of dose recovered in total tissues[a]	0.65 ± 0.05	0.10 ± 0.02	0.39 ± 0.06	0.61 ± 0.19

bw: body weight; GI: gastrointestinal; nd: not detected; sd: standard deviation

[a] Excluding plasma and carcass. Recoveries calculated assuming the following % body weight values:

Whole blood	7%
Bone	5.46%
Bone marrow	0.35%
Fat (abdominal)	7.1%
Muscle (skeletal)	45.5%

Source: Knight (2009)

Table 9. Tissue:plasma ratios of radioactivity in tissues 120 hours after administration of single oral doses of ^{14}C-(pyridyl)-pyriofenone to rats

	Tissue:plasma ratios of radioactivity											
	5 mg/kg bw						**200 mg/kg bw**					
	Males			**Females**			**Males**			**Females**		
Sample	**Mean**	**±**	**sd**	**Mean**	**±**	**sd**	**Mean**	**±**	**sd**	**Mean**	**±**	**sd**
Plasma		1			1			1			1	
Whole blood	1.54	±	0.116	2.17	±	0.084	4.06	±	0.984	2.37	±	1.03
Brain	0.093	±	0.014		nd		0.174	±	na		nd	
Heart	0.390	±	0.044		nd		0.672	±	0.042	0.492	±	0.092
Kidney	2.17	±	0.215	6.78	±	0.710	3.47	±	0.329	7.83	±	4.23
Liver	6.52	±	0.498	14.3	±	2.42	6.98	±	0.163	6.63	±	1.39
Lungs	0.481	±	0.145		nd		0.752	±	0.063	0.923	±	0.425
Spleen	0.479	±	0.054		nd		0.941	±	0.066	1.09	±	0.700
Adrenal glands	0.420	±	0.023		nd			nd			nd	
Pituitary gland		nd			nd			nd			nd	
Thyroid		nd			nd			nd			nd	
Epididymis	0.199	±	0.015		–		0.387	±	0.111		–	
Testes	0.149	±	0.020		–		0.241	±	0.022		–	
Ovaries		–			nd			–		1.32	±	na
Uterus		–			nd			–		0.546	±	0.235
Bone		nd			nd			nd			nd	
Bone marrow	0.339	±	na		nd			nd			nd	
Fat (abdominal)		nd		3.41	±	0.611	0.567	±	0.139	19.7	±	16.0
Muscle (skeletal)	0.134	±	0.017		nd		0.232	±	0.016		nd	
GI tract + contents	0.858	±	0.095	9.08	±	1.79	1.32	±	0.428	12.6	±	3.32
Residual carcass	0.242	±	0.038		nd		0.397	±	0.069	0.932	±	0.025

bw: body weight; GI: gastrointestinal; na: not applicable; nd: not detected; sd: standard deviation
Source: Knight (2009)

Similar to animals dosed with ^{14}C-(pyridyl)-pyriofenone at 5 mg/kg bw, the highest accumulation in tissues after exposure to 200 mg/kg bw was in liver, followed by blood cells, kidney, whole blood, gastrointestinal tract (including contents) and fat (females) (Table 8). Tissue:plasma ratios were highest for the fat (females) and gastrointestinal tract (females), liver (both sexes) and kidney (both sexes). Intermediate ratios were calculated for whole blood, spleen (females), gastrointestinal tract including contents (males) and ovaries (females). Tissue:plasma ratios were less than 1 for all other tissues (Table 9).

Generally, tissue concentrations were higher in males than in females and, except for blood cells, declined over time. Concentrations of radioactivity in blood cells increased with time in both sexes.

The extent of absorption was estimated by summing the radioactivity (expressed as percentage of administered dose) found in bile, urine, liver and carcass after administration of ^{14}C-(pyridyl)-

pyriofenone in bile duct–cannulated rats. On this basis, it was estimated that the extent of absorption of ^{14}C-(pyridyl)-pyriofenone at 5 mg/kg bw was 82–89% of the dose. In contrast, the extent of absorption of ^{14}C-(pyridyl)-pyriofenone at 200 mg/kg bw was estimated to be 44–53% of the dose (Table 1).

(e) Plasma and whole blood kinetics of ^{14}C-(pyridyl)-pyriofenone

Following administration of a single oral dose of ^{14}C-(pyridyl)-pyriofenone at 5 mg/kg bw, peak mean plasma concentrations of radioactivity were reached at 4 hours (males) and 12 hours (females). Peak mean whole blood concentrations of radioactivity were reached at 12 hours (both sexes). At 200 mg/kg bw, peak mean plasma and whole blood concentrations of radioactivity were reached at 6 hours (males) and 24 hours (females) (Table 6).

Similar to the situation with ^{14}C-(phenyl)-pyriofenone, the AUC_{120} was higher in males than in females, although the ratio of AUC_{120} values was greater for the low dose. The time taken to achieve maximum concentrations (T_{max}) was shorter for males than for females at both doses in plasma (but not whole blood), and the terminal half-life ($t_{½}$) in both plasma and whole blood was longer in males than in females at the low dose. The terminal half-life was similar in plasma and whole blood of the female animals at the high dose. Both the maximum concentration (C_{max}) and the extent of exposure (AUC_{120}) increased with increasing dose; however, this increase was less than proportional, indicating non-linear, dose-dependent kinetics.

Following the administration of single oral doses of ^{14}C-(phenyl)-pyriofenone to rats, the whole blood:plasma ratio (based on AUC_{120}) was less than 1, was higher in males than in females and increased with dose.

(f) Accumulation ratios

In plasma, the 24-hour accumulation ratios (AR) based on half-life (AR = 1.44*[$t_{½}$/dosing interval]) were 1.5 in male rats and 1.0 in female rats after a single dose of 5 mg/kg bw ^{14}C-(phenyl)-pyriofenone and were 2.8 in male rats and 0.8 in female rats after a single dose of 5 mg/kg bw ^{14}C-(pyridyl)-pyriofenone. After a single dose of 200 mg/kg bw ^{14}C-(phenyl)-pyriofenone, the accumulation ratios were 1.4 and 0.8 in male and female rats, respectively. After a dose of 200 mg/kg bw ^{14}C-(pyridyl)-pyriofenone, the accumulation ratios were 1.8 and 1.2 in male and female rats, respectively (Table 6).

In whole blood, the 24-hour accumulation ratios were 2.2 in male rats and 1.1 in female rats after a single dose of 5 mg/kg bw ^{14}C-(phenyl)-pyriofenone and were 1.8 in male rats and 0.8 in female rats after a single dose of 5 mg/kg bw ^{14}C-(pyridyl)-pyriofenone. After a single dose of 200 mg/kg bw ^{14}C-(phenyl)-pyriofenone, the accumulation ratios were 3.5 and 1.1 in male and female rats, respectively. After a single dose of 200 mg/kg bw ^{14}C-(pyridyl)-pyriofenone, the accumulation ratios were 3.2 and 1.3 in male and female rats, respectively (Table 6).

After repeated oral doses of ^{14}C-(phenyl)-pyriofenone, additional accumulation of radioactivity was observed. The 24-hour accumulation ratios were 2.2 and 1.6 in plasma of male and female rats, respectively, and 6.1 and 3.8 in whole blood of male and female rats, respectively. The terminal half-life also increased after repeated exposure (Table 7).

Based on these calculations, there is some evidence that pyriofenone and/or its metabolites accumulate in both plasma and whole blood in male rats after single or repeated administration at 24-hour intervals. It may also accumulate in the blood of female rats, but to a lesser extent. However, the Meeting noted that overall radioactivity recovery values 120 hours after single or repeated dosing were approximately 100%, decreasing the concern for potential accumulation.

1.2 Biotransformation

The metabolism of pyriofenone was studied in the toxicokinetics study by Knight (2009).

There were no major metabolic differences between ^{14}C-(phenyl)-pyriofenone and ^{14}C-(pyridyl)-pyriofenone. Unchanged pyriofenone was identified as the major component in faecal extracts, accounting for 18–29% of the dose at 5 mg/kg bw and 59–63% of the dose at 200 mg/kg bw (Table 10). Other components identified in faeces were 2MDPM (maximum 21% of the low dose), 3HDPM (maximum 16% of the low dose) and 4HDPM (maximum 11% of the low dose) (Table 11) and accounted for 1.9–7.7% at the high dose. All other components in faecal extracts accounted for less than 5% of the dose.

Table 10. Residues after single exposures of rats to 5 or 200 mg/kg bw of pyriofenone with either label[a]

Tissue, fluid or excreta	Principal residue(s)	Secondary residue(s)
Faeces	Pyriofenone (58.6–62.8% of the dose)	2MDPM, 3HDPM, 4HDPM (1.9–7.7% of the dose)
Urine	2MDPM conjugate (females; 9.5% of the dose, but only at 5 mg/kg bw)	Conjugates of 3HDPM and 4HDPM (<1.5% of the dose); pyriofenone (<1% of the dose)
Bile	3HDPM-glucuronide and 4HDPM-glucuronide (10.1–38.6% of the dose)	Pyriofenone (<2% of the dose)
Plasma	2MDPM (22.5–77.5% of plasma radioactivity)	Pyriofenone (<5% of plasma radioactivity)
Liver	3HDPM or 4HDPM (11.4% of liver radioactivity)	Pyriofenone (8.9% of liver radioactivity)
Kidney	Unidentified polar component (25–42% of kidney radioactivity)	Pyriofenone (7.5–39% of kidney radioactivity)
Fat	Pyriofenone (84.3–94.2% of fat radioactivity)	2MDPM, 3HDPM, 4HDPM (≤5.6% of fat radioactivity, each)

[a] Percentages reflect exposure to 200 mg/kg bw except where otherwise indicated.
Source: Data taken from Knight (2009)

Table 11. Structures of the metabolites of pyriofenone

Common name	Chemical name	Structure
2MDPM	(5-Chloro-2-methoxy-4-methyl-3-pyridyl)(3,4-dihydroxy-2-methoxy-6-methylphenyl)ketone	
3HDPM	(5-Chloro-2-methoxy-4-methyl-3-pyridyl)(3-hydroxy-2,4-dimethoxy-6-methylphenyl)ketone	

Common name	Chemical name	Structure
4HDPM	(5-Chloro-2-methoxy-4-methyl-3-pyridyl)(4-hydroxy-2,3-dimethoxy-6-methylphenyl)methanone	

In the urine of female rats, the major metabolite was an unstable conjugate of 2MDPM (maximum 9.5% of the low dose). There were no metabolites present at greater than 5% of the dose in the urine of male rats at the low dose or of either sex at the high dose. Two major metabolites in bile, each 10–39% of the dose, were identified as glucuronide conjugates of 3HDPM and 4HDPM. Parent pyriofenone accounted for less than 2% of the dose in urine and bile.

Profiles of radioactivity in plasma were similar to those obtained for urine. At the T_{max} time point, the major metabolite in plasma was a glucuronide conjugate of 2MDPM. Pyriofenone and 3HDPM and/or 4HDPM were present in extracts of liver, along with a number of other unidentified metabolites. Pyriofenone was the major identified component in extracts of kidney at 200 mg/kg bw, but was found at a lower concentration than an unidentified polar component. In fat at the high dose, parent pyriofenone was the only major component identified.

The metabolic pathway of pyriofenone in rats is shown in Fig. 3.

2. Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

Pyriofenone did not cause mortality in acute toxicity tests in rats. The lethal dose of pyriofenone was greater than 2000 mg/kg bw in an oral study, greater than 5.18 mg/L in an inhalation study and greater than 2000 mg/kg bw in a dermal study (Table 12).

Clinical signs in intoxicated animals included abnormal body position (oral), clear nasal discharge (inhalation), very slight erythema (dermal) and low body weight gain (dermal).

(b) Dermal irritation

Dermal irritation in rabbits was not observed in a study conducted in compliance with Organisation for Economic Co-operation and Development (OECD) Test Guideline 404. No signs of toxicity or ill-health were observed (Rees, 2008a).

(c) Ocular irritation

Ocular irritation was not observed in rabbits in a study conducted in compliance with OECD Test Guideline 405, except for minor conjunctival redness in two animals 1 hour after administration. The redness decreased after 24 hours and was not observed at 48 or 72 hours after administration (Rees, 2008b).

***Fig. 3. Metabolic pathway of pyriofenone in rats*[a]**

IKF-309

Faeces
Fat

3HDPM

Bile
Liver

4HDPM

Bile
Liver

Conjugates

2MDPM

Conjugates

Urine (female rats)
Plasma

Conjugates

[a] Tissue, fluid or excreta where each chemical is the principal component is indicated. IKF-309 = pyriofenone.
Source: Knight (2009)

Table 12. Acute toxicity of pyriofenone

Route	Species	Strain	Sex	Purity (%)	LD_{50}/LC_{50}	Reference
Oral	Rat	SD	F	97.88	>2 000 mg/kg bw	Moore (2008a)
Inhalation	Rat	SD	M + F	97.88	>5.18 mg/L	Hoffman (2008)
Dermal	Rat	SD	M + F	97.88	>2 000 mg/kg bw	Moore (2008b)

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

(d) Dermal sensitization

Dermal sensitization was not observed in mice in a study conducted in compliance with OECD Test Guideline 429. No signs of toxicity or ill-health were observed (Kosaka, 2009).

2.2 *Short-term studies of toxicity*

(a) Oral administration

Mice

In a 13-week toxicity study, pyriofenone (IKF-309; lot no. 0701; purity 97.88%) was administered to CD-1 mice (10 of each sex per group) in the diet at 0, 300, 1000, 3000 or 7000 parts per million (ppm) (equal to 0, 53, 176, 514 and 1318 mg/kg bw per day for males and 0, 61, 214, 695 and 1504 mg/kg bw per day for females, respectively). End-points included clinical observations (daily), body weight (weekly), feed consumption (weekly), haematology (at termination), serum biochemistry (at termination), gross pathology and histopathology.

There was no mortality during this study. There were no treatment-related effects on body weight, feed consumption, haematology or clinical chemistry. Sporadic, low-magnitude effects without evidence of a dose–response relationship were observed in a few haematology and clinical chemistry parameters, but were not considered toxicologically relevant.

Periportal hypertrophy of the liver was observed in mice treated with 7000 ppm (3/10 males; 7/10 females), and higher relative (to body weight) liver weight was observed in male mice administered 3000 or 7000 ppm and females treated with 1000, 3000 or 7000 ppm (up to a 12% increase relative to controls). The Meeting concluded that these effects were adaptive, not adverse, changes.

The no-observed-adverse-effect level (NOAEL) was 7000 ppm (equal to 1318 mg/kg bw per day), the highest dose tested (Moore, 2009).

Rats

In a 28-day dose range–finding toxicity study, Fischer rats (six of each sex per group) were administered pyriofenone (IKF-309 technical; batch no. 0602; purity 98.04%) in the diet at 0, 300, 3000, 10 000 or 20 000 ppm (equal to 0, 24.2, 251, 823 and 1657 mg/kg bw per day for males and 0, 26.1, 261, 841 and 1660 mg/kg bw per day for females, respectively). End-points included clinical signs (daily), body weights (weekly), feed consumption (weekly), feed efficiency (weekly), haematology (termination), clinical chemistry (termination), urine analysis (week 4), organ weights (termination) and histopathological analysis of the liver and kidney (termination). Ophthalmoscopy was not performed.

There was no mortality during this study. There were no significant effects on body weight, feed intake, feed efficiency or urine analysis.

At the highest dose (20 000 ppm), there were treatment-related effects on haematology and clinical chemistry, including decreased haematocrit, decreased haemoglobin concentration, decreased mean corpuscular volume and an increase in platelet count in both sexes. Additionally, the lymphocyte count was increased in males and the mean corpuscular haemoglobin was decreased in females. In clinical chemistry, there was a significant increase in gamma-glutamyl transferase (GGT), total protein, albumin, globulin and total cholesterol and a significant decrease in chloride in both sexes at the high dose. Additional sex-specific effects included increased blood urea nitrogen (males), albumin/globulin ratio (males) and calcium (males) and decreased triglycerides (males), whereas females exhibited an increase in alanine aminotransferase (ALT).

During necropsy, distended caeca were observed in all rats administered dietary concentrations of 3000 ppm or higher. Dark-coloured livers were reported in males at 10 000 ppm and in both sexes at the highest dose. There were dose-dependent increases in absolute and relative kidney and liver weights in both sexes administered dietary concentrations of 3000 ppm or higher. At 20 000 ppm, males also exhibited increased relative (to body weight) thyroid weights, and diffuse hepatocellular hypertrophy was observed in both sexes. Hyaline droplet deposition was observed in the proximal tubular cells in all males at the highest dose, and increased calcification in the corticomedullary junction was seen in 4/6 females at 20 000 ppm. Similar changes were observed in the 10 000 ppm group.

At 3000 ppm, male rats exhibited increased serum alkaline phosphatase (ALP), total protein and albumin in clinical chemistry. Distended caeca with softened contents were observed in all animals of both sexes. Liver weights (absolute and relative) were increased in male rats, whereas females showed increased relative liver weights and increased relative and absolute kidney weights. Diffuse hepatocellular hypertrophy was seen in one female rat.

No treatment-related effects were noted in the 300 ppm group in either sex.

The NOAEL was 300 ppm (equal to 24.2 mg/kg bw per day), based on increased serum ALP, total protein and albumin (males), distended caeca (both sexes), increased liver weights (both sexes) and increased kidney weights (females) at 3000 ppm (equal to 251 mg/kg bw per day) (Ohtsuka, 2010a).

In a 93-day (13-week) oral toxicity study, pyriofenone (IKF-309 technical; lot no. 0701; purity 97.88%) was administered to Fischer rats (10 of each sex per group) in the diet at 0, 300, 1000, 2500 or 5000 ppm (equal to 0, 17.9, 60.5, 150 and 305 mg/kg bw per day for males and 0, 20.6, 69.0, 171 and 350 mg/kg bw per day for females, respectively). End-points included cage-side observations (daily), detailed clinical signs (weekly), body weight, feed consumption, functional observational battery (FOB) (pretest and at 11 weeks), ophthalmology (pretest and at 13 weeks), urine analysis (pretest and at 13 weeks), haematology (13 weeks), serum biochemistry (13 weeks), gross pathology and histopathology.

Treatment-related effects on haematology (Table 13), clinical chemistry (Table 14), organ weights (Table 15) and histopathology (Table 16) were noted.

Table 13. Summary of haematology after 13 weeks of pyriofenone treatment in rats

Parameter	Sex	0 ppm	300 ppm	1 000 ppm	2 500 ppm	5 000 ppm
Mean cell volume (fL)	Males	47.2	47.1	47.1	46.8	46.3**
	Females	49.0	49.7	49.9	49.6	49.7
Mean cell haemoglobin (pg)	Males	16.6	16.6	16.7	16.5	16.4*
	Females	17.4	17.7	17.9	17.8	17.8
Platelet count (10^3/µL)	Males	865	880	865	868	927**
	Females	904	889	897	914	971
Prothrombin time (s)	Males	22.4	23.2	22.8	25.5	25.9**
	Females	17.4	17.3	16.7	16.9	16.6*
APTT (s)	Males	28.7	28.3	28.0	29.5	30.1
	Females	18.1	18.2	19.2*	19.8**	20.0**
Lymphocyte count (10^3/µL)	Males	4.94	5.66	5.86*	5.51	6.10**
	Females	4.25	4.33	4.71	4.74	4.58

APTT: activated partial thromboplastin time; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Dunnett's test)
Source: Ohtsuka (2010b)

Table 14. Summary of clinical chemistry after 13 weeks of pyriofenone treatment in rats

Parameter	Sex	0 ppm	300 ppm	1 000 ppm	2 500 ppm	5 000 ppm
AST (U/L)	Males	78	75	76	67**	63**
	Females	68	65	67	67	65
ALT (U/L)	Males	48	43*	43**	38**	39**
	Females	32	32	31	33	33
GGT (U/L)	Males	0	0	0	0	1**

Parameter	Sex	0 ppm	300 ppm	1 000 ppm	2 500 ppm	5 000 ppm
	Females	1	1	1	1	2**
Creatinine (mg/dL)	Males	0.27	0.28	0.26	0.24	0.24
	Females	0.34	0.33	0.32	0.32	0.25**
Blood urea nitrogen (mg/dL)	Males	16.1	16.6	16.8	17.3	19.3**
	Females	18.2	17.9	16.8	18.1	18.1
Total protein (g/dL)	Males	6.50	6.52	6.52	6.70**	7.02**
	Females	6.04	6.04	6.02	6.20	6.32**
Albumin (g/dL)	Males	4.47	4.43	4.44	4.58*	4.74**
	Females	4.35	4.34	4.29	4.34	4.46
Globulin (g/dL)	Males	2.03	2.09	2.08	2.13	2.28**
	Females	1.69	1.71	1.73	1.86*	1.86*
Total cholesterol (mg/dL)	Males	42	44	46	43	52**
	Females	64	66	59	65	70
Triglyceride (mg/dL)	Males	41	50	57*	44	36
	Females	10	11	9	10	9
Total bilirubin (mg/dL)	Males	0.04	0.04	0.03	0.03**	0.02**
	Females	0.04	0.04	0.03**	0.02**	0.00**
Calcium (mg/dL)	Males	10.1	10.1	10.2	10.3*	10.5**
	Females	9.6	9.5	9.5	9.6	97
Chloride (mEq/L)	Males	109.6	109.4	108.6	108.1**	107.0**
	Females	111.5	111.8	111.2	110.6	110.0

ALT: alanine aminotransferase; AST: aspartate aminotransferase; Eq: equivalents; GGT: gamma-glutamyl transferase; ppm: parts per million; U: units; *: $P < 0.05$; **: $P < 0.01$ (Dunnett's test)
Source: Ohtsuka (2010b)

Table 15. Summary of organ weights after 13 weeks of pyriofenone treatment in rats

Organ	Sex	Weight type	0 ppm	300 ppm	1 000 ppm	2 500 ppm	5 000 ppm
Liver	Males	Absolute (g)	6.83	7.01	7.22	7.81**	8.26**
		Relative[a] (%)	2.21	2.23	2.30**	2.44**	2.66*
	Females	Absolute (g)	3.83	3.74	3.83	3.96	4.31**
		Relative[a] (%)	2.21	2.19	2.26	2.34**	2.57**
Kidneys	Males	Absolute (g)	1.95	1.97	1.97	2.11**	2.25**
		Relative[a] (%)	0.63	0.63	0.63	0.66*	0.73**
	Females	Absolute (g)	1.16	1.15	1.13	1.18	1.24**
		Relative[a] (%)	0.67	0.67	0.67	0.70	0.74**
Caecum	Males	Absolute (g)	3.57	3.48	3.33	4.62	9.19**
		Relative[a] (%)	1.16	1.11	1.06	1.44	2.97**
	Females	Absolute (g)	2.72	2.82	2.71	3.78**	5.54**
		Relative[a] (%)	1.58	1.65	1.60	2.23**	3.30**

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Dunnett's test)
[a] Relative to body weight.
Source: Ohtsuka (2010b)

Table 16. Summary of treatment-related microscopic changes in the liver and kidney after 13 weeks of pyriofenone treatment in rats

	Males					Females				
Finding	**0 ppm**	**300 ppm**	**1 000 ppm**	**2 500 ppm**	**5 000 ppm**	**0 ppm**	**300 ppm**	**1 000 ppm**	**2 500 ppm**	**5 000 ppm**
Liver: Diffuse hepatocellular hypertrophy	0/10	0/10	0/10	0/10	9/10**	0/10	0/10	0/10	0/10	6/10**
Kidney: Increased hyaline droplet deposition in the proximal tubular cells	0/10	0/10	0/10	0/10	9/10**	0/10	0/10	0/10	0/10	0/10
Kidney: Basophilic change of renal tubular cells	1/10	0/10	2/10	4/10	7/10**	0/10	0/10	0/10	0/10	0/10

ppm: parts per million; **: $P < 0.01$ (Fisher's exact probability test)
Source: Ohtsuka (2010b)

At the highest dose (5000 ppm), treatment-related effects included increased motor activity (males), increased urine volume (males), yellow-brown-coloured urine (females), increased platelet (males), lymphocyte (males) and basophil counts (males), prolonged activated partial thromboplastin time (APTT) (females), increased GGT activity, serum total protein and globulin (both sexes) and decreased serum chloride (males). Distended caeca were observed in 10/10 males and 5/10 females. Absolute and relative (to body weight) organ weights were increased for liver, kidneys and caecum (both sexes). Diffuse hepatocellular hypertrophy was observed in 9/10 males and 6/10 females, and males showed an increased incidence of hyaline droplet deposition in the proximal tubular cells (9/10) and basophilic change of renal tubular cells (7/10).

At 2500 ppm, similar, but less severe, effects were seen in serum biochemistry, haematology and gross pathology. Specifically, prolonged APTT (females), increased serum total protein (males), serum albumin (males) and serum calcium (males) and decreased serum chloride (males) were observed. Increased absolute and relative kidney weights (males), absolute liver weight (males) and relative liver weight (both sexes) and absolute and relative caecum weights (females) were observed.

At 1000 ppm, there was a slight increase in relative liver weight (4%) in males and an increase in APTT in females. No other treatment-related effects were observed. There were no treatment-related effects at 300 ppm in either sex.

The NOAEL was 1000 ppm (equal to 60.5 mg/kg bw per day), based on increased liver weights in both sexes, increased caecum weights in females, increased kidney weights in males, prolonged APTT in females, increased serum total protein, albumin and calcium in males and decreased serum chloride in males at 2500 ppm (equal to 150 mg/kg bw per day) (Ohtsuka, 2010b).

Dogs

In a 90-day oral toxicity study, pyriofenone (IKF-309 technical; lot no. 0701; purity 97.88%) was administered to beagle dogs (four of each sex per group) in the diet at 0, 500, 3000 or 25 000 ppm (equal to 0, 15.0, 90.3 and 776 mg/kg bw per day, respectively) for males and at 0, 500, 3000 or 15 000 ppm (equal to 0, 15.3, 89.8 and 475 mg/kg bw per day, respectively) for females. End-points included clinical observations (daily), FOB (pretest and weekly thereafter), feed consumption (daily), water consumption (daily), body weight (weekly), ophthalmoscopy (pretest and week 13), haematology (pretest and weeks 7 and 13), serum biochemistry (pretest and weeks 7 and 13), urine analysis (pretest and weeks 7 and 13), gross pathology and histopathology.

There were no effects on mortality, clinical signs, body weight, urine analysis, haematology or gross pathology in any treated group.

At the high dose, treatment-related effects included prolonged APTT (females), increased ALP and triglycerides (both sexes), increased liver weight (both sexes), decreased spleen weight and increased incidence of centrilobular hepatocellular hypertrophy (both sexes) (Table 17). No effects of toxicological relevance were observed at any other doses.

Table 17. Summary of key findings in a 90-day oral toxicity study in dogs administered pyriofenone

Finding	0 ppm	500 ppm	3 000 ppm	15 000 ppm	25 000 ppm
APTT (s)					
Males					
Pretreatment	10.0	10.5	9.7	–	10.3
Week 7	10.0	10.6	9.2	–	9.4
Week 13	9.9	10.3	9.1*	–	9.3
Females					
Pretreatment	9.9	10.1	10.1	9.9	–
Week 7	10.0	10.0	9.5	9.1*	–
Week 13	9.9	11.1	9.6	8.9*	–
ALP (U/L)					
Males					
Pretreatment	417	353	357	–	332
Week 7	342	283	351	–	1 605
Week 13	287	279	297	–	1 673
Females					
Pretreatment	290	331	374	310	–
Week 7	238	314	486	944**	–
Week 13	226	278	492	1 111**	–
Triglycerides (mg/dL)					
Males					
Pretreatment	20	16	20	–	20
Week 7	21	14	20	–	33*
Week 13	25	20	19	–	46**
Females					
Pretreatment	16	23	22	17	–
Week 7	17	19	19	25	–
Week 13	21	23	21	26	–
Liver weight					
Males					
Absolute (g)	282	280	285	–	373**
Relative[a] (%)	2.69	2.67	2.72	–	3.77**
Females					
Absolute (g)	267	276	305	328	–
Relative[a] (%)	2.66	2.72	3.01	3.34	–

Finding	0 ppm	500 ppm	3 000 ppm	15 000 ppm	25 000 ppm
Spleen weight					
Males					
Absolute (g)	29.0	23.2	20.7**	–	24.2
Relative[a] (%)	0.28	0.22*	0.20**	–	0.25
Females					
Absolute (g)	24.8	27.8	28.1	17.8	–
Relative[a] (%)	0.25	0.27	0.28	0.18*	–
Liver histopathology					
Centrilobular hepatocellular hypertrophy					
Males	0/4	0/4	0/4	–	3/4
Females	0/4	0/4	0/4	3/4	–

–: not applicable; ALP: alkaline phosphatase; APTT: activated partial thromboplastin time; ppm: parts per million; U: units; *: $P < 0.05$; **: $P < 0.01$ (Dunnett's test or Fisher's exact probability test)

[a] Relative to body weight.

Source: Nakashima (2010a)

The NOAEL was 3000 ppm (equal to 89.8 mg/kg bw per day), based on markedly increased ALP in females at 15 000 ppm (equal to 475 mg/kg bw per day) (Nakashima, 2010a).

In a 1-year oral toxicity study, pyriofenone (IKF-309 technical; lot no. 0701; purity 97.88%) was administered to beagle dogs (four of each sex per group) at 0, 500, 3000 or 25 000 ppm (equal to 0, 13.7, 83.5 and 701 mg/kg bw per day, respectively) for males and at 0, 500, 3000 or 15 000 ppm (equal to 0, 14.1, 86.2 and 448 mg/kg bw per day, respectively) for females. End-points included clinical observations (daily), FOB (pretest and weekly thereafter), feed consumption (daily), water consumption (daily), body weight (pretest, weekly through week 13 and every 4 weeks from weeks 16 to 52), ophthalmoscopy (pretest and weeks 13, 26 and 52), haematology (pretest and weeks 13, 26 and 52), serum biochemistry (pretest and weeks 13, 26 and 52), urine analysis (pretest and weeks 13, 26 and 52), gross pathology and histopathology.

No treatment-related effects on mortality or ophthalmoscopy were observed.

Treatment-related effects on haematology (Table 18), clinical chemistry (Table 19) and organ weights (Table 20) were observed.

Table 18. Summary of haematological findings in a 52-week oral toxicity study with pyriofenone in dogs

	0 ppm	500 ppm	3 000 ppm	15 000 ppm	25 000 ppm
Haematocrit (%)					
Males					
Pretreatment	43.3	44.9	44.7	–	42.6
Week 13	47.5	49.3	49.6	–	45.4
Week 26	48.5	48.5	50.2	–	43.6**
Week 52	49.2	50.2	50.3	–	45.2
Females					

	0 ppm	500 ppm	3 000 ppm	15 000 ppm	25 000 ppm
Pretreatment	45.2	46.7	42.8	47.2	–
Week 13	47.9	50.5	48.1	52.7**	–
Week 26	47.8	49.6	48.6	51.6	–
Week 52	47.9	50.1	49.3	49.8	–
Haemoglobin concentration (g/dL)					
Males					
Pretreatment	14.4	15.0	15.1	–	14.1
Week 13	16.0	16.7	16.9	–	15.3
Week 26	16.6	16.7	17.6	–	15.1**
Week 52	17.3	17.8	18.2	–	16.1
Females					
Pretreatment	14.9	15.7	14.2	15.7	–
Week 13	16.1	16.9	16.2	17.8*	–
Week 26	16.1	16.8	16.5	17.7*	–
Week 52	16.6	17.5	17.3	17.7	–
Erythrocyte count (10^6/µL)					
Males					
Pretreatment	6.35	6.65	6.68	–	6.34
Week 13	7.30	7.56	7.60	–	6.88
Week 26	7.56	7.59	7.84	–	6.71**
Week 52	7.69	7.89	7.90	–	7.04
Females					
Pretreatment	6.71	6.86	6.14	6.90	–
Week 13	7.26	7.59	7.05	7.71	–
Week 26	7.34	7.63	7.27	7.71	–
Week 52	7.46	7.81	7.45	7.49	–
Mean cell haemoglobin (pg)					
Males					
Pretreatment	22.7	22.6	22.7	–	22.1
Week 13	21.9	22.1	22.3	–	22.3
Week 26	22.0	22.0	22.4	–	22.5
Week 52	22.5	22.6	23.1	–	22.9
Females					
Pretreatment	22.2	22.9	23.1	22.8	–
Week 13	22.2	22.3	22.9	23.1	–
Week 26	22.0	22.0	22.7	23.0*	–
Week 52	22.3	22.5	23.3	23.7*	–
APTT (s)					
Males					
Pretreatment	10.2	10.2	10.3	–	9.9
Week 13	9.9	9.6	9.7	–	8.6**
Week 26	9.8	9.6	9.5	–	8.6*
Week 52	10.0	9.7	9.7	–	8.8**
Females					

	0 ppm	500 ppm	3 000 ppm	15 000 ppm	25 000 ppm
Pretreatment	10.0	9.7	10.0	9.9	–
Week 13	10.2	10.0	9.8	9.7	–
Week 26	9.8	9.7	9.4	8.9*	–
Week 52	9.9	9.9	9.5	9.4	–

–: not applicable; APTT: activated partial thromboplastin time; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Dunnett's test)
Source: Nakashima (2010b)

Table 19. Summary of clinical chemistry findings in a 52-week oral toxicity study with pyriofenone in dogs

	0 ppm	500 ppm	3 000 ppm	15 000 ppm	25 000 ppm
ALP (U/L)					
Males					
Pretreatment	557	438	518	–	430
Week 13	344	330	618	–	1 982*
Week 26	230	265	501	–	2 294**
Week 52	200	229	505	–	2 248**
Females					
Pretreatment	391	454	494	469	–
Week 13	262	299	435	1 636*	–
Week 26	225	305	401	1 749**	–
Week 52	197	307	443	1 746**	–
GGT (U/L)					
Males					
Pretreatment	4	3	4	–	3
Week 13	4	3	4	–	6
Week 26	3	3	5	–	6*
Week 52	3	3	5	–	7*
Females					
Pretreatment	3	3	3	3	–
Week 13	3	3	3	4	–
Week 26	3	3	3	4*	–
Week 52	3	4	3	4	–
Creatinine (mg/dL)					
Males					
Pretreatment	0.51	0.51	0.46	–	0.49
Week 13	0.63	0.66	0.60	–	0.60
Week 26	0.72	0.71	0.65	–	0.60
Week 52	0.74	0.71	0.66	–	0.62*
Females					
Pretreatment	0.47	0.52	0.50	0.47	–
Week 13	0.53	0.62*	0.58	0.59	–
Week 26	0.56	0.63	0.62	0.61	–
Week 52	0.54	0.62	0.58	0.54	–

–: not applicable; ALP: alkaline phosphatase; GGT: gamma-glutamyl transferase; ppm: parts per million; U: units;
*: $P < 0.05$; **: $P < 0.01$ (Dunnett's test)
Source: Nakashima (2010b)

Table 20. Summary of organ weight findings in a 52-week oral toxicity study with pyriofenone in dogs

Organ	Sex	Weight type	0 ppm	500 ppm	3 000 ppm	15 000 ppm	25 000 ppm
Liver	Male	Absolute (g)	291	302	313	–	416*
		Relative[a] (%)	2.16	2.63	2.78	–	4.02**
	Female	Absolute (g)	310	300	321	374	–
		Relative[a] (%)	2.65	2.59	2.90	3.52*	–
Kidney	Male	Absolute (g)	45.9	43.9	49.9	–	45.5
		Relative[a] (%)	0.34	0.38	0.44**	–	0.44**
	Female	Absolute (g)	49.3	42.4	44.8	46.8	–
		Relative[a] (%)	0.42	0.37	0.41	0.44	–

–: not applicable; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Dunnett's test)
[a] Relative to body weight.
Source: Nakashima (2010b)

At the high dose, dogs exhibited vomiting (both sexes), loose stools (both sexes), decreased body weight gain (males), decreased feed consumption (both sexes), low urinary pH (males), decreased haematocrit (males), decreased haemoglobin concentration (males), decreased erythrocyte count (males), increased ALP (both sexes), increased GGT (both sexes), increased relative liver weight (both sexes), increased relative kidney weight (males; 129%), darkened liver colour (both sexes) and centrilobular hepatocellular hypertrophy (males). Of these effects, the haematological findings, clinical chemistry findings and darkened liver colour were considered toxicologically relevant.

At the intermediate dose (3000 ppm), male dogs exhibited increased relative kidney weight (129%). No treatment-related effects were observed in low-dose dogs. In the absence of other renal effects, the change in kidney weight was not considered adverse.

The NOAEL was 3000 ppm (equal to 83.5 mg/kg bw per day), based on markedly increased ALP, increased GGT and darkened liver colour at 15 000 ppm (equal to 448 mg/kg bw per day) (Nakashima, 2010b).

(b) Dermal application

In a 4-week toxicity study, CD rats (10 of each sex per group) were administered pyriofenone (batch no. 0701; purity 97.88%) at 0, 100, 300 or 1000 mg/kg bw per day by dermal occlusion. A control group was sham dosed with purified water and subjected to the same occlusion conditions as the treated animals. The dose preparations were applied for 6 hours per day for 28 days. End-points included clinical observations (daily), examination of application site (before each administration), ophthalmoscopy (pre-exposure and at week 4), body weights (weekly), feed consumption (weekly), haematology and clinical chemistry (week 4), macroscopic pathological assessment (at necropsy) and microscopic histopathological assessment (after necropsy). Urine analysis was not performed.

There were no significant findings in clinical signs, body weight, feed consumption, ophthalmoscopy, haematology, clinical chemistry, organ weights, macroscopic pathological assessment or microscopic histopathological assessment. There were occasional differences in the haematological parameters that reached statistical significance, but these were not considered biologically meaningful.

Pyriofenone did not induce any signs of systemic toxicity after dermal exposure, and it did not induce any signs of skin irritation or sensitivity (Cooper, 2010).

2.3 Long-term studies of toxicity and carcinogenicity

Mice

In a 78-week carcinogenicity study, pyriofenone (IKF-309 technical; batch no. 0701; purity 97.88%) was administered in the diet to Crl:CD-1 mice (52 of each sex per group) at 0, 600, 1800 or 5400 ppm (equal to 0, 77.6, 237 and 716 mg/kg bw per day, respectively) for males and 0, 300, 1000 or 3000 ppm (equal to 0, 49.4, 167 and 486 mg/kg bw per day, respectively) for females. End-points included clinical signs, body weight, feed consumption, haematology (leukocyte counts only), organ weights, gross pathology and histopathology.

No compound-related effects on mortality, palpable masses or leukocyte counts were noted. Survival ranged from 67% to 79%.

Treatment-related effects on liver weights (Table 21) and on neoplastic (Table 22) and non-neoplastic lesions (Table 23) were observed.

Female mice in the high-dose group exhibited reduced body weight (17% reduction). Liver and kidney effects were seen in treated mice. Increases in hepatocellular adenomas and adenocarcinomas were observed, with significance for the combined incidence reached at 5400 ppm (males only). Centrilobular hepatocytic hypertrophy and necrosis of individual hepatocytes were seen in treated male mice. Slight hepatocytic hypertrophy was seen occasionally in female mice.

In the kidney, there was an increased incidence of granular appearance in male mice in the 1800 and 5400 ppm groups and in female mice in the 1000 and 3000 ppm groups. There were an increased incidence and an increased severity of degenerative and regenerative changes (cortical tubular basophilia, cortical scarring and cortical cysts) in mid- and high-dose males. These lesions may have been the cause of increased yellow perigenital staining in male mice. In female mice given 3000 ppm, there was an increase in the incidence of chronic progressive nephropathy (moderate or marked severity), but overall incidence remained low and did not reach statistical significance (maximum of five affected female mice at 3000 ppm). Moderate or marked glomerulonephritis was observed in female mice given 1000 ppm, but occurred in only 3/52 animals and did not occur in any mice at 3000/5400 ppm (Moore, 2010a).

The NOAEL for systemic toxicity was 600 ppm (equal to 77.6 mg/kg bw per day), based on liver and kidney histopathology (hepatocellular hypertrophy and renal cortical tubular basophilia) and an increased incidence of granular appearance of the kidneys in males exposed to 1800 ppm (equal to 237 mg/kg bw per day).

The NOAEL for carcinogenicity was 1800 ppm (equal to 237 mg/kg bw per day), based on an increased incidence of combined hepatocellular adenomas and adenocarcinomas in males at 5400 ppm (equal to 716 mg/kg bw per day) (Moore, 2010a).

Table 21. Summary of liver weights after treatment of mice with pyriofenone for 78 weeks

	Males				**Females**			
	0 ppm	**600 ppm**	**1 800 ppm**	**5 400 ppm**	**0 ppm**	**300 ppm**	**1 000 ppm**	**3 000 ppm**
Number of animals examined	40	37	41	37	35	40	39	40
Terminal body weight (g)	55.6	57.8	56.7	57.4	52.0	50.5	51.1	46.5*
Absolute liver weight (g)	2.682	2.975	2.930	3.141**	2.379	2.364	2.397	2.232
Relative (to body weight) liver weight (%)	4.82	5.15	5.17	5.47**	4.58	4.68	4.69	4.80*

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Fisher's exact probability test)
Source: Moore (2010a)

Table 22. Summary of neoplastic changes in the liver after treatment of mice with pyriofenone for 78 weeks

	Incidence of neoplastic changes									
	Males					**Females**				
Finding	**0 ppm**	**600 ppm**	**1 800 ppm**	**5 400 ppm**	**HC[a]**	**0 ppm**	**300 ppm**	**1 000 ppm**	**3 000 ppm**	**HC[a]**
Number of animals examined	52	52	52	52	–	52	52	52	52	–
Hepatocellular adenoma	3	7	6	9	15.8% (7.8–26.0%)	1	0	1	2	1.07% (0–3.9%)
Hepatocellular adenocarcinoma	1	2	3	3	3.73% (0–10.0%)	0	0	0	0	0.36% (0–3.9%)
Adenoma/adenocarcinoma combined	4	9	9	12*#	18.5% (9.8–32.0%)	1	0	1	2	1.42% (0–5.0%)

HC: historical controls; ppm: parts per million; *: $P < 0.05$ (one-tailed pairwise comparison against controls); #: $P < 0.05$ (one-tailed trend test)

[a] Historical control incidence (mean and range provided). Based on 11 78-week studies in mice conducted by the contract laboratory between 1997 and 2007, with 50–60 mice per study.

Source: Moore (2010a)

Table 23. Summary of non-neoplastic changes in the liver and kidney after treatment of mice with pyriofenone for 78 weeks

	Incidence of non-neoplastic changes							
	Males				Females			
Finding	**0 ppm**	**600 ppm**	**1 800 ppm**	**5 400 ppm**	**0 ppm**	**300 ppm**	**1 000 ppm**	**3 000 ppm**
Number of animals examined	52	52	52	52	52	52	52	52
Liver								
Hepatocellular hypertrophy (centrilobular)								
Slight	0	11	13	10	0	1	2	3
Moderate	0	2	3	2	0	0	0	0
Total	0	13**	16**	12**	0	1	2	3
Necrosis of individual hepatocytes								
Minimal	0	4	3	3	0	0	0	2
Slight	1	2	5	4	0	1	0	0
Total	1	6	8*	7*	0	1	0	2
Basophilic foci								
Minimal	0	0	3	1	0	0	0	0
Slight	0	1	0	3	0	0	0	0
Total	0	1	3	4	0	0	0	0
Eosinophilic foci								
Minimal	0	1	0	3	1	0	0	0
Slight	0	0	1	1	0	0	0	0
Total	0	1	1	4	1	0	0	0
Pigment in macrophages	4	0	3	2	8	11	5	18*
Kidney								
Cortical tubular basophilia								
Minimal	32	28	30	17	18	15	16	27
Slight	4	12	15	24	8	2	4	2
Moderate	1	2	2	6	2	0	1	0
Total	37	42	47*	47*	28	17	21	29
Cortical scarring								
Minimal	14	15	11	18	6	10	9	9
Slight	2	2	9	10	5	2	1	1
Moderate	0	0	1	0	0	0	0	0
Total	16	17	21	28*	11	12	10	10
Cortical cysts								
Minimal	3	3	3	1	0	1	0	0
Slight	3	2	9	9	0	1	1	1
Moderate	1	1	0	2	0	0	0	0
Total	7	6	12	12	0	2	1	1

ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Fisher's exact probability test)
Source: Moore (2010a)

In a follow-up study, mice were administered pyriofenone (IKF-309; lot no. 0701; purity 97.88%) in the diet to evaluate its effects on enzyme induction and hepatocyte proliferation. This study aimed to provide further mechanistic information regarding the hepatocyte tumour formation observed in Moore (2010a). Two groups of male mice (12 per group) were exposed to pyriofenone in the diet at 5000 or 10 000 ppm (equal to 854 and 1714 mg/kg bw per day, respectively), whereas a control group received untreated diet. End-points included clinical signs, body weight, feed consumption, liver weight, hepatic enzyme assessment (microsomal protein concentration, cytochrome P450 [CYP] concentration, 7-ethoxyresorufin *O*-deethylase activity, testosterone 16α-, 16β- and 6β-hydroxylase activities, testosterone 17β-dehydrogenase activity, and lauric acid 11- and 12-hydroxylase activities), macropathology and histopathology for proliferating cell nuclear antigen (PCNA) as a marker for liver cell proliferation.

There were no effects on mortality, clinical signs, body weight gain or feed consumption.

There was a dose-dependent increase in relative liver weight (up to 180%), but no gross lesions were observed in the liver. There was an increase in cytochrome P450 concentration (150%) at both doses, increased activity (120%) of 7-ethoxyresorufin *O*-deethylase (CYP1A marker) at both doses, decreased activity (40%) of testosterone 17β-dehydrogenase at both doses and increased activity (120%) of testosterone 16α-hydroxylase (CYP2 marker) at the high dose.[1] There was no evidence that pyriofenone induced or suppressed hepatic cytochrome P450s. There was no evidence that pyriofenone increased the rate of hepatocyte proliferation (Table 24).

Table 24. Summary of hepatocyte liver enzymes after 28 days of treatment of mice with pyriofenone[a]

Parameter	5 000 ppm	10 000 ppm
Microsomal protein (mg/g liver)	0.9	1.0
Cytochrome P450		
nmol/mg protein per minute	1.4**	1.6**
nmol/g liver per minute	1.3**	1.5**
7-Ethoxyresorufin *O*-deethylase (CYP1A marker)		
nmol/mg protein	1.5**	1.4**
nmol/g liver	1.4*	1.4*
Testosterone 6β-hydroxylase (CYP3A marker)		
nmol/mg protein per minute	1.0	1.1
nmol/g liver per minute	0.9	1.1
Testosterone 16α-hydroxylase (CYP2 marker)		
nmol/mg protein per minute	1.1	1.2*
nmol/g liver per minute	1.0	1.2
Testosterone 16β-hydroxylase (CYP2 marker)		
nmol/mg protein per minute	b	b*
nmol/g liver per minute	b	b

[1] Of the four subgroups of pooled livers from the vehicle control group examined, only one gave a testosterone 16β-hydroxylase (CYP2 marker activity) above the level of quantification (0.175 nmol/mg protein per minute) for the assay. All four subgroups of both treated groups showed quantifiable activity. When analysed statistically, the activity expressed per gram of liver in the 10 000 ppm group was significantly greater ($P < 0.05$) than the control group activity. Owing to the prevalence of non-detects in the control group, it is not possible to accurately determine the fold increase of activity in the treated groups.

Parameter	5 000 ppm	10 000 ppm
Testosterone 17β-dehydrogenase (CYP2 marker)		
nmol/mg protein per minute	0.6**	0.6**
nmol/g liver per minute	0.5**	0.6**
Lauric acid 11-hydroxylase (CYP2E marker)		
nmol/mg protein per minute	1.1	1.0
nmol/g liver per minute	1.0	1.0
Lauric acid 12-hydroxylase (CYP4A marker)		
nmol/mg protein per minute	1.1	0.9
nmol/g liver per minute	1.0	0.9

CYP: cytochrome P450; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$

[a] Values shown are normalized to control.

[b] CYP2B activity was quantifiable in all subgroups of the pyriofenone-treated groups, but in only one subgroup of the control group. Values below the limit of quantification were taken as 0 in the calculation of the control group mean, which was then below the limit of quantification.

Source: Arrowsmith (2010a)

The author (Arrowsmith, 2010a) concluded that pyriofenone caused only a minor induction of some cytochrome P450 isoforms (CYP1A and CYP2) and had no effect on other isoforms (CYP3A, CYP2E and CYP4A). There was no evidence that pyriofenone increased the rate of cell proliferation in the liver. The minor increases in CYP1A and CYP2 activities were not considered relevant to the increased hepatic tumours seen in Moore (2010a).

Rats

In a 1-year (52-week) oral toxicity study, pyriofenone (IKF-309 technical; lot no. 0701; purity 97.88%) was administered to Fischer rats (20 of each sex per group) in the diet at 0, 200, 1000 or 5000 ppm (equal to 0, 8.51, 42.9 and 226 mg/kg bw per day for males and 0, 10.6, 53.5 and 275 mg/kg bw per day for females, respectively). End-points included cage-side observations (daily), detailed clinical signs (weekly), body weight, feed consumption, FOB (pretest and at 49 weeks), ophthalmology (pretest and at 52 weeks), urine analysis (pretest and at 13, 25 and 51 weeks), haematology (pretest and at 14, 26 and 52 weeks), serum biochemistry (pretest and at 14, 26 and 52 weeks), gross pathology and histopathology.

There were no treatment-related effects on mortality. One high-dose male and one low-dose male were found dead at weeks 40 and 46, respectively. There were no unscheduled deaths in females at any dose. There were no effects on feed efficiency, ophthalmoscopy or functional observations.

Treatment-related effects on haematology (Table 25), clinical chemistry (Table 26), organ weights (Table 27) and histopathology (Table 28) were observed.

Female rats at the high dose (5000 ppm) exhibited increased incidences of soiled fur and decreased body weight (89% of control). At the high dose, rats of both sexes exhibited decreased haematocrit, decreased haemoglobin concentration and decreased red blood cell count, accompanied by occasional decreases in mean corpuscular volume (males), mean corpuscular haemoglobin concentration (males), haemoglobin width (males) and red cell distribution width (both sexes). APTT was increased in both sexes. In clinical chemistry, there were increases in GGT, ALP, AST and ALT (both sexes) and increased total cholesterol (both sexes). In addition, sex-specific effects at the high dose included increased urine volume (males), increased urinary ketones (females) and increased blood urea nitrogen (males).

Table 25. Summary of haematology in a 52-week toxicity study with pyriofenone in rats

Parameter	Males				Females			
	0 ppm	200 ppm	1 000 ppm	5 000 ppm	0 ppm	200 ppm	1 000 ppm	5 000 ppm
Haematocrit (%)								
Week 14	46.7	46.1	46.5	45.2**	45.9	45.9	45.8	44.5*
Week 26	45.1	44.8	45.6	43.5**	45.4	45.1	45.1	43.6**
Week 52	46.3	46.3	46.1	41.7**	47.2	46.5	46.9	45.6**
Haemoglobin conc. (g/dL)								
Week 14	16.4	16.2	16.4	15.8**	16.4	16.5	16.4	15.9*
Week 26	16.2	16.1	16.4	15.6**	16.5	16.3	16.3	15.9**
Week 52	15.4	15.4	15.4	13.3**	16.2	15.9**	16.0	15.7**
Haemoglobin width (g/dL)								
Week 14	2.80	2.81	2.80	2.80	2.42	2.43	2.43	2.45
Week 26	2.65	2.60	2.65	2.63	2.19	2.15	2.18	2.22
Week 52	2.52	2.49	2.51	2.43**	2.21	2.22	2.21	2.21
RBC count (10^6/µL)								
Week 14	9.55	9.47	9.51	9.42	8.97	8.98	8.93	8.65**
Week 26	9.52	9.35	9.55	9.28*	8.84	8.75	8.82	8.57**
Week 52	9.34	9.27	9.26	8.72**	8.80	8.64	8.74	8.58**
Red cell distribution width (g/dL)								
Week 14	13.9	13.9	14.0	13.7	12.4	12.3	12.3	12.3
Week 26	14.1	14.0	13.8*	14.2	12.4	12.3	12.3	12.3
Week 52	12.9	12.7	12.9	13.3	11.7	11.7	11.7	11.3**
MCV (pg)								
Week 14	48.9	48.7	48.8	48.0*	51.1	51.1	51.3	51.4
Week 26	47.4	47.9	47.8	46.9	51.3	51.6	51.1	50.8
Week 52	49.6	49.9	49.8	47.8	53.6	53.8	53.7	53.2
MCH (pg)								
Week 14	17.1	17.1	17.3	16.8**	18.3	18.4	18.4	18.4
Week 26	17.0	17.2	17.2	16.8*	18.7	18.6	18.6	18.6
Week 52	16.5	16.6	16.6	15.2	18.5	18.4	18.4	18.3
MCHC (g/dL)								
Week 14	35.1	35.3	35.4	35.0	35.8	36.0	35.8	35.8
Week 26	35.9	36.0	35.9	35.7	36.5	36.2	36.3	36.5
Week 52	33.2	33.3	33.3	31.6*	34.4	34.2	34.2	34.3
Reticulocytes (10^9/L)								
Week 14	168.8	156.3	154.4	158.5	154.6	135.9	139.6	132.0
Week 26	201.7	190.6	182.1	200.7	178.6	172.6	176.8	159.3
Week 52	162	158	163	210	167	164	171	139**
Platelet count (10^3/µL)								
Week 14	774	755	762	824**	829	775	794	849
Week 26	776	750	741	837	722	717	723	790**

Parameter	Males 0 ppm	Males 200 ppm	Males 1 000 ppm	Males 5 000 ppm	Females 0 ppm	Females 200 ppm	Females 1 000 ppm	Females 5 000 ppm
Week 52	907	876	877	1 128	860	840	848	897
Prothrombin time (s)								
Week 52	19.1	19.0	19.6	21.9**	18.4	18.1	17.8*	17.0**
APTT (s)								
Week 52	22.9	23.0	23.5	24.6**	16.5	17.0	17.0	18.3**

APTT: activated partial thromboplastin time; conc.: concentration; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; MCV: mean corpuscular volume; ppm: parts per million; RBC: red blood cell (erythrocyte); *: $P < 0.05$; **: $P < 0.01$ (Dunnett's test)
Source: Ohtsuka (2010c)

Table 26. Summary of clinical chemistry in a 52-week toxicity study with pyriofenone in rats

Parameter	Males 0 ppm	Males 200 ppm	Males 1 000 ppm	Males 5 000 ppm	Females 0 ppm	Females 200 ppm	Females 1 000 ppm	Females 5 000 ppm
ALP (U/L)								
Week 14	448	424	452	393**	332	306	299	267**
Week 26	393	378	373	354**	264	257	269	208**
Week 52	387	347*	321**	275**	214	194	191	143**
AST (U/L)								
Week 14	93	82	76	65**	73	72	72	66*
Week 26	113	87**	91*	68**	88	93	79	63**
Week 52	99	98	94	65**	97	115	83	58**
ALT (U/L)								
Week 14	50	46	43	44	31	31	32	34*
Week 26	63	51	51*	46**	46	49	42	36
Week 52	74	70	63	41**	54	66	48	36**
GGT (U/L)								
Week 14	1	1	1	2*	1	1	1	2**
Week 26	1	1	0	1	1	2	2*	3**
Week 52	3	3	2	2**	2	3	3	2
Creatinine (mg/dL)								
Week 14	0.42	0.40	0.39	0.36**	0.38	0.36	0.37	0.31**
Week 26	0.42	0.41	0.40	0.39	0.42	0.43	0.38	0.31**
Week 52	0.34	0.33	0.33	0.29**	0.33	0.32	0.31	0.24**
Blood urea nitrogen (mg/dL)								
Week 14	16.6	15.7	16.9	20.8**	16.7	16.7	16.8	17.3
Week 26	18.6	18.4	18.6	23.2**	20.4	20.3	19.5	20.7
Week 52	15.7	15.0	15.1	16.7	18.8	17.7	18.2	19.6
Total protein (g/L)								
Week 14	6.46	6.38	6.55	7.22**	6.45	6.51	6.46	6.87**
Week 26	6.83	6.78	6.79	7.24**	6.68	6.67	6.73	7.21**

Parameter	Males				Females			
	0 ppm	200 ppm	1 000 ppm	5 000 ppm	0 ppm	200 ppm	1 000 ppm	5 000 ppm
Week 52	6.78	6.68	6.72	6.48	6.87	7.00	7.02	7.45**
Albumin (g/L)								
Week 14	4.28	4.20	4.36	4.83**	4.48	4.57	4.53	4.71**
Week 26	4.44	4.36	4.43	4.78**	4.72	4.71	4.71	4.93**
Week 52	4.26	4.18	4.22	4.17	4.83	4.90	4.92	5.17**
Globulin (g/L)								
Week 14	2.19	2.18	2.19	2.39**	1.98	1.94	1.93	2.16**
Week 26	2.39	2.42	2.35	2.46	1.96	1.96	2.02	2.28**
Week 52	2.51	2.50	2.50	2.31*	2.04	2.10	2.10	2.29**
Albumin/globulin ratio								
Week 14	1.96	1.93	1.99	2.02	2.28	2.36	2.35	2.18
Week 26	1.86	1.81	1.89	1.95	2.41	2.41	2.34	2.17**
Week 52	1.70	1.67	1.70	1.81	2.04	2.10	2.35	2.27
Total cholesterol (mg/dL)								
Week 14	48	48	47	62**	58	63	64	86**
Week 26	65	66	61	75**	88	90	91	111**
Week 52	77	77	69	63*	107	108	108	133**
Triglycerides (mg/dL)								
Week 14	66	60	56	24**	29	26	25	20
Week 26	81	80	79	59*	43	34	32*	30*
Week 52	57	55	48	45	58	59	51	43
Total bilirubin (mg/dL)								
Week 14	0.05	0.04	0.04*	0.01**	0.06	0.06	0.04*	0.02**
Week 26	0.06	0.05	0.05*	0.01**	0.07	0.07	0.05*	0.01**
Week 52	0.06	0.06	0.05**	0.01**	0.06	0.06	0.05*	0.01**
Calcium (mg/dL)								
Week 14	9.8	9.8	9.9	10.5**	9.6	9.6	9.5	9.9**
Week 26	10.1	10.1	10.1	10.5**	9.8	9.7	9.7	10.1**
Week 52	10.0	10.0	10.1	10.1	10.1	10.1	10.0	10.6**
Phosphorus (mg/dL)								
Week 14	5.4	5.8	5.3	5.9*	4.7	5.0	4.8	5.1
Week 26	4.5	4.4	4.4	4.9	3.8	3.9	3.4	4.0
Week 52	4.0	4.2	4.2	4.6**	4.0	3.9	3.6	4.6
Chloride (mEq/L)								
Week 14	107.7	107.3	107.2	106.4**	108.4	108.2	108.5	106.9**
Week 26	106.6	106.8	106.5	105.3**	107.0	106.8	107.5	105.5*
Week 52	108.6	109.0	108.6	107.9	109.8	110.1	110.3	107.7**

ALP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; Eq: equivalents; GGT: gamma-glutamyl transferase; ppm: parts per million; U: units; *: $P < 0.05$; **: $P < 0.01$ (Dunnett's test)
Source: Ohtsuka (2010c)

Table 27. Summary of organ weights in a 52-week toxicity study with pyriofenone in rats

Organ	Weight type	Males				Females			
		0 ppm	200 ppm	1 000 ppm	5 000 ppm	0 ppm	200 ppm	1 000 ppm	5 000 ppm
Brain	A (mg)	2 058	2 027	2 059	2 062	1 849	1 890*	1 855	1 861
	R-b (%)	0.50	0.51	0.50	0.52	0.87	0.87	0.89	0.95**
Heart	A (mg)	1 069	1 018	1 106	1 072	659	696*	673	691*
	R-b (%)	0.26	0.26	0.27	0.27	0.31	0.32	0.32	0.35**
Liver	A (g)	8.19	7.88	8.54	9.46**	4.34	4.51	4.48	5.06**
	R-b (%)	1.99	1.98	2.05	2.38**	2.03	2.08	2.15	2.58**
Kidneys	A (g)	2.227	2.217	2.370	2.570**	1.339	1.397	1.365	1.494**
	R-b (%)	0.54	0.56	0.57	0.65**	0.63	0.65	0.66	0.76**
Adrenals	A (mg)	41.1	40.8	40.0	45.7	50.6	51.2	50.9	52.5
	R-b (%)	0.010	0.010	0.010	0.012**	0.024	0.023	0.025	0.027**
Testes	A (mg)	3 210	3 085	3 299	3 279	–			
	R-b (%)	0.78	0.77	0.80	0.82*				
Epididymides	A (mg)	950	896	991	1 008*	–			
	R-b (%)	0.23	0.23	0.24	0.25**				
Caecum	A (g)	4.176	4.206	4.383	8.787**	3.657	3.487	3.619	6.447**
	R-b (%)	1.02	1.06	1.06	2.21**	1.71	1.61	1.74	3.29**
Spleen	A (mg)	705	678	691	647*	437	445	435	404**
	R-b (%)	0.17	0.17	0.17	0.16	0.20	0.21	0.21	0.21

A: absolute weight; ppm: parts per million; R-b: relative to body weight; *: $P < 0.05$; **: $P < 0.01$ (Dunnett's test)
Source: Ohtsuka (2010c)

Table 28. Summary of histopathology in a 52-week toxicity study with pyriofenone in rats

Site and lesion		Incidence of lesions observed							
		Males				Females			
		0 ppm	200 ppm	1 000 ppm	5 000 ppm	0 ppm	200 ppm	1 000 ppm	5 000 ppm
Bone marrow (sternum)	[*N* =]	[20]	[20]	[20]	[20]	[20]	[0]	[0]	[20]
Granuloma		0	0	0	0	10	–	–	2**
Haematopoiesis, increased		0	0	0	9**	0	–	–	0
Bone marrow (femur)	[*N* =]	[20]	[20]	[20]	[20]	[20]	[0]	[0]	[20]
Granuloma		0	0	0	0	15	–	–	6**
Haematopoiesis, increased		0	0	0	9**	0	–	–	0
Liver	[*N* =]	[20]	[20]	[20]	[20]	[20]	[20]	[20]	[20]
Hypertrophy, hepatocyte, centrilobular		0	0	0	18**	0	0	0	0
Hyperplasia, bile duct		20	19	20	0**	2	2	3	2

Site and lesion		Incidence of lesions observed							
		Males				Females			
		0 ppm	200 ppm	1 000 ppm	5 000 ppm	0 ppm	200 ppm	1 000 ppm	5 000 ppm
Foci of cellular alteration (basophilic cell type)		8	11	11	7	11	9	7	0**
Hepatodiaphragmatic nodule		2	5	2	0	2	5	8*	4
Microgranuloma		1	0	0	0	7	6	6	1*
Kidney	[N =]	[20]	[20]	[20]	[20]	[20]	[20]	[20]	[20]
Tubular basophilic change		1	1	2	10**	0	0	0	1
Increased deposition, brown pigment, renal tubular cell		0	0	0	0	0	0	0	20**
Pituitary	[N =]	[20]	[1]+	[0]+	[20]	[20]	[2]+	[3]+	[20]
Cyst, anterior lobe		1	0	–	0	6	0	0	0*

+: not subjected to statistical evaluation because only animals with macroscopic lesions were histopathologically examined; [N]: number of animals examined; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$ (Fisher's exact probability test)
Source: Ohtsuka (2010c)

At necropsy, high-dose rats of both sexes showed distended caecum with contents (five males, 10 females) and increased caecum weight. Absolute and relative (to body weight) liver weights were increased (both sexes) and were accompanied by centrilobular hepatocellular hypertrophy in males (18/20). Absolute and relative (to body weight) kidney weights were increased in both sexes and were accompanied by tubular basophilic changes (10/20 males) and increased brown pigment deposit in renal tubular cells (20/20 females). Additionally, slightly increased haematopoiesis in the bone marrow was seen in 9/20 males.

At the intermediate dose (1000 ppm), male and female rats exhibited some low-magnitude and/or sporadic (occurring at one time point but not others) effects, but these were not considered toxicologically relevant.

There were no treatment-related effects observed at the low dose (200 ppm).

The NOAEL was 1000 ppm (equal to 42.9 mg/kg bw per day), based on increased liver weight and centrilobular hypertrophy, increased APTT, decreased haemoglobin and red blood cell parameters (haematocrit, haemoglobin concentration, haemoglobin width, red blood cell count), increased GGT, ALP, AST and ALT, and increased total cholesterol in male and female rats at 5000 ppm (equal to 226 mg/kg bw per day). At 5000 ppm, nephrotoxicity was observed in rats of both sexes, including increased kidney weights, altered clinical chemistry suggestive of renal dysfunction, and renal histopathology (tubular basophilic change in 10/20 males and increased brown pigment deposition in renal tubular cells in 20/20 females) (Ohtsuka, 2010c).

In a 2-year (104-week) carcinogenicity study, pyriofenone (IKF-309 technical; batch no. 0701; purity 97.88%) was administered in the diet to Fischer rats (50 of each sex per group) at 0, 200, 1000 or 5000 ppm (equal to 0, 7.25, 36.4 and 197 mg/kg bw per day for males and 0, 9.13, 46.5 and 254 mg/kg bw per day for females, respectively). End-points included clinical signs, body weight, feed consumption, haematology (leukocyte counts; week 104), necropsy and histopathology. No clinical chemistry measurements were included.

There were no effects on total leukocyte count or differential leukocyte count.

At the high dose, there was a significant increase in mortality in males during the last 4 weeks of treatment, corresponding to a cumulative mortality rate of 34%. There was an increase in the incidence of soiled fur in both sexes, and females showed an increased incidence of fur loss. Feed consumption was increased in males, whereas body weights were decreased from weeks 11 to 104 in males and from weeks 16 to 104 in females.

Macropathological and micropathological observations at the high dose included an increased incidence of distention of the caecum (with contents) in both sexes, accompanied by soiled fur in the genital region and general loss of fur. The integumentary areas that showed a loss of fur also showed increased atrophy of hair follicles and perifolliculitis. The kidney had a coarse appearance in males. Liver, kidney and caecum weights were increased in both sexes (Table 29). Male rats also showed an increased incidence of mesenteric lymph node sinus dilatation (Table 30). Both sexes showed an increased incidence of centrilobular hepatocellular hypertrophy and centrilobular hepatocellular fatty changes. Male rats also showed an increased incidence of centrilobular hepatocellular necrosis, whereas female rats showed an increased incidence of focal hepatic congestion. Female rats also showed an increased incidence of chronic progressive nephropathy (CPN). Although the incidence of CPN was not statistically significant with respect to treatment in male rats (Table 30), there was a significant increase in the severity of CPN lesion grade in males (Table 31).

Table 29. Summary of organ weights in a carcinogenicity study in rats administered pyriofenone

		Males				**Females**			
Organ	**Weight type**	**0 ppm**	**200 ppm**	**1 000 ppm**	**5 000 ppm**	**0 ppm**	**200 ppm**	**1 000 ppm**	**5 000 ppm**
Body weight (final)	A (g)	449	441	436	377**	267	271	276	228**
Brain	A (g)	2.12	2.14	2.12	2.14	1.96	1.94	1.96	1.94
	R-b (%)	0.47	0.48	0.49	0.57**	0.74	0.72	0.71	0.85**
Heart	A (g)	1.16	1.19	1.15	1.23	0.835	0.803	0.896	0.892
	R-b (%)	0.26	0.27	0.27	0.33**	0.32	0.30	0.33	0.38**
Liver	A (g)	9.84	11.2	10.3	10.6	6.14	5.81	6.85	6.96*
	R-b (%)	2.19	2.54	2.36	2.85**	2.31	2.16	2.49	3.07**
Kidneys	A (g)	2.50	2.60	2.54	2.93**	1.75	1.67	1.81	1.89*
	R-b (%)	0.56	0.59	0.58	0.78**	0.66	0.62	0.66	0.83**
Caecum	A (g)	4.75	4.70	4.25	7.17**	3.59	3.70	3.41	7.63**
	R-b (%)	1.06	1.06	0.98	1.92**	1.35	1.37	1.25	3.36**
Adrenals	A (g)	0.058	0.056 7	0.056 3	0.070 8	0.066 4	0.062 1	0.069 0	0.064 3
	R-b (%)	0.013	0.013	0.013	0.019*	0.025	0.023	0.025	0.029
Testes	A (g)	2.65	3.60	3.43	3.57	–			
	R-b (%)	0.59	0.82	0.79	0.96*				
Epididymides	A (g)	0.696	0.528**	0.682	0.586	–			
	R-b (%)	0.16	0.12**	0.16	0.16				

A: absolute weight; ppm: parts per million; R-b: relative to body weight; *: $P < 0.05$; **: $P < 0.01$ (Dunnett's test)
Source: Ohtsuka (2010d)

Table 30. Summary of non-neoplastic lesions in a rat carcinogenicity study

		Incidence[a] of non-neoplastic lesions							
		Males				Females			
Findings	**Fate**	**0 ppm**	**200 ppm[b]**	**1 000 ppm[b]**	**5 000 ppm**	**0 ppm**	**200 ppm[b]**	**1 000 ppm[b]**	**5 000 ppm**
Atrophy of hair follicle (lumbo-dorsal)	tk	0/43	1/2	2/4	4*/33	0/35	0/2	0/6	1/38
	ke/fd	0/7	0/11	0/7	2/17	2/15	0/16	0/14	2/12
	all	0/50	1/13	2/11	6*/50	2/50	0/18	0/20	3/50
Perifolliculitis (lumbo-dorsal)	tk	0/43	0/2	0/4	2/33	2/35	2/2	5/6	8/38
	ke/fd	0/7	0/11	0/7	0/17	0/15	0/16	0/14	0/12
	all	0/50	0/13	0/11	2/50	2/50	2/18	5/20	8*/50
Increased extramedullary haematopoiesis in the spleen	tk	2/43	1/4	1/3	5/33	4/35	0/4	0/3	3/38
	ke/fd	4/7	4/11	0/7	7/17	2/15	5/16	5/14	1/12
	all	6/50	5/15	1/10	12/50	6/50	5/20	5/17	4/50
Dilatation of sinus in mesenteric lymph nodes	tk	0/43	0/39	1/43	12**/33	0/35	–	–	1/38
	ke/fd	0/7	1/11	0/7	5/16	0/15	0/16	0/14	0/12
	all	0/50	1/50	1/50	17**/49	0/50	0/16	0/14	1/50
Hyperplasia of alveolar epithelial cells	tk	10/43	1/5	1/1	3/33	0/35	1/1	1/1	3/38
	ke/fd	0/7	1/11	1/7	1/17	0/15	1/16	0/14	1/12
	all	10/50	2/16	2/8	3*/50	0/50	2/17	1/15	4/50
Focal hepatic congestion	tk	0/43	0/39	0/43	0/33	1/35	1/34	2/36	12**/38
	ke/fd	0/7	0/11	0/7	0/17	0/15	0/16	0/14	1/12
	all	0/50	0/50	0/50	0/50	1/50	1/50	2/50	13**/50
Centrilobular hepatocyte fatty change	tk	0/43	0/39	0/43	19**/33	0/35	0/34	0/36	27**/38
	ke/fd	2/7	1/11	0/7	4/17	7/15	5/16	4/14	6/12
	all	2/50	1/50	0/50	23**/50	7/50	5/50	4/50	33**/50
Centrilobular hepatocyte necrosis	tk	0/43	0/39	0/43	0/33	0/35	0/34	0/36	0/38
	ke/fd	0/7	1/11	0/7	8*/17	1/15	2/16	0/14	0/12
	all	0/50	1/50	0/50	8**/50	1/50	2/50	0/50	0/50
Centrilobular hepatocyte hypertrophy	tk	0/43	0/39	0/43	33**/33	0/35	0/34	0/36	36**/38
	ke/fd	0/7	0/11	0/7	1/17	0/15	0/16	0/14	1/12
	all	0/50	0/50	0/50	34**/50	0/50	0/50	0/50	37**/50
Hyperplasia of bile duct	tk	43/43	39/39	4*/43	21**/33	14/35	13/34	17/36	5**/38
	ke/fd	6/7	10/11	7/7	6*/17	7/15	5/16	5/14	1*/12
	all	49/50	49/50	50/50	27**/50	21/50	18/50	22/50	6**/50
Hepato-diaphragmatic nodule	tk	11/43	6/39	4*/43	4/33	7/35	5/34	7/36	6/38
	ke/fd	2/7	2/11	1/7	1/17	4/15	4/16	5/14	3/12
	all	13/50	8/50	5*/50	5*/50	11/50	9/50	12/50	9/50
	tk	6/43	3/39	1/43	0*/33	16/35	21/34	20/36	15/38

Findings	Fate	Incidence[a] of non-neoplastic lesions							
		Males				Females			
		0 ppm	200 ppm[b]	1 000 ppm[b]	5 000 ppm	0 ppm	200 ppm[b]	1 000 ppm[b]	5 000 ppm
Hepatic microgranuloma	ke/fd	0/7	0/11	1/7	0/17	2/15	3/16	5/14	2/12
	all	6/50	3/50	2/50	0*/50	18/50	24/50	25/50	17/50
Oedema of renal papilla	tk	22/43	24/39	24/43	8*/33	13/35	15/34	16/36	19/38
	ke/fd	1/7	5/11	4/7	2/17	3/15	5/16	6/14	5/12
	all	23/50	29/50	28/50	10*/50	16/50	20/50	22/50	24/50
Chronic progressive nephropathy	tk	38/43	34/39	42/43	31/33	16/35	20/34	30**/36	36**/38
	ke/fd	3/7	6/11	4/7	13/17	1/15	5/16	5/14	9**/12
	all	41/50	40/50	46/50	44/50	17/50	25/50	35**/50	45**/50
Luminal dilatation of the uterine horn	tk					8/35	0/6	1/11	2*/38
	ke/fd		–			0/15	3/16	2/14	0/12
	all					8/50	3/22	3/25	2*/50
Hyperplasia of anterior cell in the pituitary	tk	17/43	2/15	3/13	8/33	13/35	7/24	1/23	15/38
	ke/fd	1/7	3/11	0/7	3/17	7/15	1*/16	1*/14	2/12
	all	18/50	5/26	3/20	11/50	20/50	8/40	2/37	17/50
Hyperplasia of adrenal medullary cells	tk	10/43	1/3	0/3	16/33	4/35	–	0/1	2/38
	ke/fd	3/7	0*/11	0/7	5/17	0/15	0/16	0/14	0/12
	all	13/50	1/14	0/10	21/50	4/50	0/16	0/15	2/50

–: no data or not applicable; all: all animals examined; ke/fd: killed in extremis or found dead during treatment period; ppm: parts per million; tk: terminal kill after 104-week treatment; *: $P < 0.05$; **: $P < 0.01$ (Fisher's exact probability test)

[a] Figures show the incidence of findings observed/number of animals examined.

[b] For the 200 and 1000 ppm groups, with the exception of liver and kidneys, results were not subject to statistical analysis, as not all animals were examined.

Source: Ohtsuka (2010d)

Table 31. Statistical analysis of grade of CPN in rats in a carcinogenicity study

Fate	Grade	Incidence[a] of CPN, by grade							
		Males				Females			
		0 ppm	200 ppm	1 000 ppm	5 000 ppm	0 ppm	200 ppm	1 000 ppm	5 000 ppm
tk	−	5	5	1	2	19	14	6	2
	+	32	28	32	11	11	16	23	22
	++	5	4	10	9	5	3	5	14
	+++	1	2	0	11	0	1	2	0
	Incidence	38/43	34/39	42/43	31/33	16/35	20/34	30/36	36/38
	Significance				**			*	**
ke/fd	−	4	5	3	4	14	11	9	3
	+	1	6	3	8	1	4	2	5
	++	0	0	1	2	0	1	3	4

Fate	Grade	Incidence[a] of CPN, by grade							
		Males				Females			
		0 ppm	200 ppm	1 000 ppm	5 000 ppm	0 ppm	200 ppm	1 000 ppm	5 000 ppm
	+++	2	0	0	3	0	0	0	0
	Incidence	3/7	6/11	4/7	13/17	1/15	5/16	5/14	9/12
	Significance								**
all	−	9	10	4	6	33	25	15	5
	+	33	34	35	19	12	20	25	27
	++	5	4	11	11	5	4	8	18
	+++	3	2	0	14	0	1	2	0
	Incidence	41/50	40/50	46/50	44/50	17/50	25/50	35/50	45/50
	Significance				**			**	**

all: all animals examined; ke/fd: killed in extremis or found dead during treatment period; tk: terminal kill after 104-week treatment; *: $P < 0.05$; **: $P < 0.01$ (Dunnett type mean rank test)
[a] Figures show the incidence of findings observed/number of animals examined.
Source: Ohtsuka (2010d)

There were no toxicologically relevant effects on the incidence of neoplastic lesions in treated rats (Table 32). The combined incidence of hepatocellular adenomas and carcinomas in treated rats did not reach statistical significance.

Table 32. Summary of neoplastic lesions in a rat carcinogenicity study

Findings	Fate	Incidence[a] of neoplastic lesions							
		Males				Females			
		0 ppm	200 ppm	1 000 ppm	5 000 ppm	0 ppm	200 ppm	1 000 ppm	5 000 ppm
Mononuclear cell leukaemia	tk	3/43	3/39	1/43	0/33	3/35	5/34	3/36	3/38
	ke/fd	1/7	3/11	1/7	3/17	9/15	6/16	3*/14	6/12
	all	4/50	6/50	2/50	3/50	12/50	11/50	6/50	9/50
Interstitial testicular cell tumour	tk	38/43	32/39	31/43	25/33				
	ke/fd	3/6	7/11	1/7	4/16			–	
	all	41/49	39/50	32/50	29*/49				
Hepatocellular adenoma/ carcinoma	tk	4/43	2/39	3/43	4/33	0/35	0/34	0/36	1/38
	ke/fd	0/7	0/11	0/7	4/17	0/15	0/16	0/14	0/12
	all	4/50	2/50	3/50	8/50	0/50	0/50	0/50	1/50

all: all animals examined; ke/fd: killed in extremis or found dead during treatment period; tk: terminal kill after 104-week treatment; *: $P < 0.05$ (Fisher's exact probability test)
[a] Figures show the incidence of findings observed/number of animals examined.
Source: Ohtsuka (2010d)

Peto's test was applied to analyse the occurrence of neoplastic lesions in males with an incidence of four or more in at least one dose group (Table 33). This analysis was undertaken because the male 5000 ppm group showed a statistically significant increase in mortality during the final stage of the study. Peto's test for the combined incidence of hepatocellular adenoma and carcinoma and the incidence of systemic mononuclear cell leukaemia was performed on the data from all male dose groups. Peto's test for other neoplastic lesions was performed on the data only from the control and 5000 ppm groups, as all animals in the 200 and 1000 ppm groups were not examined histopathologically for the organs/tissues concerned. No dose-related increasing trends were observed in the neoplastic lesions analysed by Peto's test.

Table 33. Summary of Peto's test in a rat carcinogenicity study with pyriofenone

Sex	Site	Neoplastic lesion	*P*-value	Judgement
Male	Systemic	Mononuclear cell leukaemia	0.56	Not significant
	Skin	Fibroma	1.00	Not significant
	Pancreas	Islet cell adenoma/carcinoma	0.13	Not significant
	Liver	Hepatocellular adenoma/carcinoma	0.14	Not significant
	Pituitary	Anterior adenoma/carcinoma	0.26	Not significant
	Thyroid	C-cell adenoma/carcinoma	1.00	Not significant
	Adrenal	Phaeochromocytoma (benign/malignant)	1.00	Not significant

Source: Ohtsuka (2010d)

At the intermediate dose (1000 ppm), the only test article–related effect was an increased incidence of CPN in female rats. No adverse effects were noted in male rats.

There were no effects related to treatment observed at the low dose.

The NOAEL was 200 ppm (equal to 9.13 mg/kg bw per day), based on CPN in female rats at 1000 ppm (equal to 46.5 mg/kg bw per day). The nephropathy in female rats at 1000 ppm and in both sexes at 5000 ppm (Ohtsuka, 2010d) was considered related to the nephrotoxicity observed in both sexes at 5000 ppm in the 52-week rat study (Ohtsuka, 2010c). There was no evidence of increased neoplasia or tumour incidence after exposure to pyriofenone (Ohtsuka, 2010d).

2.4 Genotoxicity

Pyriofenone was tested for genotoxicity in an adequate range of in vitro and in vivo assays. It did not produce evidence of genotoxicity in any assay (Table 34).

Table 34. Summary of genotoxicity studies with pyriofenone[a,b,c]

End-point	Test object	Concentration	Purity (%)	Results	Reference
In vitro					
Reverse mutation	*Salmonella typhimurium* and *Escherichia coli*	5, 15, 50, 150, 500, 1 500 or 5 000 µg/plate ±S9	97.88	Negative	May (2007)[d]
Mammalian cell gene mutation	Mouse lymphoma L5178Y cells (*tk* locus)	5, 9.93, 19.86, 20, 39.72, 40, 50, 60, 70, 79.44, 80, 90, 100, 125,	97.88	Negative	Hynes (2008a)[e]

End-point	Test object	Concentration	Purity (%)	Results	Reference
		158.88, 317.75, 635.5 or 1 271 µg/mL ±S9			
Chromosomal aberration	CHL cells	0, 20, 30, 60, 65, 70, 90, 100, 110, 120 or 130 µg/mL ±S9	97.88	Negative	Pritchard (2008)[f]
In vivo					
Mouse micronucleus test (oral gavage)	CD-1 mice, 5/sex per dose	0, 500, 1 000 or 2 000 mg/kg bw	97.88	Negative	Hynes (2008b)[g]
Comet assay	F344 CrlCrlj rats, male, 5/dose	0, 500, 1 000 or 2 000 mg/kg bw	97.88	Negative	Wada (2017)[h]

bw: body weight; CHL: Chinese hamster lung; GLP: good laboratory practice; OECD: Organisation for Economic Co-operation and Development; S9: 9000 × *g* supernatant fraction from rat liver homogenate; *tk*: thymidine kinase

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

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

[c] All studies used pyriofenone batch no. 0701.

[d] Performed in accordance with OECD Test Guideline No. 471.

[e] Performed in accordance with OECD Test Guideline No. 476.

[f] Performed in accordance with OECD Test Guideline No. 473.

[g] Performed in accordance with OECD Test Guideline No. 474.

[h] Performed in accordance with OECD Test Guideline No. 489.

2.5 *Reproductive and developmental toxicity*

(a) Multigeneration studies

In a multigeneration reproductive toxicity study, pyriofenone (IKF-309 technical; batch no. 0701; purity 97.88%) was administered in the diet to Wistar Hannover rats (24 of each sex per group) at 0, 150, 1000 or 5000 ppm for two generations. These dietary concentrations were equal to 0, 7.29, 47.8 and 257 mg/kg bw per day for parental (F_0) males, 0, 9.5, 58.1 and 301 mg/kg bw per day for F_0 females, 0, 7.73, 52.2 and 267 mg/kg bw per day for first filial generation (F_1) males and 0, 9.3, 59.5 and 311 mg/kg bw per day for F_1 females, respectively. Rats were exposed for 18 weeks from premating through mating, gestation and postnatal growth of offspring. Parental animals of both sexes were exposed to the test article for 10 weeks prior to mating and during mating, gestation and lactation, and offspring were exposed from weaning for 10 weeks until mating to produce the second generation. F_0 adults were terminated after weaning of the F_1 offspring. Adult F_1 rats and second filial generation (F_2) pups were terminated after weaning of the F_2 generation.

Measurements in parental animals (F_0 and adult F_1 rats) included clinical signs, urine analysis, haematology, feed and water consumption, body weight, estrous cyclicity, reproductive performance, pregnancy rate, gestation duration, sperm evaluation and necropsy. Histopathology was performed on reproductive organs, pituitary, adrenals, liver, kidney, thyroid and caecum.

Litter measurements included clinical observation of pups, number of pups per litter, sex ratio of pups, external abnormalities and survival to postnatal day 21. F_1 rats were evaluated for physical development and sexual maturation (preputial separation and vaginal opening).

There were no treatment-related effects on reproductive performance, sexual development, estrous cyclicity, gestation index, number of implantation sites or sperm parameters in either generation.

No adverse effects related to treatment were observed in either sex of F_0 or F_1 parental animals at 150 or 1000 ppm. At the high dose (5000 ppm), treatment-related effects included decreased haematocrit (F_0 and F_1 females; F_1 males), decreased haemoglobin concentration (F_0 and F_1 females; F_1 males) and decreased erythrocyte count (F_1 females). There was also a significant increase in haemoglobin distribution width and platelet count in F_1 females. High-dose rats also showed increased

kidney, thyroid and caecum weights (F_0 and F_1) and distention of the caecum (F_1) (Table 35). F_2 pups at the high dose showed decreased spleen weights (14.4% decrease in males; 13.3% decrease in females), but no histopathology in the spleen.

Table 35. Summary of dietary two-generation reproductive toxicity study in rats administered pyriofenone

	Males				Females			
Parameter	0 ppm	150 ppm	1 000 ppm	5 000 ppm	0 ppm	150 ppm	1 000 ppm	5 000 ppm
F_0 generation								
Number of animals/group	24	24	24	24	24	24	24	24
Mortality	0	0	1	0	0	0	1	0
Body weight (g)								
At pairing								
Week 10	394	395	389	397	231	233	232	238
Gain: weeks 0–10	238	239	233	242	109	111	110	116
Gestation								
Day 0	–	–	–	–	232	234	234	238
Gain, days 0–20	–	–	–	–	102	106	100	103
Lactation								
Day 0	–	–	–	–	246	251	254	254
Day 21	–	–	–	–	280	284	286	296**
Feed consumption (g/rat per day)								
Pre-pairing								
Weeks 1–10	19.5	19.8	19.7	20.9	15.2	15.6	15.5	15.8
Gestation								
Days 0–7	–	–	–	–	15.3	15.8	15.7	15.3
Days 7–14	–	–	–	–	17.7	18.4	18.2	18.0
Days 14–20	–	–	–	–	18.7	19.3	19.4	20.5**
Lactation								
Days 0–7	–	–	–	–	31.7	30.6	31.8	32.0
Days 7–14	–	–	–	–	49.2	51.0	48.1	47.4
Days 14–20[a]	–	–	–	–	59.7	61.1	56.5	60.2
Litter data (F_1 litters)								
Live litters born	–	–	–	–	22	20	23	23
Live litter size, day 0	–	–	–	–	12.0	12.7	11.3	11.8
Fertility index (%)	–	–	–	–	91.7	83.3	100	95.8
Gestation index (%)	–	–	–	–	100	100	100	100
Offspring body weight (g)								
Day 0	5.9	5.7	6.0	5.8	5.6	5.4	5.6	5.5
Day 4	10.1	9.6	10.4	10.0	9.8	9.4	9.8	9.6
Day 21 (weaning)	54.9	54.4	54.3	51.7*	52.9	53.1	51.8	49.6*

Parameter	Males 0 ppm	Males 150 ppm	Males 1 000 ppm	Males 5 000 ppm	Females 0 ppm	Females 150 ppm	Females 1 000 ppm	Females 5 000 ppm
% of control	–	99	99	94	–	100	98	94
Terminal observations: F_0 adults[b]								
Number examined	24	24	23	24	24	24	23	24
Terminal body weight (g)	447	439	439	440	261	266	265	268
Organ weights								
Relative liver weight (%)	2.94	3.03	3.13*	3.78**	3.90	4.33**	4.26**	5.40**
Relative kidney weight (%)	0.266	0.276	0.281	0.330**	0.350	0.360	0.363	0.386**
Relative thyroid weight (% × 1 000)	5.32	5.99	5.82	6.54**	8.87	8.64	7.21	9.39*
Relative caecum weight (%)	1.72	1.80	1.87	3.18**	2.39	2.42	2.59	3.56**
Gross pathology								
Dark liver	0	0	0	13**	0	0	0	19**
Histopathology								
Number examined	22	20	23	23	21	20	23	23
Liver								
Centrilobular hypertrophy	0	0	0	0	0	0	0	20**
Diffuse hypertrophy	0	0	0	22**	0	0	0	0
Lipofuscin in Glisson’s capsule	0	0	0	13**	0	0	0	0
Thyroid								
Follicular cell hypertrophy	13	–	–	15	3	5	5	14**
Kidney								
Hyaline droplets in proximal tubule	0	0	0	11**	0	0	0	0
Haematology								
Haematocrit (%)	44.6	44.1	44.1	43	47.6	48.7	47.0	43.4*
Haemoglobin concentration (g/dL)	15.0	14.9	14.9	14.5	16.2	16.4	16.0	14.5*
RBC count (10^6/µL)	8.37	8.25	8.27	8.02	8.26	8.23	8.16	7.56
Sperm morphology								
Sperm head count (× 10^6/g testis)	127	127	131	131	–	–	–	–
Sperm count (× 10^6/g epididymis)	544	556	586	568	–	–	–	–
% motile sperm	91.0	92.3	93.0	91.4	–	–	–	–
% normal sperm	97.0	98.3	98.7	97.8	–	–	–	–
F_1 generation								
Number of animals/group	24	24	24	24	24	24	24	24
Mortality	0	0	0	0	0	0	0	0
Body weight (g)								
At pairing								
Week 10	383	386	381	393	230	225	228	229
Gain, weeks 0–10	320	324	320	330	171	167	170	170

Parameter	Males 0 ppm	Males 150 ppm	Males 1 000 ppm	Males 5 000 ppm	Females 0 ppm	Females 150 ppm	Females 1 000 ppm	Females 5 000 ppm
Gestation								
Day 0	–	–	–	–	232	229	232	232
Gain, days 0–20	–	–	–	–	98	95	95	102
Lactation								
Day 0	–	–	–	–	253	256	258	256
Day 21	–	–	–	–	281	281	289	296**
Feed consumption (g/rat per day)								
Pre-pairing								
Weeks 1–10	18.8	19.0	17.9	19.9	14.5	14.6	14.2	14.7
Gestation								
Days 0–7	–	–	–	–	14.8	15.4	14.7	15.6
Days 7–14	–	–	–	–	17.6	18.3	17.7	18.5
Days 14–20	–	–	–	–	18.9	20.1	19.7	21.1**
Lactation								
Days 0–7	–	–	–	–	32.1	30.2	29.6	33.9
Days 7–14	–	–	–	–	48.7	48.7	46.0	49.1
Days 14–20[a]	–	–	–	–	58.0	57.3	54.5	61.5
Sexual maturation								
Preputial separation								
Age	42.3	41.9	42.4	42.0	–	–	–	–
Body weight (g)	182	182	182	181	–	–	–	–
Vaginal opening								
Age	–	–	–	–	31.9	31.6	31.2	31.6
Body weight (g)	–	–	–	–	101	100	97	100
Litter data (F_2 litters)								
Live litters born	–	–	–	–	22	24	22	24
Live litter size, day 0	–	–	–	–	11.6	10.5	9.9	10.7
Fertility index (%)	–	–	–	–	95.7	100	91.7	100
Gestation index (%)	–	–	–	–	100	100	100	100
Offspring body weight (g)								
Day 0	6.0	6.1	6.0	6.0	5.7	5.8	5.7	5.9
Day 4	10.3	10.7	10.7	10.6	10.1	10.5	10.3	10.4
Day 21 (weaning)	55.0	55.3	54.6	53.1	52.7	53.2	52.8	51.8
% of control	–	101	99	97	–	101	100	98
Terminal observations: F_1 adults[c]								
Number examined	24	24	24	24	24	24	24	24
Terminal body weight (g)	446	448	449	459	265	266	270	277
Organ weights								

Parameter	Males 0 ppm	Males 150 ppm	Males 1 000 ppm	Males 5 000 ppm	Females 0 ppm	Females 150 ppm	Females 1 000 ppm	Females 5 000 ppm
Relative liver weight (%)	3.06	3.22	3.16	3.90**	4.32	4.46	4.63	6.41**
Relative kidney weight (%)	0.268	0.282	0.282	0.325**	0.357	0.366	0.367	0.403**
Relative thyroid weight (% × 1 000)	5.57	6.77	5.38	6.45*	7.64	7.61	7.03	8.89*
Relative caecum weight (%)	1.68	1.75	1.79	3.17**	2.20	2.42	2.39	3.38**
Histopathology								
Number examined	22	24	22	24	22	24	23	23
Liver								
Centrilobular hepatocellular hypertrophy	0	0	0	0	0	0	0	21**
Diffuse hepatocellular hypertrophy	0	0	0	22**	0	0	0	0
Lipofuscin in Glisson's capsule	0	0	0	8**	0	0	0	0
Thyroid								
Follicular cell hypertrophy	5	7	5	19**	5	7	4	16**
Kidney								
Hyaline droplets in proximal tubule	0	0	0	11**	0	0	0	0
Haematology								
Haematocrit (%)	43.9	43.6	44.4	41.6**	47.5	46.4	46.7	44.4*
Haemoglobin concentration (g/dL)	14.9	14.7	14.9	14.1*	16.2	15.7	15.9	15.3*
RBC count (10^6/µL)	8.59	8.45	8.58	8.19	8.52	8.31	8.47	8.05*

–: no data or not applicable; F_0: parental generation; F_1: first filial generation; ppm: parts per million; RBC: red blood cells; *: $P < 0.05$; **: $P < 0.01$
[a] Dam + litter.
[b] For F_0 males after 18 weeks of treatment; for F_0 females after weaning of litters (day 26 postpartum).
[c] For F_1 males after 18 weeks of treatment; for F_1 females after weaning of litters (day 26 postpartum).
Source: Hojo (2009a)

The NOAEL for parental toxicity was 1000 ppm (equal to 47.8 mg/kg bw per day), based on altered haematological parameters and changes in organ weights in F_0 and F_1 rats at 5000 ppm (equal to 257 mg/kg bw per day).

The NOAEL for reproductive toxicity was 5000 ppm (equal to 257 mg/kg bw per day), the highest dose tested.

The NOAEL for offspring toxicity was 5000 ppm (equal to 257 mg/kg bw per day), the highest dose tested (Hojo, 2009a).

(b) Developmental toxicity

Rats

In a developmental toxicity study, pyriofenone (IKF-309 technical; batch no. 0701; purity 97.88%) was administered by gavage to pregnant Wistar Hannover rats (24 per group) from gestation day (GD) 6 to GD 19 at 0, 30, 300 or 1000 mg/kg bw per day. The vehicle was 1% carboxymethylcellulose. End-points included clinical signs, maternal body weight (GDs 0, 6, 9, 12, 15, 18 and 20), feed consumption, necropsy (maternal animals), histopathology of liver and kidney, gravid

uterine weight, numbers of corpora lutea and implantations, resorptions (early/late), placental weights, and number and position of live and dead fetuses in the uterus. Live fetuses were sexed, weighed, terminated and examined for external and orifice abnormalities. Half of the fetuses were used to evaluate visceral abnormalities, and the other half were used to evaluate skeletal abnormalities after appropriate fixation techniques.

Rats were observed daily throughout the dosing period for mortality and clinical signs. Body weight was determined on GDs 1, 3, 6, 10, 14, 17 and 20. All rats were terminated on GD 20; macroscopic examination was performed, with emphasis on ovaries and uteri.

No deaths occurred on study. There were no treatment-related effects on clinical signs, gravid uterine weights, numbers of corpora lutea or implantations, or preimplantation loss. There were no treatment-related effects on the number of live fetuses, resorptions, fetal weight, placental weight, sex ratio or external, visceral or skeletal malformations or variations.

Although there were increases in skeletal variations (supernumerary rib, discontinuous rib cartilage, presacral vertebrae) in fetuses of the 300 and 1000 mg/kg bw per day groups when considering fetal incidence (Table 36), after comparing the litter incidence of skeletal variations across dose groups, there were no significant trends related to dose.

Table 36. Summary of rat developmental toxicity study with pyriofenone: malformations and variations

Parameter	Incidence of finding			
	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**
Number of fetuses examined	149	150	148	157
External malformations	No treatment-related effect			
Visceral examinations	No treatment-related effect			
Skeletal malformations	No treatment-related effect			
Number of fetuses with skeletal variation	84	83	105*	121**
Supernumerary ribs	64	51	85*	98**
Discontinuous rib cartilage	38	45	47	62*
27 presacral vertebrae	6	3	19	14
Number of litters per group	24	24	24	24
Skeletal variation (litters affected)	22	22	23	23
Supernumerary ribs (litters affected)	21	18	23	22
Discontinuous rib cartilage (litters affected)	15	17	20	23*
27 presacral vertebrae (litters affected)	6	2	10	7
Skeletal variation (% litters affected)	91.6	91.6	95.8	95.8
Supernumerary ribs (% litters affected)	87.5	75.0	95.8	91.6
Discontinuous rib cartilage (% litters affected)	62.5	70.8	83.3	95.8
27 presacral vertebrae (% litters affected)	25.0	8.3	41.6	29.1

bw: body weight; *: $P < 0.05$; **: $P < 0.01$
Source: Hojo (2009b)

The only treatment-related effects in maternal animals were increased absolute and relative weights of liver and caecum at 300 and 1000 mg/kg bw per day and decreased feed consumption during GDs 6–9 and 9–12 at 1000 mg/kg bw per day (Table 37).

Table 37. Summary of rat developmental toxicity study with pyriofenone: effects in maternal animals

Parameter	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
Number of females mated	24	24	24	24
Number of females pregnant	24	24	24	24
Number of females that aborted	0	0	0	0
Number of females that were killed prematurely	0	0	0	0
Number of females with live young at termination	24	24	24	24
Number of litters examined	24	24	24	24
Clinical signs	No treatment-related effect			
Body weight (g)				
Day 0	210	210	210	210
Day 6	229	231	231	229
Day 20	319	329	324	324
Body weight change (g)				
Days 6–20	90	98	93	95
Feed consumption (g/day)				
Days 6–9	16.6	16.4	15.3	12.8**
Days 9–12	17.7	17.9	17.2	15.6*
Days 12–15	18.0	19.1	18.6	18.0
Days 15–18	20.5	21.7	20.7	21.1
Days 18–20	19.1	20.2	20.0	20.4
Absolute gravid uterine weight (g)	64	66	64	66
Adjusted body weight, day 20 (g)	255	263	260	258
Organ weights				
Liver				
Absolute (g)	12.3	13.0	13.1*	14.3**
Relative to body weight (%)	4.84	4.94	5.03*	5.54**
Kidney				
Absolute (g)	0.778	0.818	0.799	0.806
Relative to body weight (%)	0.305	0.312	0.308	0.313
Caecum				
Absolute (g)	4.83	4.99	5.61*	6.52**
Relative to body weight (%)	1.90	1.90	2.17	2.53**
Number of litters	24	24	24	24
Number of corpora lutea	13.5	14.4	13.8	14.1
Number of implantations	12.8	13.0	12.8	13.3
% resorptions/fetal deaths	7.5	6.4	7.8	5.9

Parameter	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
Number of live fetuses	11.8	12.1	11.8	12.5
Sex ratio (% male)	49.6	45.9	46.1	49.5
Preimplantation loss (%)	5.4	8.9	6.7	5.3
Fetal weight (g)				
Males	3.61	3.63	3.56	3.52
Females	3.43	3.47	3.40	3.34
Placental weight (g)	0.431	0.441	0.435	0.419

bw: body weight; *: $P < 0.05$; **: $P < 0.01$
Source: Hojo (2009b)

The NOAEL for maternal toxicity was 30 mg/kg bw per day, based on increased liver and caecum weights in dams at 300 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 1000 mg/kg bw per day, the highest dose tested (Hojo, 2009b).

Rabbits

In a developmental toxicity study, pyriofenone (IKF-309 technical; batch no. 0701; purity 97.88%) was administered by gavage to pregnant Japanese white rabbits (24 per group) from GDs 6 to 27 at 0, 30, 300 or 1000 mg/kg bw per day. The vehicle was 1% carboxymethylcellulose. End-points included clinical signs, maternal body weight (GDs 0, 6, 9, 12, 15, 18, 21, 24, 27 and 28), feed consumption, necropsy (maternal animals), gravid uterine weight, numbers of corpora lutea and implantations, placental weights, and number and position of live and dead fetuses in the uterus. Live fetuses were sexed, weighed, scarified and examined for external and orifice abnormalities. Half of the fetuses were used to evaluate visceral abnormalities, and the other half were used to evaluate skeletal abnormalities after appropriate fixation techniques.

There were no treatment-related effects on gravid uterine weight, numbers of corpora lutea or implantations, preimplantation loss, number of live fetuses, resorptions or fetal deaths, sex ratio, fetal weight or placental weight. There were no treatment-related trends in external, visceral or skeletal malformations or variations.

Two high-dose females aborted. There was a reduction in feed intake from GD 12 to GD 15 at 300 mg/kg bw per day. There were no other treatment-related effects observed in the study.

In a preliminary study, enlarged livers and elevated relative (to body weight) liver weights were observed in gravid female rabbits dosed at 1000 mg/kg bw per day, but no histopathology was performed. Despite the liver being identified as a potential target organ, the comprehensive developmental toxicity study in rabbits did not include an assessment of the liver.

The NOAEL for maternal and embryo/fetal toxicity was 1000 mg/kg bw per day, the highest dose tested (Takahashi, 2009).

2.6 Special studies

(a) Neurotoxicity

In an acute neurotoxicity study in rats, pyriofenone (IKF-309 technical; batch no. 0701; purity 97.88%) was administered once by oral gavage to Sprague Dawley rats (10 of each sex per group) at 0, 125, 500 or 2000 mg/kg bw. The vehicle was 1% carboxymethylcellulose. The rats were observed for

14 days after dosing. End-points included clinical signs, home cage observations, feed consumption, brain weight and anatomical measurement, macropathology and neurohistopathology.

There was no mortality on study. There were no effects of treatment on feed consumption, neurobehaviour, brain anatomical measurements, brain weight, or macroscopic or microscopic investigations.

Reduced body tone (three males, two females) and piloerection (two males, seven females) were observed at the time of peak effect on the day of administration in rats given 2000 mg/kg bw. These signs were not observed on day 8 or 15 of the observation period.

Pyriofenone was not neurotoxic in this study. The NOAEL for neurotoxicity was 2000 mg/kg bw, the highest dose tested (Powell, 2010).

In a 13-week subchronic neurotoxicity study in rats, pyriofenone (IKF-309 technical; batch no. 0701; purity 97.88%) was administered in the diet to Sprague Dawley rats (10 of each sex per group) at 0, 1000, 4000 or 15 000 ppm (equal to 0, 62, 310 and 927 mg/kg bw per day for males and 0, 77, 378 and 1147 mg/kg bw per day for females, respectively). End-points included clinical signs, home cage observations, feed consumption, brain weight and anatomical measurement, macropathology and neurohistopathology.

There was no mortality reported on study. There were no effects of treatment on clinical signs, feed consumption, neurobehaviour, brain anatomical measurements, brain weight, or macroscopic or microscopic investigations.

The only treatment-related effects were decreased body weight gain in males at 4000 ppm (11%) and in both sexes at 15 000 ppm (11–19%).

Pyriofenone was not neurotoxic in this study. The NOAEL for neurotoxicity was 15 000 ppm (equal to 927 mg/kg bw per day), the highest dose tested (Arrowsmith, 2010b).

(b) Immunotoxicity

Mice

In a 4-week immunotoxicity study in mice, pyriofenone (IKF-309 technical; batch no. 0701; purity 97.88%) was administered through the diet to female CD-1 mice (10 per group) at 0, 1000, 3000 or 7000 ppm (equal to 0, 192, 553 and 1270 mg/kg bw per day, respectively). A separate group of eight female mice was set aside as the cyclophosphamide (positive control) group. Four days prior to necropsy, all treatment groups were administered a single intravenous dose of sheep red blood cells (2×10^8 cells/mL) in 0.9% saline. The positive control group was given a single intraperitoneal dose of cyclophosphamide at 20 mg/kg bw for 5 days (study days 22–26). The positive control group was administered sheep red blood cells after the fourth consecutive dose of cyclophosphamide and terminated 4 days later. Study end-points included clinical condition, body weight, feed and water consumption, spleen and thymus weights, macropathology and plaque-forming cell assays. Spleen samples were taken for immunotoxicology investigations, but no other tissues were retained.

There were no unscheduled deaths on study. There were no treatment-related effects on body weight, feed intake, water consumption, spleen or thymus weights, or macropathology findings.

There were no treatment-related effects on the number of cells per spleen or plaque-forming cells (per million cells or per spleen) in the pyriofenone treatment groups. The cyclophosphamide positive control group showed significant decreases in plaque-forming cells, demonstrating the sensitivity of the assay.

Pyriofenone did not affect immune function in this study, as assessed by the measurement of antigen-specific, T-cell-dependent antibody formation. The NOAEL for immunotoxicity was 7000 ppm (equal to 1270 mg/kg bw per day), the highest dose tested (Laurent, 2010).

Rats

In a 4-week immunotoxicity study in rats, pyriofenone (IKF-309 technical; batch no. 0701; purity 97.88%) was administered through the diet to female Sprague Dawley rats (10 per group) at 0, 2000, 6000 or 20 000 ppm (equal to 0, 179, 505 and 1690 mg/kg bw per day, respectively). A separate group of eight female rats was set aside as the cyclophosphamide (positive control) group. Four days prior to necropsy, all treatment groups were administered a single intravenous dose of sheep red blood cells (2×10^8 cells/mL) in 0.9% saline. The positive control group was given a single intraperitoneal dose of cyclophosphamide at 50 mg/kg bw (5 mg/mL solution at 10 mL/kg bw) 2 days prior to necropsy. Study end-points included clinical condition, body weight, feed and water consumption, spleen and thymus weights, macropathology and plaque-forming cell assays. Spleen samples were taken for immunotoxicology investigations, but no other tissues were retained.

Spleen samples were used to create single-cell suspensions for each animal. The cells were mixed with an agar matrix containing a suspension of sheep red blood cells and guinea-pig serum complement. The mixture was poured into petri dishes, allowed to set and incubated for up to 3 hours. The number of lytic plaques for each animal was determined, and the number of plaque-forming cells per one million splenocytes, the number of plaque-forming cells per spleen and total number of white blood cells per spleen were calculated for each rat.

There were no unscheduled deaths on study. There were no treatment-related effects on clinical signs, water intake, spleen or thymus weights, or necropsy findings.

There was a significant decrease in body weight gain (72% of control) in high-dose rats. Feed consumption was decreased relative to controls for these animals. Transiently lower body weights were observed in rats exposed to 2000 or 6000 ppm during study days 1–4, but no general trend was observed.

There were no treatment-related effects on the number of white blood cells per spleen or the number of plaque-forming cells (per million cells or per spleen) in the pyriofenone treatment groups. The cyclophosphamide positive control group showed significant decreases in all parameters, demonstrating the sensitivity of the assay.

Pyriofenone did not affect immune function in this study, as assessed by the measurement of antigen-specific, T-cell-dependent antibody formation. The NOAEL for immunotoxicity was 20 000 ppm (equal to 1690 mg/kg bw per day), the highest dose tested (Chambers, 2010).

(c) Mechanistic studies

In a non-GLP-compliant 7-day mechanistic study, pyriofenone (IKF-309 technical; batch no. 0701; purity 97.88%) was administered to wild-type constitutive androstane receptor (CAR) or CAR knockout rats (five males per group) in the diet at 0 or 5000 ppm (equal to 0 and 363.56 mg/kg bw per day in wild-type CAR rats and 0 and 357.17 mg/kg bw per day in CAR knockout rats, respectively). End-points included clinical signs, feed and water intake, body weight, clinical chemistry (at termination), liver weight and liver histopathology. Additional end-points included liver Ki-67 antibody staining, CYP2B1 gene expression measured through quantitative reverse transcription polymerase chain reaction, and CYP2B1 (testosterone 16β-hydroxylase) activity.

There were no treatment-related effects on clinical signs, body weight or necropsy findings.

Treatment-related effects included higher serum total cholesterol (wild-type CAR), lower serum triglycerides (wild-type CAR), higher total serum protein (CAR knockout) and lower serum albumin/globulin ratio (CAR knockout). Liver weights were increased in both wild-type CAR and CAR knockout treated rats. Diffuse hepatocellular hypertrophy was observed in both wild-type CAR and

CAR knockout treated rats. Additionally, both wild-type CAR and CAR knockout rats showed a decreased ki-67 ratio, suggesting a decrease in hepatocyte proliferation.

CYP2B1 gene expression and activity were increased in treated wild-type CAR rats. In contrast, in CAR knockout rats, CYP2B1 gene expression increased, but there was no increase in CYP2B1 activity. The authors concluded that pyriofenone induced CYP2B1 activity through CAR activation (Yamamoto, 2017).

(d) Studies on metabolites

Acute toxicity

The acute oral toxicity of 2MDPM, 3HDPM and 4HDPM was studied by the acute toxic class method (Table 38). All three metabolites showed low oral toxicity. No deaths or treatment-related clinical signs were reported in any of the studies.

Table 38. Acute oral toxicity of 2MDPM, 3HDPM and 4HDPM in rats

Test article	Strain	Sex	Purity (%)	LD_{50}	Reference
2MDPM	SD	F	99.2–99.8	>2 000 mg/kg bw	Moore (2010b)
3HDPM	SD	F	96.1	>2 000 mg/kg bw	Moore (2010c)
4HDPM	SD	F	99.28	>2 000 mg/kg bw	Fukuyama (2010)

bw: body weight; F: female; LD_{50}: median lethal dose; SD: Sprague Dawley

Genotoxicity

2MDPM, 3HDPM and 4HDPM were tested in bacterial reverse mutation assays (Table 39). None of the studies indicated a potential for mutagenicity.

Table 39. Summary of genotoxicity of pyriofenone metabolites[a,b]

Test substance	End-point	Test object	Concentration	Purity (%)	Results	Reference
2MDPM	Reverse mutation	*Salmonella typhimurium* and *Escherichia coli*	Up to 5 000 µg/plate ±S9	99.4	Negative	May (2010a)[c]
3HDPM			Up to 5 000 µg/plate ±S9	94.8	Negative	May (2010b)[d]
4HDPM			Up to 1 250 µg/plate ±S9 in *S. typhimurium* Up to 5 000 µg/plate ±S9 in *E. coli*	99.28	Negative	Wada (2010)[e]

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.

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

[c] Batch no. 155-100318-1; performed in accordance with OECD Test Guideline No. 471.

[d] Batch no. 155-100316-4; performed in accordance with OECD Test Guideline No. 471.

[e] Batch no. 155-100324-5; performed in accordance with OECD Test Guideline No. 471.

(e) Microbiological effects

The fungicide pyriofenone was evaluated at the 2018 JMPR to determine its impact on microbiota in the gastrointestinal tract. As no data were submitted by the sponsors, a literature search was performed using a number of search engines. These included BioOne (http://www.bioone.org/), Google (https://www.google.com/), Google Scholar (http://scholar.google.com/), PubMed (http://www.ncbi.nlm.nih.gov/pubmed), ScienceDirect (http://www.sciencedirect.com/) and Web of Science (https://apps.webofknowledge.com).

The search strategy used the input keywords of the fungicide chemical name (pyriofenone), chemical structure, antimicrobial mode of action, antimicrobial spectrum of activity, antimicrobial resistance, resistance mechanisms and genetics, microbiome, microbiota, gut microbiota, gut microbiome, gastrointestinal microbiota, gastrointestinal microbiome, etc., as well as the Boolean operators AND, OR and NOT.

The extensive search and review of the scientific literature did not find any reports on the effects of pyriofenone on the intestinal microbiome to include in the toxicological risk assessment.

3. Observations in humans

The sponsor provided some workplace monitoring information. Annual medical examinations (laboratory tests, chest X-rays, clinical examinations, lung function, skin and eye examinations) have been carried out by the Occupational Medical Advisor for Ishihara Sangyo Kaisha on manufacturing plant personnel since the start of manufacture of pyriofenone. In these reports on manufacturing plant personnel, no adverse health effects have been noted (Ishihara Sangyo Kaisha, Ltd, 2017). No information on accidental or intentional poisoning in humans is available.

Comments

Biochemical aspects

Following oral administration of ^{14}C-(phenyl)-pyriofenone or ^{14}C-(pyridyl)-pyriofenone to bile duct–cannulated rats at 5 or 200 mg/kg bw, the compound was rapidly absorbed; absorption 48 hours after dosing ranged from 76% to 89% of the dose at 5 mg/kg bw and from 36% to 53% of the dose at 200 mg/kg bw for the two radiolabels. The compound was largely excreted into bile (at the low dose, 65–81%; at the high dose, 33–49%) and urine (<13%, regardless of dose, sex or label) within 48 hours. In intact animals, most of the radiolabel was excreted in the faeces, probably via the bile, within 120 hours. Based on half-life considerations, there was some evidence of accumulation of radioactivity in plasma and whole blood. Pharmacokinetic parameters after single oral doses indicated that the rate and extent of exposure were higher in males than in females and that this difference was greater at 200 mg/kg bw than at 5 mg/kg bw (Knight, 2009).

In a metabolic study, unchanged pyriofenone represented maxima of 29% of the low dose and 63% of the high dose in faeces. Other components identified in faeces were 2MDPM (maximum 21% of the low dose), 3HDPM (maximum 16% of the low dose) and 4HDPM (maximum 11% of the low dose). In bile, two major metabolites were identified as glucuronide conjugates of 3HDPM and 4HDPM, each accounting for a maximum of 39% of the low dose. All other metabolites in bile accounted for 2% of the dose or less. In the urine of female rats at the low dose, there was one major metabolite at a maximum of 9.5% of the dose, which was identified as an unstable conjugate of 2MDPM. In male and female rats, no other metabolites accounted for more than 2% of the dose in urine (Knight, 2009).

At the T_{max}, the major metabolite in plasma was identified as a glucuronide conjugate of 2MDPM. Parent pyriofenone, 3HDPM and/or 4HDPM were present in extracts of livers from treated rats. Parent pyriofenone was the major identified component in extracts of kidney at the high dose (Knight, 2009).

Toxicological data

The acute toxicity of pyriofenone was studied by oral (LD_{50} > 2000 mg/kg bw), inhalation (LC_{50} > 5.18 mg/L) and dermal (LD_{50} > 2000 mg/kg bw) administration in rats (Hoffman, 2008; Moore, 2008a,b). Pyriofenone was not irritating to the skin of rabbits (Rees, 2008a), but it induced slight ocular irritation in rabbits (Rees, 2008b). Pyriofenone was not sensitizing in mice (Kosaka, 2009).

In a 13-week study in mice using dietary concentrations of pyriofenone of 0, 300, 1000, 3000 and 7000 ppm (equal to 0, 53, 176, 514 and 1318 mg/kg bw per day for males and 0, 61, 214, 695 and 1504 mg/kg bw per day for females, respectively), the NOAEL was 7000 ppm (equal to 1318 mg/kg bw per day), the highest dose tested (Moore, 2009).

In a 28-day dose range–finding study in rats using dietary concentrations of pyriofenone of 0, 300, 3000, 10 000 and 20 000 ppm (equal to 0, 24.2, 251, 823 and 1657 mg/kg bw per day for males and 0, 26.1, 261, 841 and 1660 mg/kg bw per day for females, respectively), the NOAEL was 300 ppm (equal to 24.2 mg/kg bw per day), based on increased serum ALP, total protein and albumin (males), distended caeca (both sexes), increased liver weights (both sexes) and increased kidney weights (females) at 3000 ppm (equal to 251 mg/kg bw per day) (Ohtsuka, 2010a).

In a 93-day study in which rats were administered a dietary concentration of pyriofenone of 0, 300, 1000, 2500 or 5000 ppm (equal to 0, 17.9, 60.5, 150 and 305 mg/kg bw per day for males and 0, 20.6, 69.0, 171 and 350 mg/kg bw per day for females, respectively), the NOAEL was 1000 ppm (equal to 60.5 mg/kg bw per day), based on increased liver weights in both sexes, increased kidney weights in males, increased caecum weights in females, altered clinical chemistry in both sexes and prolonged APTT in females at 2500 ppm (equal to 150 mg/kg bw per day) (Ohtsuka, 2010b).

In a 90-day study in dogs administered a dietary concentration of pyriofenone of 0, 500, 3000 or 25 000 ppm (equal to 0, 15.0, 90.3 and 776 mg/kg bw per day, respectively) for males and 0, 500, 3000 or 15 000 ppm (equal to 0, 15.3, 89.8 and 475 mg/kg bw per day, respectively) for females, the NOAEL was 3000 ppm (equal to 89.8 mg/kg bw per day), based on a marked increase in ALP in females at 15 000 ppm (equal to 475 mg/kg bw per day) (Nakashima, 2010a).

In a 1-year study in dogs administered a dietary concentration of pyriofenone of 0, 500, 3000 or 25 000 ppm (equal to 0, 13.7, 83.5 and 701 mg/kg bw per day, respectively) for males and 0, 500, 3000 or 15 000 ppm (equal to 0, 14.1, 86.2 and 448 mg/kg bw per day, respectively) for females, the NOAEL was 3000 ppm (equal to 83.5 mg/kg bw per day), based on markedly increased ALP, increased GGT and darkened liver colour at 15 000 ppm (equal to 448 mg/kg bw per day) (Nakashima, 2010b).

The overall NOAEL for pyriofenone in dogs was 3000 ppm (equal to 89.8 mg/kg bw per day), and the overall lowest-observed-adverse-effect level (LOAEL) was 15 000 ppm (equal to 448 mg/kg bw per day).

In a 78-week carcinogenicity study in mice administered a dietary concentration of pyriofenone of 0, 600, 1800 or 5400 ppm (equal to 0, 77.6, 237 and 716 mg/kg bw per day, respectively) for males and 0, 300, 1000 or 3000 ppm (equal to 0, 49.4, 167 and 486 mg/kg bw per day, respectively) for females, the NOAEL for systemic toxicity was 600 ppm (equal to 77.6 mg/kg bw per day), based on liver and kidney histopathology (hepatocellular hypertrophy and single-cell necrosis and renal cortical tubular basophilia) in males at 1800 ppm (equal to 237 mg/kg bw per day). The NOAEL for carcinogenicity was 1800 ppm (equal to 237 mg/kg bw per day), based on an increase in the incidence of combined hepatocellular adenomas and adenocarcinomas in males at 5400 ppm (equal to 716 mg/kg bw per day) (Moore, 2010a). The Meeting noted that the incidence, while significant in comparison with concurrent controls, was within the historical control range.

In a 52-week study in rats using dietary administration of pyriofenone at 0, 200, 1000 and 5000 ppm (equal to 0, 8.51, 42.9 and 226 mg/kg bw per day for males and 0, 10.6, 53.5 and 275 mg/kg bw per day for females, respectively), the NOAEL was 1000 ppm (equal to 42.9 mg/kg bw per day), based on increased liver weight and centrilobular hypertrophy, increased APTT, decreased haemoglobin and red blood cell parameters (haematocrit, haemoglobin concentration, haemoglobin width, red blood cell

count), increased GGT, ALP, AST and ALT, and increased total cholesterol in rats of both sexes at 5000 ppm (equal to 226 mg/kg bw per day). Additionally, at 5000 ppm, nephrotoxicity was observed in rats of both sexes, including increased kidney weights, altered clinical chemistry suggestive of renal dysfunction, and renal histopathology (tubular basophilic change in 10/20 males and increased brown pigment deposition in renal tubular cells in 20/20 females) (Ohtsuka, 2010c).

In a 2-year carcinogenicity study in rats administered a dietary concentration of pyriofenone of 0, 200, 1000 or 5000 ppm (equal to 0, 7.25, 36.4 and 197 mg/kg bw per day for males and 0, 9.13, 46.5 and 254 mg/kg bw per day for females, respectively), the NOAEL was 200 ppm (equal to 9.13 mg/kg bw per day), based on CPN in female rats at 1000 ppm (equal to 46.5 mg/kg bw per day). The nephropathy in female rats at 1000 ppm and in both sexes at 5000 ppm was considered related to the nephrotoxicity observed in the 52-week rat study in both sexes at 5000 ppm (Ohtsuka, 2010c). There was no evidence of increased neoplasia or tumour incidence after exposure to pyriofenone (Ohtsuka, 2010d).

The Meeting concluded that there was limited evidence of carcinogenicity in the livers of male mice and that pyriofenone was not carcinogenic in female mice or rats.

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

The Meeting concluded that pyriofenone is unlikely to be genotoxic.

In view of the lack of genotoxicity, the absence of carcinogenicity in rats and the fact that hepatocellular adenomas and adenocarcinomas were increased only in male mice at the highest dose tested, the Meeting concluded that pyriofenone is unlikely to pose a carcinogenic risk to humans from the diet.

In a two-generation study of reproductive toxicity in rats, pyriofenone was administered in the diet to rats at 0, 150, 1000 or 5000 ppm (equal to 0, 7.29, 47.8 and 257 mg/kg bw per day for F_0 males and 0, 9.5, 58.1 and 301 mg/kg bw per day for F_0 females, respectively). The NOAEL for parental toxicity was 1000 ppm (equal to 47.8 mg/kg bw per day), based on altered haematological parameters and changes in liver, kidney, thyroid and caecum weights in F_0 and F_1 rats at 5000 ppm (equal to 257 mg/kg bw per day). The NOAEL for reproductive toxicity and offspring toxicity was 5000 ppm (equal to 257 mg/kg bw per day), the highest dose tested (Hojo, 2009a).

In a study of developmental toxicity, rats were dosed with pyriofenone by oral gavage from GD 6 to GD 19 at 0, 30, 300 or 1000 mg/kg bw per day. The NOAEL for maternal toxicity was 30 mg/kg bw per day, based on increased liver and caecum weights in dams at 300 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 1000 mg/kg bw per day, the highest dose tested (Hojo, 2009b).

In a study of developmental toxicity, rabbits were dosed with pyriofenone by gavage from GDs 6 to 27 at 0, 30, 300 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 (Takahashi, 2009).

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

Pyriofenone did not produce neurotoxicity in acute studies in rats at doses up to 2000 mg/kg bw or in subchronic studies in rats at doses up to 927 mg/kg bw per day (Arrowsmith, 2010b; Powell, 2010).

The Meeting concluded that pyriofenone is not neurotoxic.

Pyriofenone did not produce immunotoxicity in 4-week studies in female mice at doses up to 1270 mg/kg bw per day or in female rats at doses up to 1690 mg/kg bw per day (Chambers, 2010; Laurent, 2010).

The Meeting concluded that pyriofenone is not immunotoxic.

No information on the potential effects of pyriofenone on the microbiome of the human gastrointestinal tract is available.

Toxicological data on metabolites and/or degradates

The main metabolites of pyriofenone were tested in acute toxicity studies and genotoxicity studies. The acute oral toxicity of 2MDPM, 3HDPM and 4HDPM was studied by the acute toxic class method in rats (all LD_{50}s > 2000 mg/kg bw) (Fukuyama, 2010; Moore, 2010b,c). All three metabolites tested negative for mutagenicity in bacterial reverse mutation assays (May, 2010a,b; Wada, 2010).

Human data

In reports on manufacturing plant personnel, no adverse health effects were noted. No information on accidental or intentional poisonings in humans was identified.

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

Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) of 0–0.09 mg/kg bw on the basis of the NOAEL of 9.13 mg/kg bw per day from the 2-year carcinogenicity study in rats for CPN in females at 46.5 mg/kg bw per day. A safety factor of 100 was applied. The upper bound of the ADI provides a margin of about 8000 relative to the LOAEL for carcinogenicity in male mice.

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

Levels relevant to risk assessment of pyriofenone

Species	Study	Effect	NOAEL	LOAEL
Mouse	Seventy-eight-week study of carcinogenicity[a]	Toxicity	600 ppm, equal to 77.6 mg/kg bw per day	1 800 ppm, equal to 237 mg/kg bw per day
		Carcinogenicity	1 800 ppm, equal to 237 mg/kg bw per day	5 400 ppm, equal to 716 mg/kg bw per day
Rat	Two-year study of carcinogenicity[a]	Toxicity	200 ppm, equal to 9.13 mg/kg bw per day	1 000 ppm, equal to 46.5 mg/kg bw per day
		Carcinogenicity	5 000 ppm, equal to 197 mg/kg bw per day[b]	–
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	5 000 ppm, equal to 257 mg/kg bw per day[b]	–
		Parental toxicity	1 000 ppm, equal to 47.8 mg/kg bw per day	5 000 ppm, equal to 257 mg/kg bw per day
		Offspring toxicity	5 000 ppm, equal to 257 mg/kg bw per day[b]	–
	Developmental toxicity study[c]	Maternal toxicity	30 mg/kg bw per day	300 mg/kg bw per day

Species	Study	Effect	NOAEL	LOAEL
		Embryo and fetal toxicity	1 000 mg/kg bw per day[b]	–
Rabbit	Developmental toxicity study[c]	Maternal toxicity	1 000 mg/kg bw per day[b]	–
		Embryo and fetal toxicity	1 000 mg/kg bw per day[b]	–
Dog	Three-month and 1-year studies of toxicity[a,d]	Toxicity	3 000 ppm, equal to 89.8 mg/kg bw per day	15 000 ppm, equal to 448 mg/kg bw per day

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

Acceptable daily intake (ADI)

0–0.09 mg/kg bw per day

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 pyriofenone

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid; 76–89% of the dose at 5 mg/kg bw and 36–53% of the dose at 200 mg/kg bw in bile duct–cannulated rats
Dermal absorption	No data
Distribution	Widespread: highest concentrations in liver, kidney, plasma, whole blood and blood cells
Potential for accumulation	Some evidence of accumulation in plasma and whole blood
Rate and extent of excretion	Rapid: Largely excreted into bile and urine by 48 hours
Metabolism in animals	Converted to 2MDPM, 3HDPM and 4HDPM
Toxicologically significant compounds in animals and plants	Pyriofenone
Acute toxicity	
Rat, LD_{50}, oral	>2 000 mg/kg bw
Rat, LD_{50}, dermal	>2 000 mg/kg bw
Rat, LC_{50}, inhalation	>5.18 mg/L
Rabbit, dermal irritation	Not irritating
Rabbit, ocular irritation	Slightly irritating

Mouse, dermal sensitization	Not sensitizing
Short-term studies of toxicity	
Target/critical effect	Liver, kidney, caecum / increased APTT, increased serum protein, albumin and calcium, and decreased serum chloride
Lowest relevant oral NOAEL	60.5 mg/kg bw per day (rat)[a]
Lowest relevant dermal NOAEL	1 000 mg/kg bw per day, highest dose tested (rat)
Lowest relevant inhalation NOAEC	No data
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Kidney/CPN in female rats
Lowest relevant NOAEL	9.13 mg/kg bw per day (rat)
Carcinogenicity	Limited evidence of carcinogenicity in the liver of male mice, not carcinogenic in female mice or rats[b]
Genotoxicity	
	No evidence of genotoxicity[b]
Reproductive toxicity	
Target/critical effect	No reproductive effects / increased liver and caecum weights, anaemia in parental animals
Lowest relevant reproductive NOAEL	257 mg/kg bw per day, highest dose tested (rat)
Lowest relevant parental NOAEL	47.8 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	257 mg/kg bw per day, highest dose tested (rat)
Developmental toxicity	
Target/critical effect	No embryo/fetal effects / increased liver and caecum weights in maternal rats
Lowest relevant maternal NOAEL	30 mg/kg bw per day (rat)
Lowest relevant developmental NOAEL	1 000 mg/kg bw per day, highest dose tested (rat and rabbit)
Neurotoxicity/delayed neurotoxicity	
Acute neurotoxicity	2 000 mg/kg bw, highest dose tested (rat)
Subchronic neurotoxicity	927 mg/kg bw, highest dose tested (rat)
Other toxicological studies	
Immunotoxicity	Negative
Studies with metabolites 2MDPM, 3HDPM and 4HDPM	Oral LD_{50} > 2 000 mg/kg bw (rats) Negative in bacterial mutagenesis assays
Human data	
	None identified

[a] A NOAEL of 24.2 mg/kg bw per day was noted in a 28-day dose range–finding study in rats.
[b] Unlikely to pose a carcinogenic risk to humans via exposure from the diet.

Summary

	Value	Study	Safety factor
ADI	0–0.09 mg/kg bw per day	Two-year carcinogenicity study in rats	100
ARfD	Unnecessary	–	–

References

Arrowsmith W (2010a). IKF-309: assessment of effects on hepatic drug metabolising enzyme induction and hepatocyte proliferation by dietary administration to male CD1 mice for 4 weeks. Unpublished report no. JSM0045. Huntingdon Life Sciences, Alconbury, United Kingdom. Submitted to WHO by Ishihara Sangyo Kaisha, Ltd.

Arrowsmith W (2010b). IKF-309: neurotoxicity study by dietary administration to CD rats for 13 weeks. Unpublished report no. JSM0027. Huntingdon Life Sciences, Alconbury, United Kingdom. Submitted to WHO by Ishihara Sangyo Kaisha, Ltd.

Chambers PR (2010). IKF-309: 4-week dietary immunotoxicity study in the female rat. Unpublished report no. JSM0037. Huntingdon Life Sciences, Alconbury, United Kingdom. Submitted to WHO by Ishihara Sangyo Kaisha, Ltd.

Cooper S (2010). Toxicity study by dermal administration to CD rats for 4 weeks. Unpublished report no. JSM0033. Huntingdon Life Sciences, Alconbury, United Kingdom. Submitted to WHO by Ishihara Sangyo Kaisha, Ltd.

Fukuyama T (2010). 4HDPM: acute oral toxicity study in rats. Unpublished report no. IET 10-0093. The Institute of Environmental Toxicology, Uchimoriya-machi, Japan. Submitted to WHO by Ishihara Sangyo Kaisha, Ltd.

Hoffman GM (2008). IKF-309 technical: an acute (4-hour) inhalation toxicity study in the rat via nose-only exposure. Unpublished report no. 07-5532. Huntingdon Life Sciences, East Millstone, USA. Submitted to WHO by Ishihara Sangyo Kaisha, Ltd.

Hojo H (2009a). IKF-309 technical: a reproduction toxicity study in rats. Unpublished report no. IET 06-0112. The Institute of Environmental Toxicology, Uchimoriya-machi, Japan. Submitted to WHO by Ishihara Sangyo Kaisha, Ltd.

Hojo H (2009b). IKF-309 technical: a teratogenicity study in rats. Unpublished report no. IET 06-0114. The Institute of Environmental Toxicology, Uchimoriya-machi, Japan. Submitted to WHO by Ishihara Sangyo Kaisha, Ltd.

Hynes L (2008a). IKF-309 technical: in vitro mutation test using mouse lymphoma L5178Y cells. Unpublished report no. ISK0310. Huntingdon Life Sciences, Alconbury, United Kingdom. Submitted to WHO by Ishihara Sangyo Kaisha, Ltd.

Hynes G (2008b). IKF-309 technical: mouse micronucleus test. Unpublished report no. ISK0327. Huntingdon Life Sciences, Alconbury, United Kingdom. Submitted to WHO by Ishihara Sangyo Kaisha, Ltd.

Ishihara Sangyo Kaisha, Ltd (2017). Pyriofenone. Toxicological data for JMPR evaluation 2018. Osaka, Japan. Submitted to WHO by Ishihara Sangyo Kaisha, Ltd.

Knight LJL (2009). IKF-309: metabolism in rats. Unpublished report no. ISK0281. Huntingdon Life Sciences, Alconbury, United Kingdom. Submitted to WHO by Ishihara Sangyo Kaisha, Ltd.

Kosaka T (2009). IKF 309 technical: skin sensitisation study in mice – local lymph node assay. Unpublished report no. IET 07-0113. The Institute of Environmental Toxicology, Uchimoriya-machi, Japan. Submitted to WHO by Ishihara Sangyo Kaisha, Ltd.

Laurent MC (2010). IKF-309: 4-week dietary immunotoxicity study in the female mouse. Unpublished report no. JSM0036. Huntingdon Life Sciences, Alconbury, United Kingdom. Submitted to WHO by Ishihara Sangyo Kaisha, Ltd.

May K (2007). IKF-309 technical: bacterial reverse mutation test. Unpublished report no. ISK0311/073744. Huntingdon Life Sciences, Alconbury, United Kingdom. Submitted to WHO by Ishihara Sangyo Kaisha, Ltd.

May K (2010a). 2MDPM: bacterial reverse mutation test. Unpublished report no. JSM0127. Huntingdon Life Sciences, Alconbury, United Kingdom. Submitted to WHO by Ishihara Sangyo Kaisha, Ltd.

May K (2010b). 3HDPM: bacterial reverse mutation test. Unpublished report no. JSM0128 by Huntingdon Life Sciences, Alconbury, United Kingdom. Submitted to WHO by Ishihara Sangyo Kaisha, Ltd.

Moore EL (2008a). IKF-309 technical: acute oral toxicity to the rat (acute toxic class method). Unpublished report no. ISK0313. Huntingdon Life Sciences, Alconbury, United Kingdom. Submitted to WHO by Ishihara Sangyo Kaisha, Ltd.

Moore EL (2008b). IKF-309 technical: acute dermal toxicity to the rat. Unpublished report no. ISK0312. Huntingdon Life Sciences, Alconbury, United Kingdom. Submitted to WHO by Ishihara Sangyo Kaisha, Ltd.

Moore EL (2009). IKF-309: toxicity study by dietary administration to CD1 mice for 13 weeks. Unpublished report no. ISK0291. Huntingdon Life Sciences, Alconbury, United Kingdom. Submitted to WHO by Ishihara Sangyo Kaisha, Ltd.

Moore EL (2010a). IKF-309: carcinogenicity study by dietary administration to CD1 mice for 78 weeks. Unpublished report no. ISK0305. Huntingdon Life Sciences, Alconbury, United Kingdom. Submitted to WHO by Ishihara Sangyo Kaisha, Ltd.

Moore ML (2010b). 2MDPM: acute oral toxicity to the rat (acute toxic class method). Unpublished report no. JSM0115. Huntingdon Life Sciences, Alconbury, United Kingdom. Submitted to WHO by Ishihara Sangyo Kaisha, Ltd.

Moore ML (2010c). 3HDPM: acute oral toxicity to the rat (acute toxic class method). Unpublished report no. JSM0145. Huntingdon Life Sciences, Alconbury, United Kingdom. Submitted to WHO by Ishihara Sangyo Kaisha, Ltd.

Nakashima N (2010a). IKF-309 technical: repeated dose 90-day oral toxicity study in dogs. Unpublished report no. IET 06-0109. The Institute of Environmental Toxicology, Uchimoriya-machi, Japan. Submitted to WHO by Ishihara Sangyo Kaisha, Ltd.

Nakashima N (2010b). IKF-309 technical: repeated dose 1-year oral toxicity study in dogs. Unpublished report no. IET 06-0110. The Institute of Environmental Toxicology, Uchimoriya-machi, Japan. Submitted to WHO by Ishihara Sangyo Kaisha, Ltd.

Ohtsuka R (2010a). IKF-309 technical: repeated dose 28-day oral toxicity study in rats. Unpublished report no. IET 06-0014. The Institute of Environmental Toxicology, Uchimoriya-machi, Japan. Submitted to WHO by Ishihara Sangyo Kaisha, Ltd.

Ohtsuka R (2010b). IKF-309 technical: repeated dose 90-day oral toxicity study in rats. Unpublished report no. IET 06-0015. The Institute of Environmental Toxicology, Uchimoriya-machi, Japan. Submitted to WHO by Ishihara Sangyo Kaisha, Ltd.

Ohtsuka R (2010c). IKF-309 technical: repeated dose 1-year toxicity study in rats. Unpublished report no. IET 06-0085. The Institute of Environmental Toxicology, Uchimoriya-machi, Japan. Submitted to WHO by Ishihara Sangyo Kaisha, Ltd.

Ohtsuka R (2010d). IKF-309 technical: carcinogenicity study in rats. Unpublished report no. IET 06-0086. The Institute of Environmental Toxicology, Uchimoriya-machi, Japan. Submitted to WHO by Ishihara Sangyo Kaisha, Ltd.

Powell LAJ (2010). IKF-309: neurotoxicity study by a single oral gavage administration to CD rats followed by a 14-day observation period. Unpublished report no. ISK0407. Huntingdon Life Sciences, Alconbury, United Kingdom. Submitted to WHO by Ishihara Sangyo Kaisha, Ltd.

Pritchard L (2008). IKF-309 technical: in vitro mammalian chromosome aberration test in CHL cells. Unpublished report no. ISK0322. Huntingdon Life Sciences, Alconbury, United Kingdom. Submitted to WHO by Ishihara Sangyo Kaisha, Ltd.

Rees P (2008a). IKF-309 technical: skin irritation to the rabbit. Unpublished report no. ISK0315. Huntingdon Life Sciences, Alconbury, United Kingdom. Submitted to WHO by Ishihara Sangyo Kaisha, Ltd.

Rees P (2008b). IKF-309 technical: eye irritation to the rabbit. Unpublished report no. ISK0314. Huntingdon Life Sciences, Alconbury, United Kingdom. Submitted to WHO by Ishihara Sangyo Kaisha, Ltd.

Takahashi K (2009). IKF-309 technical: teratogenicity study in rabbits. Unpublished report no. IET 06-0116. The Institute of Environmental Toxicology, Uchimoriya-machi, Japan. Submitted to WHO by Ishihara Sangyo Kaisha, Ltd.

Wada K (2010). 4HDPM: bacterial reverse mutation test. Unpublished report no. IET 10-0094. The Institute of Environmental Toxicology, Uchimoriya-machi, Japan. Submitted to WHO by Ishihara Sangyo Kaisha, Ltd.

Wada K (2017). IKF-309 technical: comet assay in rats. Unpublished report no. IET 16-0074. The Institute of Environmental Toxicology, Uchimoriya-machi, Japan. Submitted to WHO by Ishihara Sangyo Kaisha, Ltd.

Yamamoto D (2017). A liver carcinogenic mechanism analysis study of IKF-309 in CAR KO rats. Unpublished report no. B160815. LSI Medience Corporation, Tokyo, Japan. Submitted to WHO by Ishihara Sangyo Kaisha, Ltd.

TIOXAZAFEN

First draft prepared by
G. Wolterink[1] *and A.R. Boobis*[2]

[1] *Centre for Nutrition, Prevention and Health Services, National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands*
[2] *Department of Medicine, Imperial College London, London, United Kingdom*

Explanation 711
Evaluation for acceptable intake 712
1. Biochemical aspects 712
1.1 Absorption, distribution and excretion 712
(a) Oral route 712
(b) Dermal route 718
1.2 Biotransformation 719
2. Toxicological studies 723
2.1 Acute toxicity 723
(a) Lethal doses 723
(b) Dermal irritation 723
(c) Ocular irritation 723
(d) Dermal sensitization 724
2.2 Short-term studies of toxicity 724
(a) Oral administration 724
(b) Dermal application 730
(c) Exposure by inhalation 731
2.3 Long-term studies of toxicity and carcinogenicity 733
2.4 Genotoxicity 739
2.5 Reproductive and developmental toxicity 740
(a) Multigeneration studies 740
(b) Developmental toxicity 741
2.6 Special studies 742
(a) Immunotoxicity 742
(b) Mode of action 743
(c) Neurotoxicity 753
(d) Studies with metabolites and impurities 754
3. Observations in humans 758
Comments 758
Toxicological evaluation 761
References 764

Explanation

Tioxazafen (Fig. 1) is the International Organization for Standardization–approved common name for 3-phenyl-5-thiophen-2-yl-1,2,4-oxadiazole (International Union of Pure and Applied Chemistry), which has the Chemical Abstracts Service number 330459-31-9.

Tioxazafen is a seed treatment nematicide for use on corn, soy and cotton. It appears to act through interaction with a nematode-specific insertion of the L3 subunit of the mitochondrial ribosome, leading to disruption of ribosomal translation in nematodes.

Tioxazafen 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.

Fig. 1. Chemical structure of tioxazafen

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

Evaluation for acceptable intake

1. Biochemical aspects

Absorption, distribution, metabolism and excretion (ADME) studies were conducted in mice and rats using tioxazafen radiolabelled with ^{14}C in the phenyl or the thiophene ring (Fig. 2).

Fig. 2. Position of ^{14}C label in tioxazafen used in rat ADME studies

[oxadiazole-3-^{13}C, phenyl-UL-^{14}C]tioxazafen (PH-tioxazafen)

[oxadiazole-5-^{13}C, thiophene-2-^{14}C]tioxazafen (TH-tioxazafen)

* Denotes the position of the radiolabel.

Source: Thomas (2014)

1.1 Absorption, distribution and excretion

(a) Oral route

Mice

Groups of 25 male and 25 female CD-1 mice received non-radiolabelled tioxazafen (batch no. GLP-1106-21504-T; purity 98.23%) for 3 days at a dietary concentration of 100, 250, 750 or 1750 parts per million (ppm) (equal to 22, 61, 159 and 451 mg/kg body weight [bw] per day, respectively) for males and 50, 100, 250 or 750 ppm (equal to 12, 28, 63 and 167 mg/kg bw per day, respectively) for females prior to radiolabel administration. On the morning of the fourth day, all animals were also

administered a small dose of [oxadiazole-3-^{13}C, phenyl-UL-^{14}C]tioxazafen (PH-tioxazafen) (batch no. 69757-1-95-1; radiochemical purity 98.3%) by oral gavage (see Table 1). All animals remained on treated diets until scheduled euthanasia.

Table 1. Study design for absorption, distribution and excretion study in mice

	Dietary concentration of tioxazafen (ppm)		**Oral gavage dose of [^{14}C]tioxazafen**	
Group	**Males**	**Females**	**mg/kg bw**	**μCi[a]/kg bw**
1	100	50	1.07	80
2	250	100	1.60	120
3	750	250	1.60	120
4	1 750	750	2.14	160

bw: body weight; ppm: parts per million
[a] 1 μCi = 37 kBq.
Source: Godsey (2015)

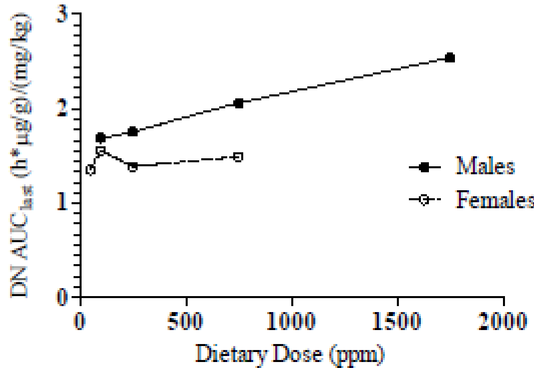
Blood was sampled from five animals of each sex per dose euthanized at approximately 1, 2, 4, 8 and 24 hours following radiolabel administration. Urine and faeces were collected for 24 hours from those animals designated for the 24-hour blood sampling. Plasma, urine and faeces were analysed for total radioactivity by liquid scintillation counting. Metabolism data are presented in section 1.2.

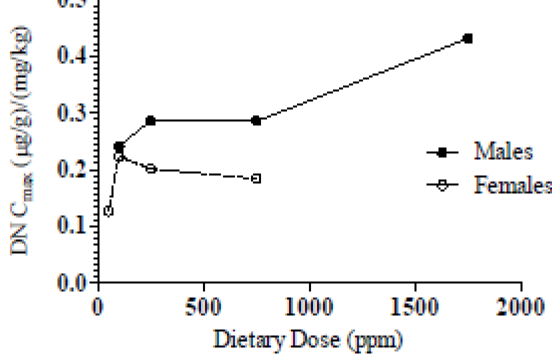
A 13% loss in body weight occurred in 1750 ppm males during the 3 days prior to radiolabel administration, whereas body weight gain was slightly reduced in 750 ppm males. Feed consumption was not affected. Total recovery of radioactivity in excreta was 72–98%. Because the doses of radiolabelled tioxazafen were not equivalent for all unlabelled tioxazafen dose groups, pharmacokinetic parameters (i.e. maximum concentration [C_{max}] and the area under the plasma concentration–time curve [AUC]) were normalized by dividing AUC and C_{max} by the actual dose of radiolabelled tioxazafen administered for each group. Consistent increases in dose-normalized AUC and dose-normalized C_{max} were observed in 1750 ppm males; these findings suggest that systemic exposure in this group increased in a greater-than-proportional manner relative to the dose administered (Fig. 3). Slight differences in dose-normalized AUC and C_{max} in the lower-dose males were not considered to be meaningful owing to the small magnitude of the differences or the lack of a clear dose–response relationship. No meaningful dose-related changes in AUC or C_{max} were noted in females. However, it is noted that the maximum dose administered to females was 750 ppm.

For all dose groups, the peak concentration was observed at 1 hour post-dosing, with the exception of the 50 ppm females, for which the peak concentration was observed at 2 hours post-dosing. Plasma half-lives ($t_{½}$) were similar across dose groups, ranging from 8.15 to 9.86 hours for males and from 9.52 to 11.9 hours for females.

Following 3 days of dietary exposure to non-radiolabelled tioxazafen and a single oral gavage dose of [^{14}C]tioxazafen, an approximately equal portion of the administered dose was excreted in the urine and faeces over 24 hours post-dosing, except for the 1750 ppm males, for which excretion in faeces and overall recovery of radioactivity in the excreta were somewhat low (Table 2). This may be due to slightly delayed faecal elimination at the highest dose (Godsey, 2015).

Fig. 3. Dose-normalized AUC in plasma and C_{max} for tioxazafen following oral administration of [14C]tioxazafen to mice





AUC_{last}: area under the plasma concentration–time curve at the last time point; C_{max}: maximum concentration; DN: dose-normalized; ppm: parts per million

Source: Godsey (2015)

Table 2. Recovery of radioactivity in excreta following oral administration of [14C]tioxazafen to mice

	Recovery of radioactivity (% of administered radiolabel)							
	Males[a]				Females[a]			
Excreta	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Urine	42.3	58.9	39.7	43.0	35.6	42.7	47.9	47.6
Faeces	43.2	34.1	37.9	26.1	45.8	51.3	47.0	44.6
Cage rinse	0.58	4.61	1.00	2.46	3.22	2.12	2.76	4.30
Total recovery	86.1	97.5	78.6	71.6	84.7	96.1	97.7	96.5

ppm: parts per million

[a] Groups: males, 1 = 100 ppm, 2 = 250 ppm, 3 = 750 ppm, 4 = 1750 ppm; females, 1 = 50 ppm, 2 = 100 ppm, 3 = 250 ppm, 4 = 750 ppm.

Source: Godsey (2015)

Rats

Various experiments were conducted in Sprague Dawley (Crl:CD(SD)) rats to investigate the toxicokinetics and metabolism of tioxazafen (Table 3). The rats received either a low dose (3 mg/kg bw) or a high dose (100 mg/kg bw) of either PH-tioxazafen (radiochemical purity 98.5–99.4%; "A"

groups) or TH-tioxazafen (radiochemical purity 99.0%; “B” groups). Oral doses were administered by gavage as a suspension in 0.5% carboxymethylcellulose in water. Intravenous doses were single bolus doses administered as a solution in ethanol:Cremophor EL:saline. Animals were not fasted prior to dosing.

Table 3. Summary of experiments performed to investigate the toxicokinetics and metabolism of tioxazafen in rats

Group	Route	^{14}C label	Dose (mg/kg bw)	Number of animals	Sample collection
Pilot phase					
1A	Oral	PH	3	2 M	Expired air, excreta
1B	Oral	TH	3	2 M	Expired air, excreta
Pharmacokinetics phase					
2A	iv	PH	3	8 M	Blood
2B	iv	TH	3	8 M	Blood
3A	Oral	PH	3	8 M	Blood
3B	Oral	TH	3	8 M	Blood
4A	Oral	PH	100	8 M	Blood
4B	Oral	TH	100	8 M	Blood
Disposition and metabolite identification phase					
5A	iv	PH	3	4 M	Excreta, blood, tissues, carcass
5B	iv	TH	3	4 M	Excreta, blood, tissues, carcass
6A	Oral	PH	3	4 M / 4 F	Excreta, blood, tissues, carcass
6B	Oral	TH	3	4 M / 4 F	Excreta, blood, tissues, carcass
7A	Oral	PH	100	4 M	Excreta, blood, tissues, carcass
7B	Oral	TH	100	4 M	Excreta, blood, tissues, carcass
8A	Repeat oral	PH	3	4 M	Excreta, blood, tissues, carcass
8B	Repeat oral	TH	3	4 M	Excreta, blood, tissues, carcass
11A (bile duct cannulation)	Oral	PH	100	4 M	Bile, excreta, carcass
11B (bile duct cannulation)	Oral	TH	100	4 M	Bile, excreta, carcass
12A	Oral	PH	3	4 M / 4 F	Excreta, blood, tissues, carcass
13A	Repeat oral	TH	3	4 M	Excreta, blood, tissues, carcass

Group	Route	^{14}C label	Dose (mg/kg bw)	Number of animals	Sample collection
Quantitative whole-body autoradiography phase					
9A	Oral	PH	3	4 M / 4 F	Blood, carcass
9B	Oral	TH	3	4 M / 4 F	Blood, carcass
10A	Oral	PH	100	4 M / 4 F	Blood, carcass
10B	Oral	TH	100	4 M / 4 F	Blood, carcass

bw: body weight; F: females; iv: intravenous; M: males; PH: phenyl label; TH: thiophene label
Source: Thomas (2014)

Metabolite profiles in excreta were identified using radio–high-performance liquid chromatography (HPLC) and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). These data are presented in section 1.2.

In the pilot phase (Groups 1A and 1B), expired air components, urine and faeces were collected up to 48 hours post-dosing, and total radioactivity was analysed.

In the pharmacokinetics phase (Groups 2A, 2B, 3A, 3B, 4A and 4B), blood samples were collected from four animals per group per time point for up to 96 hours post-dosing for Groups 2A and 2B and up to 168 hours post-dosing for Groups 3A, 3B, 4A and 4B.

In the disposition and metabolite identification (see section 1.2) phase (Groups 5A, 5B, 6A, 6B, 7A, 7B, 8A, 8B, 12A and 13A), urine and faeces were collected up to 168 hours post-dosing. Groups 8B and 13A received a daily gavage dose (3 mg/kg bw) of non-labelled tioxazafen for 14 consecutive days followed by a single gavage dose of PH-tioxazafen or TH-tioxazafen. Following necropsy, specified tissues and the carcasses were collected and analysed for total radioactivity. Animals in Groups 11A and 11B received a single gavage dose (100 mg/kg bw) of PH-tioxazafen or TH-tioxazafen, and urine, faeces and bile were collected up to 48 hours post-dosing.

In the quantitative whole-body autoradiography phase, blood was sampled from two animals of each sex from Groups 9A and 9B at 2 and 48 hours post-dosing and from two animals of each sex from Groups 10A and 10B at 4 and 48 hours post-dosing. Subsequently, the carcasses were embedded in carboxymethylcellulose for quantitative whole-body autoradiography sectioning and analysis.

In the pilot phase, following a single oral dose at 3 mg/kg bw, the total recovery of administered radioactivity in the urine, faeces, cage rinse, expired air, activated carbon and cage wash after 48 hours was 67.6% and 76.9% of the dose for PH-tioxazafen and TH-tioxazafen, respectively, with 21–23% of the dose in urine, 35–45% in faeces and less than 1% in expired air.

The plasma pharmacokinetics of tioxazafen following a single intravenous dose at 3 mg/kg bw or a single oral dose at either 3 or 100 mg/kg bw in male rats is shown in Table 4. The plasma C_{max} values (time to reach C_{max} [T_{max}] = 2 hours) were 0.5–0.8 µg/g following an oral dose of 3 mg/kg bw, and the AUC_{∞} was slightly lower for the oral dose (8–12 µg·h/g) than for the intravenous dose (15–17 µg·h/g). The $t_{½}$ in plasma was 44–47 hours following the low oral dose and 38–42 hours following the high oral dose. Bioavailability (% oral absorption) calculated from the intravenous dose and the low oral dose was approximately 58% for PH-tioxazafen and 73% for TH-tioxazafen. At the high oral dose (100 mg/kg bw), the plasma C_{max} values (T_{max} = 4 hours) were 23–25 µg/g. The AUC_{∞} was 543–587 µg·h/g and may have been slightly more than dose proportionate. However, this is likely due to binding of tioxazafen to the dosing syringe at the low dose.

In general, more than 95% of the total amount of radioactivity recovered was collected in the excreta in the first 48 hours following dosing. There was generally no difference in excretion pattern related to the position of the radiolabel, dose, dose route, dosing regimen (single or repeated administration) or sex of the rats. As shown in Table 5, in all groups, about 45–69% of the administered radioactivity was recovered from faeces and 24–38% of the dose was recovered from

urine. There was possibly a slight difference in the pattern of excretion when comparing the intravenous PH- and TH-label doses, for which urinary elimination was 38% and 30% of the eliminated dose, respectively.

Table 4. Pharmacokinetics of tioxazafen equivalents in male rats following administration of [^{14}C]tioxazafen

Parameter	Intravenous administration			
	PH-tioxazafen 3 mg/kg bw		TH-tioxazafen 3 mg/kg bw	
AUC_∞ (µg·h/g)	14.6		17.1	
$t_{½}$ (h)	34.3		35.4	
C_0 (µg/g)	1.98		2.41	
	Oral administration			
	PH-tioxazafen		**TH-tioxazafen**	
	3 mg/kg bw	**100 mg/kg bw**	**3 mg/kg bw**	**100 mg/kg bw**
AUC_∞ (µg·h/g)	8.40	587	12.4	543
$t_{½}$ (h)	44.2	41.7	46.8	37.9
C_{max} (µg/g)	0.541	25.2	0.800	23.2
T_{max} (h)	2	4	2	4
Bioavailability (%)	57.5	121	72.7	95.1

AUC_∞: estimated area under the concentration–time curve from the time of dosing to infinity; bw: body weight; C_0: initial concentration; C_{max}: maximum concentration; PH-tioxazafen: [oxadiazole-3-^{13}C, phenyl-UL-^{14}C]tioxazafen; $t_{½}$: half-life; TH-tioxazafen: [oxadiazole-5-^{13}C, thiophene-2-^{14}C]tioxazafen; T_{max}: time to reach maximum concentration; UL: uniformly labelled

Source: Thomas (2014)

Table 5. Recovery of [^{14}C]tioxazafen in excreta of rats over 168 hours after intravenous and oral administration

	Mean % of administered dose									
	3 mg/kg bw (single dose)						100 mg/kg bw (single dose)		3 mg/kg bw (repeated dose)	
	Intravenous		Oral				Oral		Oral	
	PH	TH	PH		TH		PH	TH	PH[a]	TH[b]
Matrix	**Males**	**Males**	**Males**	**Females**	**Males**	**Females**	**Males**	**Males**	**Males**	**Males**
Urine	38.1	29.9	31.5	24.4	29.7	34.9	31.3	31.1	32.9	30.5
Faeces	51.4	60.4	58.7	44.8	68.6	65.0	53.1	62.0	61.1	63.1
Cage rinse	3.91	2.90	2.88	3.27	3.10	3.85	2.88	2.90	2.84	2.87
Cage wash DI/MeOH	0.03/ 0.01	0.06/ 0.01	0.03/ 0.02	0.05/ 0.04	0.02/ 0.01	0.13/ 0.02	0.03/ 0.01	0.04/ 0.01	0.02/ 0.04	0.02/ 0.02
Total	93.4	93.3	93.2	72.6	101.5	103.9	87.3	96.1	97.0	96.5

bw: body weight; DI: deionized water; MeOH: methanol; PH: [phenyl-UL-^{14}C, oxadiazole-3-^{13}C]tioxazafen; TH: [thiophene-2-^{14}C, oxadiazole-5-^{13}C]tioxazafen; UL: uniformly labelled

[a] Non-labelled tioxazafen for 14 consecutive days followed by a single gavage dose of PH-tioxazafen.

[b] Non-labelled tioxazafen for 14 consecutive days followed by a single gavage dose of TH-tioxazafen.

Source: Thomas (2014)

In bile duct–cannulated rats, approximately 60% of PH-tioxazafen was recovered in the bile, 21% in the urine and 3.3% in the faeces over 48 hours post-dosing, indicating that greater than 97% of the dose was absorbed. The TH-tioxazafen dose was primarily recovered in the urine (45%) and the bile (32.2%), followed by the faeces (11%), indicating that greater than 89% of the dose was absorbed.

Tissue distribution was not affected by the position of the radiolabel, sex of the rats or single-dose versus repeated-dose administration. The concentration in tissues at 168 hours post-dosing following a 3 mg/kg bw dose was generally less than 0.1 µg/g, with the exception of the adrenal gland (mean concentration across all groups 0.3–0.73 µg/g), kidneys (0.14–0.32 µg/g), liver (0.08–0.16 µg/g) and thyroid (0.07–0.23 µg/g). At 168 hours following a 100 mg/kg bw dose, highest concentrations were found in the adrenal gland (14–16 µg/g), followed by the kidneys (4.6–7.0 µg/g), liver (3.0–3.6 µg/g) and thyroid (2.8–3.6 µg/g). All other tissues had mean concentrations ranging from 0.07 to 1.7 µg/g. Tissue concentrations were proportionate to dose in the 3 and 100 mg/kg bw dose groups.

The greatest recovery was in the liver (up to 0.23% of the dose). In the remaining carcass, 0.43–0.73% of the administered dose was recovered.

The quantitative whole-body autoradiography showed that tioxazafen is widely distributed and that the radioactivity rapidly declined over the 48-hour period. Across doses (3 or 100 mg/kg bw) and collection times (T_{max} or 48 hours), tissue-to-plasma ratios were generally significantly higher than unity in the adrenal gland, liver, total kidney, kidney cortex, thyroid gland, stomach and small intestine (at T_{max}), fat (100 mg/kg bw dose) and urinary bladder.

At T_{max} (2 hours post-dosing), the highest concentrations following administration of the 3 mg/kg bw dose were in the stomach, kidney cortex, liver, total kidney and adrenal gland (generally greater than 2.5 µg/g), followed by the bladder, kidney medulla, Harderian gland, small intestine, thyroid and lung (generally greater than 0.5 µg/g). There appeared to be a sex difference in the stomach concentration – i.e. 9–26 µg/g in males compared with 0.8–5.6 µg/g in females at T_{max}. The lowest concentrations were in the eye and bone (<0.1 µg/g). Forty-eight hours after dosing at 3 mg/kg bw, the concentrations had decreased to less than or equal to 1.6 µg/g for the adrenal gland, less than 0.5 µg/g for the next highest tissues (total kidney and kidney cortex, liver, urinary bladder and thyroid) and less than 0.1 µg/g for the remaining tissues.

At T_{max} (4 hours post-dosing), the highest concentrations following administration of the 100 mg/kg bw dose were in the adrenal gland and urinary bladder of males and females after PH-tioxazafen treatment and in the adrenal gland, kidney cortex, liver and stomach of males and adrenal gland and fat of females after TH-tioxazafen treatment (100–500 µg/g). Concentrations in the Harderian gland, small intestine, thyroid, total kidney, fat and liver in the other groups were about 30–80 µg/g. The lowest concentrations were in the eye and bone (<5 µg/g). For the remaining tissues, the concentrations were less than 30 µg/g. Forty-eight hours after dosing at 100 mg/kg bw, the concentrations had decreased to less than or equal to 70 µg/g for the adrenal gland, less than 15 µg/g (PH label) or 20 µg/g (TH label) for the next highest tissues (liver, total kidney, kidney cortex, thyroid) and less than 7.5 µg/g for the remaining tissues (Thomas, 2014).

(b) Dermal route

In vivo

The dermal absorption of PH-tioxazafen (batch no. 69757-1-91-1; radiochemical purity 97.9%) was studied in vivo in male Sprague Dawley rats (four per dose per time point). Tioxazafen was applied to the skin at a concentration of 4.5, 45 or 450 g/L for 8 hours. One group was terminated at 8, 24, 48, 72, 144 and 168 hours post-dosing to provide additional data on the stratum corneum reservoir and long-term absorption, distribution and excretion of tioxazafen. Following dose administration, the application site was protected by an O-ring and dressing. Urine and faeces were collected, and cage wash was retained. At 8 hours post-dosing, the exposed area was gently wiped

clean (twice), then dried with cotton wool. The distribution of total radioactivity in the urine, faeces, cage wash, whole blood, plasma, dose site washes, dressing, dose site, stratum corneum, hair, gastrointestinal tract and carcass was analysed at each time point. The dislodgeable dose, absorbed dose (urine, faeces, cage wash, gastrointestinal tract and carcass), dermal delivery (absorbed dose and radioactivity in exposed skin) and potentially absorbable dose (sum of dermal delivery plus the stratum corneum tape strips 3–20) were determined.

Total recovery was 96–103%. Following 8 hours of dermal exposure, 90–102% of the applied radioactivity was readily removed from the skin surface by a mild aqueous wash. The maximum potentially absorbable dose for the concentrations of 4.5, 45 and 450 g/L were 7.45%, 1.59% and 1.65%, respectively (Hutton, 2014).

In vitro

The dermal absorption of PH-tioxazafen (radiochemical purity 97.9%) was studied in vitro in human and rat skin membranes. Tioxazafen was applied at a concentration of 4.5, 45 or 450 g/L. Each test preparation was applied to 20 rat and 10 human split-thickness skin membranes at an application rate of approximately 10 μL/cm^2. Dermal absorption was assessed by collecting receptor fluid in hourly fractions from 0 to 8 hours post-application and then in 2-hourly fractions from 8 to 24 hours post-application. At 8 hours post-application, exposure was terminated by washing and drying the skin surface. The experiment was then terminated for half of the rat skin samples for each test preparation. At 24 hours post-application (i.e. after a 16-hour post-exposure monitoring period), the remaining rat and all human skin samples were terminated as described above. All samples were analysed by liquid scintillation counting. The potentially absorbable dose was defined as the sum of dermal delivery (absorbed dose and radioactivity in exposed skin) plus the stratum corneum tape strips 3–10.

Total recovery of radioactivity was 95–100%. The majority (58–96%) of the applied dose was removed by washing at 8 hours post-application for all test groups. At the concentration of 450 g/L, the potentially absorbable dose was 0.52% of the applied dose in the human skin, with termination conducted at 24 hours post-application, and 29% and 8% of the applied dose in the rat skin, with termination conducted at 8 hours and 24 hours post-application, respectively. At the concentration of 45 g/L, the potentially absorbable dose was 3.6% of the applied dose in the human skin, with termination conducted at 24 hours post-application, and 32% and 14% of the applied dose in the rat skin, with termination conducted at 8 hours and 24 hours post-application, respectively. At the concentration of 4.5 g/L, the potentially absorbable dose was 4.5% of the applied dose in the human skin, with termination conducted at 24 hours post-application, and 33.04% and 32.17% of the applied dose in the rat skin, with termination conducted at 8 hours and 24 hours post-application, respectively (Craig & Prisk, 2014).

1.2 Biotransformation

Mice

The metabolism of tioxazafen was studied in groups of CD-1 mice that received non-radiolabelled tioxazafen for 3 days followed by a small dose of PH-tioxazafen. Study details and toxicokinetics data are presented in section 1.1 above. Plasma, urine and faeces were analysed for total radioactivity. Samples with sufficient radioactivity were pooled, and the metabolite profile was determined by radio-HPLC. Metabolites were not further identified.

Tioxazafen was extensively metabolized. No parent tioxazafen was detected in the plasma, and only minor amounts (<5%) were found in the urine. Parent compound was detected in only a few faecal samples (Godsey, 2015).

Rats

The metabolism of PH-tioxazafen and TH-tioxazafen was studied in groups of Sprague Dawley rats treated with low (3 mg/kg bw) oral or intravenous doses or high (100 mg/kg bw) oral doses. The study design and toxicokinetics are described in section 1.1. Urine was collected on ice over the following intervals: approximately 0–6, 6–12 and 12–24 hours post-dosing and then daily through 168 hours post-dosing. Only in the oral dosing groups, bile was collected on ice over the following intervals: 0–4, 4–8, 8–12, 12–18, 18–24 and 24–48 hours post-dosing. Faeces were collected on ice over the following intervals: approximately 0–12 and 12–24 hours post-dosing and then daily through 168 hours post-dosing. Based on total radioactivity results, selected urine and bile samples and faecal extracts were pooled and profiled for metabolites using radio-HPLC and LC-MS/MS.

Tioxazafen was extensively and completely metabolized in the rat. Unchanged tioxazafen was not observed in urine, faeces or bile. Differences in metabolite profiles between PH-tioxazafen and TH-tioxazafen were related to the metabolites unique to each label. There were no significant differences in the metabolite profiles of urine and faecal samples collected from animals administered tioxazafen, regardless of route of administration (oral or intravenous), sex or dosing regimen (single or repeated dose).

PH-tioxazafen and TH-tioxazafen were extensively metabolized to approximately 30 and 29 components, respectively. Most metabolites represented less than 5% of the radioactivity in any individual sample and less than 1% of the administered dose. Structurally identified metabolites (≥1% of the administered dose in urine or approximately ≥2% of the administered dose in bile) are shown in **Error! Reference source not found.**6. After PH-tioxazafen dosing, the major metabolite in urine was benzamidine (M1) (4.1–12.6%), followed by 5-hydroxy-tioxazafen glucuronide (M39) (1.2–4.6%) and hippuric acid (M7) (1.0–2.6%). After TH-tioxazafen dosing, the major metabolite in urine was 2-thenoylglycine (M57) (0.7–6.2%), followed by unidentified metabolite M26 (0.6–4.0%, also present in PH-label urine at a maximum of 1.4%) and 5-hydroxy-tioxazafen glucuronide (3.6–4.7%). Benzamidine was also the major metabolite in the faeces from all groups treated with PH-tioxazafen. Smaller, poorly resolved peaks were present in all faecal chromatograms in the same regions identified in the urine profiles of either label, but they were minor contributors to the total overall radioactivity.

Table 6. Names and structures of identified metabolites of tioxazafen in rats that constituted more than 1% or more than 2% of the administered dose in the urine or bile, respectively

Metabolite (matrix)	Trivial name	Chemical name	Structure
M1 (urine, faeces)	Benzamidine	Benzenecarboximidamide	NH$_2$ NH
M57 (urine)	2-Thenoylglycine	*N*-(2-Thienylcarbonyl)glycine	HO$_2$C H N S O
M39 (urine, bile)	5-Hydroxy-tioxazafen glucuronide	[5-(3-Phenyl-1,2,4-oxadiazol-5-yl)thiophen-2-yl]-β-D-glucopyranosiduronic acid	HO OH O OH CO$_2$H O S N N–O

Metabolite (matrix)	Trivial name	Chemical name	Structure
M7 (urine)	Hippuric acid	*N*-Benzoylglycine	
M4 (urine)	4-Hydroxy-iminoamide glucuronide	[2-[(Iminophenylmethyl)-aminocarbonyl]-thiophen-4-yl]-β-D-glucopyranosiduronic acid	
M3 (urine, bile)	5-Hydroxy-iminoamide glucuronide	[5-[(Iminophenylmethyl)-aminocarbonyl]-thiophen-2-yl]-β-D-glucopyranosiduronic acid	
M24 (urine)	Butenoic acid sulfinate	4-(3-Phenyl-1,2,4-oxadiazol-5-yl)-4-sulfinobut-3-enoic acid	(tentative structure, other structures are possible)
M34 (urine, bile)	Dihydroxy-tioxazafen glucoside sulfate	Sulfuric acid mono-[2-(β-D-glucopyranosyloxy)-5-(3-phenyl-1,2,4-oxadiazol-5-yl)thiophen-3-yl] ester	
M22 (urine, bile)	Dihydroxy-tioxazafen diglucuronide	[3-(β-D-Glucopyranuronosyloxy)-5-(3-phenyl-1,2,4-oxadiazol-5-yl)thiophen-2-yl]-β-D-glucopyranosiduronic acid	
M30 (urine, bile)	Butenoic acid sulfinate glutathione conjugate	*N*-[*S*-[4-(3-Phenyl-1,2,4-oxadiazol-5-yl)-4-sulfinobut-3-enoyl]-*N*-L-γ-glutamyl-L-cysteinyl]glycine	(tentative structure, other structures are possible)

Source: Thomas (2014)

In bile, the primary metabolite was M39 (23–27% of the administered dose) following a single oral dose of PH-tioxazafen or TH-tioxazafen to male rats. Further investigation by LC-MS/MS characterized up to 29 PH-tioxazafen metabolites and 20 TH-tioxazafen metabolites in bile.

The major proposed pathways for the metabolism of tioxazafen in the rat are:

1) Reductive cleavage of the N–O bond of the oxadiazole ring, leading to tioxazafen iminoamide, a transient metabolite that is not observed as a free metabolite in any matrix. The iminoamide metabolite is hydrolysed (almost certainly enzyme mediated) to benzamidine, the major urinary and faecal metabolite, which is also further hydrolysed to benzoic acid (eliminated in urine as the glycine conjugate, hippuric acid). Hydrolysis

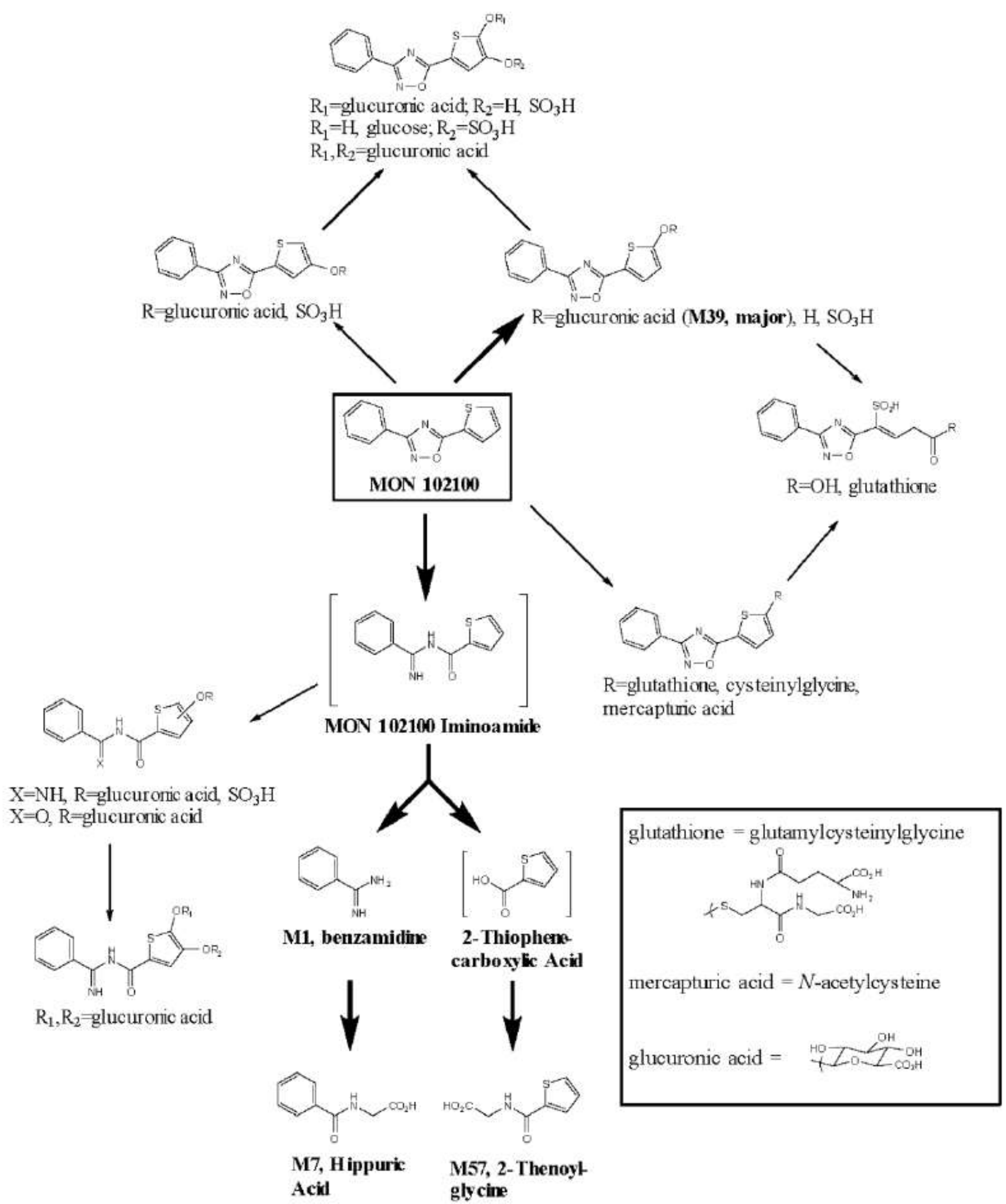
of the iminoamide also gives 2-thiophenecarboxylic acid (eliminated in urine as the glycine conjugate, 2-thenoylglycine).

2) Hydroxylation of the thiophene ring (primarily at the 5-position of the ring, adjacent to the sulfur atom) and conjugation as the glucuronide (major) or sulfate.

Additional proposed pathways of metabolism are dihydroxylation of the thiophene ring and conjugation with glucuronic acid, sulfate or glucose; glutathione substitution on the thiophene ring and catabolism to the mercapturate; oxidative ring-opening of the thiophene ring; and oxidation of the sulfur atom of the thiophene ring, forming the thiophene-*S*-oxide (Thomas, 2014).

Proposed metabolic pathways for tioxazafen in rats are shown in Fig. 4.

Fig. 4. Proposed pathways of metabolism of tioxazafen (MON 102100) in rats



Source: Thomas (2014)

2. Toxicological studies

2.1 Acute toxicity

(a) Lethal doses

The results of studies of acute toxicity with tioxazafen are summarized in Table 7.

Table 7. Summary of acute toxicity studies with tioxazafen

Species	Strain	Sex	Route	Vehicle	Purity (%)	LD_{50}/LC_{50}	Reference
Rat	Sprague Dawley	F	Oral	Distilled water	98.8	>5 000 mg/kg bw (M/F)	Durando (2011a)[a]
Rat	Sprague Dawley	M/F	Dermal	Moistened with distilled water	98.8	>5 000 mg/kg bw (M/F)	Durando (2011b)[b]
Rat	Wistar (HsdCpb:WU (SPF))	M/F	Inhalation	–	98.8	>5.2 mg/L (M/F)	Durando (2011c)[c]

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

[a] No deaths, clinical signs or gross pathological findings were observed during or at the end of the 14-day observation period. Batch no. GLP-1106-21504-T.

[b] No deaths were observed. Following patch removal, all rats exhibited ocular and/or nasal discharge on day 1 or 2. Three females showed signs of anogenital staining from days 1 through 13. Dermal irritation was noted at the dose site and/or mechanical damage due to unwrapping around the dose site for four of five males on day 1. Although three females lost body weight through day 7, all animals gained weight over the entire observation period. No gross abnormalities were noted for any of the animals when necropsied at the conclusion of the 14-day observation period. Batch no. GLP-1106-21504-T.

[c] Rats were exposed nose only to tioxazafen at a gravimetric chamber concentration of 5.2 mg/L. No deaths were observed. Following exposure, three of five males and all five females exhibited irregular respiration. All affected rats recovered from this symptom by day 9. Although all rats lost body weight by day 1, all animals showed a weight gain thereafter and over the entire 14-day study. Macroscopic examination at the end of the 14-day observation period showed no treatment-related effects. Mass median aerodynamic diameter was 3.1 (± 2.3) µm. Batch no. GLP-1106-21504-T.

(b) Dermal irritation

In an acute dermal irritation study, the intact skin of three male Himalayan rabbits was exposed for 4 hours under semi-occlusion to 0.5 g tioxazafen (moistened with distilled water to achieve a dry paste containing 65% w/w tioxazafen, 0.77 g of test mixture; batch no. GLP-1106-21504-T; purity 98.8%). Dermal irritation was scored according to the Draize method at 0.5–1, 24, 48 and 72 hours after patch removal.

No skin irritation was observed at any time point (Durando, 2011d).

(c) Ocular irritation

In an acute eye irritation study, 60 mg of tioxazafen (equivalent to 0.1 mL; batch no. GLP-1106-21504-T; purity 98.8%) was instilled into the conjunctival sac of the right eye of each of three female New Zealand rabbits. The untreated eye served as a control. The eyes were examined macroscopically according to the Draize method for signs of irritation at 1, 24, 48 and 72 hours and days 4 and 7 post-instillation.

Corneal opacity and iritis were not observed. Minimal conjunctivitis was observed from 1 to 72 hours post-instillation. In this study, tioxazafen was mildly irritating to the eye (Durando, 2011e).

(d) Dermal sensitization

In a dermal sensitization study using the Buehler test, tioxazafen (batch no. GLP-1106-21504-T; purity 98.8%) formulated in 2% carboxymethylcellulose in distilled water was tested in 20 male Hartley guinea-pigs. The vehicle control group consisted of 10 animals. Tioxazafen was topically applied once per week for 3 weeks. The doses were based on the results of a range-finding study in which two animals of each sex were treated topically with 4%, 9%, 18%, 35%, 53% or 70% weight per weight of the test substance in vehicle. In the induction phase, the animals received three topical inductions with 70% tioxazafen once per week, followed by a topical challenge (6-hour exposure under occlusion) with 18% tioxazafen 28 days after the first induction dose. Mineral oil was used as a positive control.

Very faint erythema (score of 0.5) was noted for four of 19 test sites 24 hours after challenge. Irritation persisted at one of these sites through 48 hours. Very faint erythema (score of 0.5) was noted for four of 10 naive control sites 24 hours after challenge. Irritation cleared from the affected sites by 48 hours. In this study, tioxazafen was not a contact sensitizer (Durando, 2011f).

2.2 Short-term studies of toxicity

(a) Oral administration

Mice

In a 28-day dietary range-finding toxicity study, tioxazafen (batch no. GLP-1005-20603-T; purity 99.7%) was administered to groups of 10 male and 10 female Crl:CD-1(ICR) mice at 0, 20, 100, 300, 1000 or 3000 ppm (equal to 0, 4, 19, 58, 184 and 437 mg/kg bw per day for males and 0, 5, 25, 70, 219 and 399 mg/kg bw per day for females, respectively). The animals were checked daily for mortality and clinical signs of toxicity. Body weights and feed consumption were measured twice per week. Prior to termination, blood samples were taken from five animals of each sex per group for haematology and blood biochemistry. All the mice underwent complete necropsy. Brain, kidneys, adrenals, spleen, heart, liver, ovaries with oviduct, testes, epididymides, thyroid with parathyroid, and uterus were weighed. Liver was examined microscopically from all control, 300 and 1000 ppm group animals. Heart was examined microscopically from the control and 1000 ppm group males.

At 3000 ppm, four females and one male died, and two females were killed in extremis. The remaining females and males in this group were euthanized on study days 5 and 8, respectively. Prior to death or early termination, these animals showed hypoactivity, continuous tremors, body and extremities pale, body and extremities cool to touch, respiration rate decreased, respiration laboured, dermal atonia, thin, partial closure of the eye(s), decreased defecation, faeces smaller than normal and/or yellow material on the urogenital and/or anogenital area. Necropsy revealed dark red contents in the gall bladder in three females. As these animals were not examined microscopically, the cause of death was not determined. All high-dose mice showed body weight losses, reduced feed consumption and reduced feed efficiency.

At 1000 ppm, one female was euthanized in extremis on study day 5. Limited microscopic examination showed mild hepatocellular single-cell necrosis. Prior to euthanasia, this animal showed intermittent tremors, body and extremities pale, dermal atonia, thin, defecation decreased and partial closure of the eye(s).

In the 1000 ppm group, decreased defecation was observed in females during the first week of the study. Transiently lower body weights (on day 3: 9% reduction in body weight compared with day 0, and 15% lower body weight compared with controls), feed consumption (37% reduction compared with controls) and feed efficiency were noted in the 1000 ppm group females from study days 0 to 3. These values recovered by study days 3–7 and were similar to those of the control group for the remainder of the study. Higher bilirubin values were noted in the 1000 ppm group males (93%) and females (60%), and higher cholesterol (72%) and gamma-glutamyl transferase (GGT) values (200%) were noted in the 1000 ppm group females. Higher liver weights (absolute 19–23%; relative 18–21%)

in the 1000 ppm group males and females correlated with minimal to mild centrilobular hepatocellular hypertrophy in the liver.

There were no test substance–related clinical observations, effects on body weight, feed consumption, feed efficiency, haematology or serum chemistry values, or macroscopic or microscopic findings in the 20, 100 or 300 ppm group males and females.

The no-observed-adverse-effect level (NOAEL) was 300 ppm (equal to 58 mg/kg bw per day), based on increased bilirubin, liver weights and centrilobular hepatocellular hypertrophy in both sexes, increased cholesterol and GGT levels in females and termination of one female in extremis at 1000 ppm (equal to 184 mg/kg bw per day) (Kirkpatrick, 2013a).

In a 90-day dietary toxicity study, tioxazafen (batch no. GLP-1007-20811-T; purity 98.5%) was administered to groups of 10 male and 10 female Crl:CD-1(ICR) mice at 0, 10, 50, 200, 600 or 1250 ppm (equal to 0, 2.1, 10.3, 42, 125 and 260 mg/kg bw per day for males and 0, 2.6, 13.8, 54, 174 and 319 mg/kg bw per day for females, respectively). The animals were checked daily for mortality and clinical signs of toxicity. Body weights and feed consumption were measured weekly. Ophthalmologic examination was performed pretest and during week 12. Prior to termination, blood samples were taken from five animals of each sex per group for haematology and blood biochemistry. All mice underwent complete necropsy. Brain, kidneys, adrenals, spleen, heart, liver, ovaries with oviduct, testes, epididymides, thymus, thyroid with parathyroid, and uterus were weighed. An extensive range of organs and tissues was examined microscopically from all animals found dead or euthanized in extremis and from all control and 1250 ppm group animals at scheduled termination.

At 1250 ppm, one female was killed in extremis on study day 3. This animal displayed partial closure of both eyes, dermal atonia, body cool to touch and hypoactivity. Histopathology showed moderate centrilobular to midzonal hepatocellular necrosis, mild hepatocellular hypertrophy and moderate cortical lymphoid necrosis in the thymus. Hepatocellular necrosis was considered to be the treatment-related cause of death. One female in the 600 ppm group died on study day 70. Examination showed mild to moderate haemorrhages in the brain (cerebellum) and spinal cord (cervical and thoracic). This was considered to be the result of a cage accident and not treatment related.

In high-dose males (1250 ppm), a slightly higher incidence of yellow staining of the urogenital area was observed from day 17 onward. No other clinical signs were reported.

Transiently lower mean body weight gains (5%), feed consumption (13%) and feed efficiency were noted in the 1250 ppm group females during study week 0–1, but these recovered during the second study week. Body weights, feed consumption and feed efficiency in the other treatment groups were not affected. Ophthalmological and haematological examination showed no effect of treatment in any groups. Higher total bilirubin levels were noted in females of the 600 and 1250 ppm groups (36% and 50%, respectively). Higher mean cholesterol levels (80%) were observed in the 1250 ppm group females. Higher mean absolute (13–24%) and relative (17–22%) liver weights were noted in the 1250 ppm group males and females and correlated with centrilobular hepatocellular hypertrophy. Slightly higher absolute and relative liver weights (10%) were noted in the 600 ppm group males. Minimal to mild centrilobular hepatocellular hypertrophy was noted in the liver at 200 (males only), 600 and 1250 ppm.

The NOAEL was 600 ppm (equal to 125 mg/kg bw per day), based on increased bilirubin and cholesterol levels in females, increased liver weights and hepatocellular hypertrophy in both sexes and termination of one female in extremis at 1250 ppm (equal to 260 mg/kg bw per day) (Kirkpatrick, 2013b).

Rats

In a 28-day dietary range-finding toxicity study, tioxazafen (batch no. GLP-1005-20603-T; purity 99.7%) was administered to groups of six male and six female Sprague Dawley (Crl:CD(SD))

rats at 0, 50, 200, 1000, 3000 or 10 000 ppm (equal to 0, 4, 15, 76, 201 and 628 mg/kg bw per day for males and 0, 5, 18, 89, 221 and 760 mg/kg bw per day for females, respectively). The animals were checked daily for mortality and clinical signs of toxicity. Body weights and feed consumption were measured twice weekly. Blood was sampled on the day of necropsy for haematological and clinical biochemistry examinations. All the rats were macroscopically examined, and weights of liver, spleen, kidneys, heart, brain, thymus, thyroid with parathyroids, uterus, ovaries with oviducts, testes, epididymides and adrenals were determined. The adrenals (females only), coagulating glands, epididymides, kidneys, liver, ovaries, prostate, sternal bone and marrow, testes and uterus from all animals in the control and 10 000 ppm groups were examined microscopically. Gross lesions and femur bone and marrow were examined from all animals. In addition, the adrenals (females only), sternal bone and marrow, kidneys, liver, coagulating gland, epididymides, prostate and testes were examined in descending group order beginning with the 3000 ppm group until a no-effect level was determined for each tissue.

No deaths were observed. Yellow, red and/or brown material around the urogenital and/or anogenital areas was noted in the 10 000 ppm group males and females. Body weight loss was observed during the first week of treatment in males and females at 3000 ppm (5% and 4%, respectively) and 10 000 ppm (14% and 13%, respectively). Although these animals gained weight during the remainder of the study, final body weights were 17–25% lower than those of controls at 3000 ppm and 27–39% lower than those of controls at 10 000 ppm. In males at 1000 ppm, final body weights were 12% lower than those of controls. Lower feed consumption was noted in the 3000 and 10 000 ppm group males and females throughout the study, compared with the control group. In addition, lower feed consumption was noted in the 1000 ppm group males throughout the study and in the 1000 ppm group females from study days 0 to 3. Lower and/or negative mean feed efficiency values were noted in the 1000 ppm and higher groups from study days 0 to 3 and 3 to 7 (except for the 1000 ppm group females from study days 3 to 7).

At 10 000 ppm, statistically significant reductions in red blood cell count (8% males, 14% females), haemoglobin (13% males, 20% females), haematocrit (12% males, 18% females), mean corpuscular volume (4% males), mean corpuscular haemoglobin (5% males, 7% females), platelet count (24% females) and lymphocyte count (37% females) and increases in activated partial thromboplastin time (14% females), reticulocyte count (53% males, 90% females), red cell distribution width (17% males, 22% females) and haemoglobin distribution width (32% males, 48% females) were observed. In the females of the 3000 ppm group, reductions in haemoglobin (8%), haematocrit (9%) and activated partial thromboplastin time (22%) and increased haemoglobin distribution width (23%) were observed. Higher total bilirubin (45% males, 82% females) and sorbitol dehydrogenase levels (70% males, 85% females) were noted in the 10 000 ppm group, and higher cholesterol values were noted in the 3000 ppm (68% males, 70% females) and 10 000 ppm (150% males, 131% females) groups.

Macroscopically small seminal vesicles, coagulating glands, prostate and epididymides and small and/or soft testes were observed at 10 000 ppm.

Higher relative liver weights in the 3000 ppm (19–21%) and 10 000 ppm (61–68%) groups were considered treatment related. Lower mean absolute adrenal gland weights were noted in the 3000 and 10 000 ppm group males and females. Significantly lower absolute epididymides weights were noted in the 10 000 ppm group. In addition, a decrease in spermatogenesis was correlated with lower absolute testes weight in the 10 000 ppm group males. In the 3000 and 10 000 ppm group females, lower mean absolute ovarian and uterine weights were noted. Other reductions in absolute organ weights at 1000 (males only), 3000 and 10 000 ppm were considered secondary to the lower final body weights.

Histopathology showed cytoplasmic alteration and karyomegaly in the kidneys, tinctorial variations in hepatocellular staining characteristics in the liver and premature closure of the growth plate of the sternum in the 1000 ppm (liver findings only; males only), 3000 ppm and 10 000 ppm groups; hyperostosis (bone thickening) in the femur and increased adipose tissue of the sternal bone marrow in males and females in the 1000 ppm group and above; increased adipose of the femoral

bone marrow and diffuse cytoplasmic vacuolization and atrophy of the adrenal cortex in the 3000 and 10 000 ppm group females; hypospermia and increased number/amount of germ cells and cellular debris in the epididymides, hypospermatogenesis and seminiferous tubule degeneration in the testes, and decreased amounts of secretory material in the prostate, coagulating glands and seminal vesicles of the 10 000 ppm group males.

The NOAEL was 200 ppm (equal to 15 mg/kg bw per day), based on decreases in body weight gain and histopathological changes in the liver in males and decreased feed consumption and feed efficiency, hyperostosis in the femur and increased adipose tissue of the sternal bone marrow in both sexes at 1000 ppm (equal to 76 mg/kg bw per day) (Kirkpatrick, 2013c).

In a 90-day dietary toxicity study, tioxazafen (batch no. GLP-1007-20811-T; purity 100%) was administered to groups of 10 male and 10 female Sprague Dawley (Crl:CD(SD)) rats at 0, 10, 50, 250, 750 or 1500 ppm (equal to 0, 1, 3, 16, 47 and 91 mg/kg bw per day for males and 0, 1, 4, 19, 55 and 113 mg/kg bw per day for females, respectively). The animals were checked daily for mortality and clinical signs of toxicity. Body weights and feed consumption were measured weekly throughout the study, except for week 12, when they were measured twice weekly. The rats were subjected to ophthalmoscopy and tested in a functional observational battery (FOB), including a locomotor activity test, prior to treatment and during week 12 of treatment. Blood and urine were sampled on the day of necropsy for haematological and clinical biochemistry examinations and urine analysis. All the rats were macroscopically examined, and weights of liver, spleen, kidneys, heart, brain, thymus, thyroid with parathyroids, uterus, ovaries with oviducts, testes, epididymides and adrenals were determined. An extensive range of organs and tissues of all control and high-dose rats and all gross lesions from rats in the other treatment groups were microscopically examined.

No deaths were observed. Low incidences of brown material around the anogenital area were noted in the 1500 ppm males. During the study, body weight gains were up to 7.5% lower in the 1500 ppm males and up to 10% lower in the 750 and 1500 ppm females, when compared with the control group. Lower feed consumption (in g/animal per day; up to 19% in males and up to 21% in females) was noted from the beginning of the study and generally throughout the study, and lower mean feed efficiency values were noted from study week 0–1 in the 1500 ppm group males and females and from study week 1–2 in the 750 ppm group females. There were no treatment-related effects on ophthalmoscopy or in the FOB. Slight treatment-related reductions in red blood cell counts (7%) and haemoglobin (6%) and haematocrit (6%) levels were noted in the 1500 ppm group females. Statistically significantly higher mean white blood cell and absolute lymphocyte counts in the 750 ppm group females were considered incidental in the absence of a dose–response relationship. In the 1500 ppm group males and females, cholesterol levels were increased (25–31%). At this dose, a slightly lower urinary pH (6.3 vs 6.7 in controls) was also noted in the males, and variable urine colour (yellow, dark yellow and/or red) was noted in males and females. No treatment-related macroscopic changes were observed. Relative liver weights were slightly increased in males (9%) and females (15%) at 1500 ppm. However, no histopathological changes in the liver were found. Relative kidney weight was increased in high-dose males (11%) and females (13%). In the kidney, foreign material within the tubular lumina in the cortex was noted at 250 ppm and higher in both sexes. The surrounding tubular epithelium appeared normal. Additionally, two males from the 1500 ppm group and one male from the 750 ppm group had small amounts of a coarsely granular brown pigment within the tubular epithelial cells. In the 1500 ppm group males and females, renal tubular hyperplasia, as characterized by small aggregates of basophilic tubules with epithelial cells that were enlarged and often piled upon each other, was found in the outer stripe of the outer medulla and was morphologically distinct from chronic progressive nephropathy. Minimal to mild metaphyseal hyperostosis was noted in both sexes at 750 and 1500 ppm. The hyperostosis was characterized by increased amounts of metaphyseal trabeculae filling the marrow space.

The NOAEL was 250 ppm (equal to 16 mg/kg bw per day), based on a reduction in body weight gain in females and metaphyseal hyperostosis in both sexes at 750 ppm (equal to 47 mg/kg bw per day) (Kirkpatrick, 2013d).

Dogs

In a 28-day range-finding oral toxicity study, tioxazafen (batch no. GLP-1009-20903-T; purity 99.2%) was administered by gelatine capsule to groups of two male and two female beagle dogs at a dose of 0, 10, 100, 250 or 500 mg/kg bw per day. The animals were checked daily for mortality and clinical signs of toxicity. Body weights were measured pretreatment and on study days 3, 6, 10, 13, 17, 20, 24 and 27. Feed consumption was recorded daily. Blood was sampled prior to the initiation of dose administration (study day −6), prior to early termination for high-dose animals (study day 16) and at the scheduled necropsy (study day 28) for haematological and clinical biochemistry examinations. All dogs were macroscopically examined, and weights of liver with drained gall bladder, spleen, kidneys, heart, brain, pituitary, thymus, thyroid with parathyroids, uterus with cervix, ovaries, testes, epididymides and adrenals were determined. Microscopic examination was performed on gross lesions as well as the adrenal glands, kidney, liver and testes from all males and the adrenal glands, heart, kidney, liver and thymus from the females of the 500 mg/kg bw per day group that were euthanized in extremis or terminated early.

One female of the 500 mg/kg bw per day group was euthanized in extremis on day 16. As the other dogs in this dose group also showed signs of excessive toxicity, these animals were also euthanized on this study day. Prior to early termination, decreased defecation (females only), red mucoid faeces, soft faeces, diarrhoea (males only), diarrhoea containing red material and emesis containing red material were observed. At 250 mg/kg bw per day, decreased defecation, red mucoid faeces, faeces containing red material, soft faeces and diarrhoea were noted from study day 2 onwards. One female at this dose also showed emesis containing red material, dermal atonia and low incidences of decreased activity and was noted as being thin. At 100 mg/kg bw per day, faeces containing red material and emesis containing yellow material were observed.

Over the duration of the dosing period, body weight gain was observed in control males (1.1 kg) and females (0.9 kg). Body weight loss was observed in males at 500 mg/kg bw per day (0.9 kg) and females at 250 mg/kg bw per day (0.9 kg) and 500 mg/kg bw per day (0.6 kg). This body weight effect became noticeable as early as study day 3. Males at 250 mg/kg bw per day showed a slight loss of body weight (up to 0.3 kg) during treatment, whereas their terminal body weight was equal to their weight at the start of treatment. In the 100 mg/kg bw per day group animals, one male maintained its weight, one male gained 1.4 kg, one female lost 0.2 kg and one female gained 1.1 kg. Lower feed consumption was noted in the 250 and 500 mg/kg bw per day group males and females. Feed consumption was markedly lower in the 500 mg/kg bw per day group males (up to 76%) and females (up to 78%) during the first study week and continuing throughout the study when compared with the control group. Transiently (but generally) lower feed consumption was noted in the 250 mg/kg bw per day group males and females throughout the study.

At the time of early termination for the 500 mg/kg bw per day group (study day 16), increases in total white cell counts (males 148%; females 254%), absolute neutrophil counts (males 311%; females 488%) and absolute monocyte counts (males 324%; females 341%) and decreased eosinophil cell counts (64%) were seen when compared with pretest levels. At the scheduled necropsy (study day 28), in the 250 mg/kg bw per day group, an increase in absolute eosinophil counts in males (55%) and higher total white cell counts in females (69%) were seen. At the time of early termination for the 500 mg/kg bw per day group, decreased albumin (31–46%), calcium (12–24%) and total protein (23–34%) levels, decreased albumin/globulin ratio (25–37%) and increased urea nitrogen (51–81%) and triglyceride levels (89–169%) were seen in both sexes relative to pretest levels. Additionally, females had decreased phosphorus levels (8%). These changes were considered to be test substance–related changes, except for the decreased calcium levels, which were considered to be related to the low albumin levels and not to be a direct effect of test substance administration. At the scheduled study termination, in both sexes at 250 mg/kg bw per day, lower total protein (25–28%) and albumin levels (32–36%) and albumin/globulin ratio (27–28%) were seen. In females at 100 mg/kg bw per day, reductions in total protein (15%) and albumin (24%) were also found. A higher urea nitrogen level was seen in the 250 mg/kg bw per day group males. Reductions in alkaline phosphatase, alanine aminotransferase (ALT), aspartate aminotransferase (AST), GGT and total bilirubin observed at 250 mg/kg bw per day were considered not to be of toxicological relevance.

In the males at 500 mg/kg bw per day, lower absolute and relative weights of epididymides (absolute 43%, relative 35%), prostate (absolute 53%, relative 39%), testes (absolute 54%, relative 48%) and thymus (absolute 60%, relative 55%) were noted at early termination. In the 500 mg/kg bw per day females, lower absolute and relative weights of heart (absolute 37%, relative 24%) and thymus (absolute 83%, relative 78%) were observed. It is noted that the organ weights of the early terminated animals were compared with those of controls terminated at scheduled necropsy at study day 28. In females at 250 mg/kg bw per day, lower absolute and relative weights of heart (absolute 20%, relative 12%) and thymus (absolute 64%, relative 57%) were found. In females at 100 mg/kg bw per day, thymus weights were reduced (absolute 32%, relative 28%). Necropsy showed one male and one female at 500 mg/kg bw per day with oedema in multiple tissues (mesentery, pancreas and/or clear fluid within the abdominal cavity). Microscopic examination showed increased glycogen in the liver of the 500 mg/kg bw per day males and females, thymic lymphoid depletion in females at doses of 250 mg/kg bw per day and higher, and single-cell lymphoid necrosis in the thymus of one female from each of the 100 and 500 mg/kg bw per day groups (Kirkpatrick, 2012).

In a 90-day oral toxicity study, tioxazafen (batch no. GLP-1106-21504-T; purity 98.8%) was administered by gelatine capsule to groups of five male and five female beagle dogs at a dose of 0, 1, 3, 10, 40 or 120 mg/kg bw per day. The animals were checked daily for mortality and clinical signs of toxicity. A detailed clinical examination was performed weekly. Ophthalmoscopy was performed prior to treatment and during the last week of treatment. Body weights were measured pretreatment and at least weekly during treatment. Feed consumption was recorded daily. Blood and urine were sampled prior to treatment, after about 6 weeks of treatment and prior to the scheduled necropsy for haematological and clinical biochemistry examinations and urine analysis. After termination, all dogs were macroscopically examined, and weights of liver with drained gall bladder, spleen, kidneys, heart, lungs, brain, pituitary, pancreas, thymus, thyroid with parathyroids, uterus, ovaries, prostate, testes, epididymides and adrenals were determined. Microscopic examination was performed on all gross lesions and a wide range of organs and tissues of the control and high-dose animals. If treatment-related findings were observed in a tissue in the high-dose group, the same tissues were evaluated in lower-dose groups.

One female of the high-dose group was found dead on day 3 of treatment. Necropsy showed red streaking of the mucosa in small and large intestines and multiple black discoloured zones in the lung. Ulceration of the internal surface of the larynx was also noted. This is an unusual lesion and suggests a physical or irritant cause, such as might result from a dosing error or regurgitation/ aspiration. Microscopic changes in the respiratory tract consisted only of minimal focal acute inflammatory exudates and peribronchial/perivascular oedema, as well as common background changes. Clinical observations prior to death for this female on study days 0–2 included red diarrhoea with white substance (study day 1) and red faeces with white substance (study day 2). Emesis was found in the cage on the day of death. These observations were not found in controls, but were also recorded on the same days (study days 0–2) in other males and females at 120 mg/kg bw per day. It was noted that feed consumption for this animal was the highest of any female at this dose on study days 0–2. Although equivocal, the death was considered potentially treatment related.

Clinical observations in the other animals were mainly limited to soft or mucoid faeces, sometimes containing white or red material, and vomitus containing white material was noted in the majority of treated groups. The red material noted in faeces was considered likely to be an excreted metabolite. There were no test substance–related effects on body weight gain, feed consumption, feed efficiency, clinical chemistry, urine analysis or ophthalmoscopy. Higher leukocyte counts were noted in males at 40 and 120 mg/kg bw per day (12 900 and 13 900/mm^3, compared with 8600/mm^3 in controls) and females at 120 mg/kg bw per day (11 900/mm^3 compared with 8300/mm^3 in controls) prior to termination. These increases were largely due to significant increases in absolute neutrophil counts. These slight increases were considered to be a stress response to treatment or inflammation and not considered to be an immunotoxic effect, as these effects are normally expressed as decreased (not increased) white blood cell counts.

Higher absolute and relative liver weights were noted in the 120 mg/kg bw per day group females (19% and 27%, respectively). The increased liver weights were not accompanied by haematological, clinical chemistry or histopathological changes indicative of liver injury; therefore, the isolated change in liver weights in the high-dose females was not considered biologically relevant. Higher absolute and relative lung weights were noted in the 120 mg/kg bw per day group males (21% and 18%, respectively) and females (15% and 22%, respectively). No histopathological changes in the lung were noted. Although the significance of the lung weight increases was considered equivocal, owing to the concordance of relative lung weight increases in both sexes, the increased lung weights at 120 mg/kg bw per day were considered treatment related and adverse.

The NOAEL was 40 mg/kg bw per day, based on an increase in lung weights in both sexes and one female mortality at 120 mg/kg bw per day (Cada, 2013).

(b) Dermal application

In a 28-day dermal toxicity study, tioxazafen (batch no. GLP-1106-21504-T; purity 98.23%) was administered to groups of 10 male and 10 female Sprague Dawley (Crl:CD(SD)) rats at 0, 100, 300 or 1000 mg/kg bw per day. The animals were checked daily for mortality and clinical signs of toxicity. Dermal observations of the dosing site were conducted prior to treatment, weekly during the treatment period and on the day of the scheduled necropsy for erythema and oedema in accordance with a four-step grading system (very slight, slight, moderate and severe). Body weights and feed consumption were measured prior to treatment, weekly throughout the study and on the day of the scheduled necropsy. The rats were subjected to ophthalmoscopy prior to treatment and near the end of the treatment period. Blood and urine were sampled on the day of necropsy for haematological and clinical biochemistry examinations and urine analysis. All the rats were macroscopically examined, and weights of liver, spleen, kidneys, heart, lungs, brain, thymus, thyroid with parathyroids, uterus, ovaries with oviducts, testes, epididymides and adrenals were determined. An extensive range of organs and tissues of all control and high-dose rats and all gross lesions from rats in the other treatment groups were microscopically examined. In addition, adrenal glands, liver and skin (treated and untreated) were examined from rats in the 100 and 300 mg/kg bw per day groups.

On study day 0, in the mid-dose group, one female died during dose application and one male and one female were found dead prior to completion of the 6-hour (± 30 minutes) dosing period. These deaths were not considered to be test substance related, but instead a result of the dose application and wrapping procedures. These animals were replaced. No other mortalities occurred. Yellow material around the urogenital area was noted in the high-dose males and in the mid- and high-dose females. Yellow material around the anogenital area was noted in the high-dose females. Local dermal effects on the application site were not observed. Statistically significantly lower mean body weight gains were noted in the 300 and 1000 mg/kg bw per day animals during certain intervals after treatment. In high-dose males, reductions in final body weights (9%) and occasionally in feed consumption (up to 10%) compared with controls were observed. No treatment-related effects on haematology were observed. Clinical chemistry showed higher bilirubin (225%) in the high-dose males and higher cholesterol in the mid-dose (29%) and high-dose females (42%). A lower ALT level in the high-dose females was considered not to be toxicologically relevant. Urinary pH was slightly lower (6.4 compared with 6.7 in controls) in mid- and high-dose males. Urobilinogen was increased (150%) in high-dose males. Red urinary discoloration in one low-dose male and in mid- and high-dose males and females was believed to be a result of urinary excretion of test substance or a coloured metabolite.

In high-dose males, increases in relative heart weight (17%) and absolute (13%) and relative lung weights (23%) were found. Thymus weights were lower in the 300 mg/kg bw per day (absolute 23%, relative 20%) and 1000 mg/kg bw per day males (absolute 28%, relative 22%). In high-dose females, increased weights of kidney (relative 10%), liver (relative 12%), lung (absolute 16%, relative 21%) and thyroid/parathyroid (relative 17%) were found. Minimal to mild epidermal hyperplasia at the site of application was observed in both sexes of all treatment groups. Minimal to mild cytoplasmic alteration of centrilobular hepatocytes was observed in both sexes at the middle and high

doses and in females at the low dose. However, hypertrophy of centrilobular hepatocytes was not detected. The mild increases in liver weights and corresponding histological changes were considered related to liver enzyme induction and not adverse. In the adrenal cortex, an increased incidence and severity of cytoplasmic vacuolization (minimal to moderate) was observed in the 300 and 1000 mg/kg bw per day group males, and minimal vacuolization with minimal brown pigment was also observed in two high-dose females.

The NOAEL was 100 mg/kg bw per day, based on a reduction in body weight gain in both sexes and adrenal cortical cytoplasmic vacuolization in males at 300 mg/kg bw per day (Kirkpatrick, 2013e).

(c) Exposure by inhalation

In a 4-week inhalation toxicity study, groups of 10 male and 10 female Sprague Dawley (Crl:CD®(SD)) rats were exposed nose only by inhalation to tioxazafen (batch no. GLP-1401-23093-T; purity 98.8%) as a dust aerosol at an actual mean analytical concentration of 0, 15.3, 50.0 or 288 mg/m^3 air for 6 hours per day, 5 days per week. The average mass median aerodynamic diameter was 2.2–2.4 µm (geometric standard deviation 1.8–2.1). The rats were checked daily for mortality and clinical signs. Detailed physical examinations were performed prior to treatment, weekly during treatment and on the day of termination. Ophthalmoscopy was performed before treatment and towards termination. Body weights and feed consumption were determined prior to treatment, weekly during treatment and at termination (body weight only). At termination, blood and urine were sampled for haematology, clinical chemistry and urine analysis. All the animals underwent necropsy, and the weights of adrenals, heart, spleen, brain, liver, kidneys, thymus, thyroid with parathyroids, ovaries with oviducts, uterus, testes, epididymides and lungs were determined. Histopathological examinations were performed on a wide range of organs and tissues from the control and high-dose animals and on all gross lesions. Adrenal glands and nasal sections I–VI of the nasal cavity were examined from all animals at the scheduled necropsy.

There were no test substance–related effects on survival, haematology or ophthalmic findings. Red, purple and/or yellow material on the anogenital area, urogenital area, ventral trunk and/or hindlimbs was noted in the 50.0 and 288 mg/m^3 group males and females prior to exposure and on non-exposure days. Purple material was noted on study day 23 or later. Red colour was observed around the eyes of one male in the 50.0 mg/m^3 group. Compared with the control group, the mean body weight gains were statistically significantly lower (>10%) in the 50.0 and 288 mg/m^3 group males and the 288 mg/m^3 females, from study days 0 to 7. Final body weights were lower in males and females at 50.0 mg/m^3 (6% and 7%, respectively) and 288 mg/m^3 (10% and 8%, respectively). Feed consumption was lower (11% on average) throughout the study for both sexes at 288 mg/m^3. Occasionally, slightly lower feed consumption was observed in both sexes at 50.0 mg/m^3. Higher total bilirubin levels were noted in males at the middle and high doses (0.05 and 0.08 mg/dL, respectively, below detection limit in controls) and in high-dose females (0.14 mg/dL compared with 0.05 mg/dL in controls). Cholesterol levels were increased in females at the middle dose (24%) and high dose (53%) and in males at the high dose (26%). Red discoloration of the urine in high-dose females was most likely attributed to urinary excretion of the test substance or a metabolite and was considered not to be indicative of renal or systemic toxicity, as there was no correlation with blood in the urine. In both sexes at the high dose, lower adrenal weights (absolute 16–25%, relative 11–16%) were found. In high-dose females, relative liver weight was increased (15%). The higher total bilirubin and serum cholesterol levels had no clear correlation with higher liver weights and did not correlate with microscopic findings. The Meeting noted, however, that higher total bilirubin levels and higher liver weights were also found in other studies with tioxazafen. Minimal to mild atrophy and vacuolization of the adrenal glands were observed in mid-dose females and in both sexes at the high dose. Chronic inflammation of the caecum was noted in males and females at 288 mg/m^3. Hyperplasia of the respiratory epithelium lining the septum of nasal sections II and III was observed in all treatment groups. Minimal to mild squamous metaplasia of the epithelium of nasal sections II and III was noted in one mid-dose male, two high-dose males and one high-dose female. Minimal to mild exudate was

found present in mid- and high-dose males in nasal sections II, III and VI. In the 15.3, 50.0 and 288 mg/m^3 group males and 15.3 and 288 mg/m^3 group females, there was minimal subacute inflammation in nasal section II or III in males at all treatment levels and in females at the middle and high doses. These changes in respiratory epithelium in nasal sections II and III are common and adaptive responses following repeated inhalation exposures. Degeneration of respiratory epithelium in nasal sections II and III was observed in mid- and high-dose males. Degeneration of respiratory epithelium was not considered to be a normal adaptive tissue response, but rather was considered to be a response to tissue injury. In nasal section IV, there was minimal to mild degeneration of olfactory epithelium lining the dorsal meatus in the 288 mg/m^3 group males and females.

The no-observed-adverse-effect concentration (NOAEC) was 15.3 mg/m^3, based on decreased body weight gains in males, adrenal atrophy in females and degenerative changes in the respiratory and olfactory epithelium of males at 50.0 mg/m^3 (Randazzo, 2014a).

In a 13-week inhalation toxicity study, groups of 10 male and 10 female Sprague Dawley (Crl:CD®(SD)) rats were exposed nose only by inhalation to tioxazafen (batch no. GLP-1401-23093-T; purity 98.8%) as a dust aerosol at an actual mean analytical concentration of 0, 15.1, 49.7 or 284 mg/m^3 air for 6 hours per day, 5 days per week. The average mass median aerodynamic diameter was 2.1–2.6 µm (geometric standard deviation 1.9–2.1). The rats were checked daily for mortality and clinical signs. Detailed physical examinations were performed prior to treatment, weekly during treatment and on the day of termination. Ophthalmoscopy was performed before treatment and towards termination. Body weights and feed consumption were determined prior to treatment, weekly during treatment and at termination (body weight only). At termination, blood and urine were sampled for haematology, clinical chemistry and urine analysis. All the animals underwent necropsy, and the weights of adrenals, heart, spleen, brain, pituitary, liver, kidneys, thymus, thyroid with parathyroids, ovaries with oviducts, uterus, testes, epididymides, prostate and lungs were determined. Histopathological examinations were performed on a wide range of organs and tissues from the control and high-dose animals and on all gross lesions. Adrenal glands and nasal sections I–VI of the nasal cavity of all animals, testes of all males and liver of all females were examined at the scheduled necropsy.

There were no test substance–related effects on survival, haematology or ophthalmic findings. Red, purple and/or yellow material on various body surfaces was noted in both sexes at 284 mg/m^3. Final body weights were lower in males (14%) and females (8%) at the high dose. Feed consumption was lower (11% on average) throughout the study in males at the high dose. Occasionally, slightly lower feed consumption was observed in females at the high dose. Higher levels of serum albumin (8%), total bilirubin (0.12 mg/dL compared with 0.02 mg/dL in controls), creatinine (26%) and cholesterol (41%) and lower chloride levels (2%) were noted in the 284 mg/m^3 group males. In high-dose females, higher levels of total bilirubin (0.18 mg/dL compared with 0.08 mg/dL in controls) and cholesterol (63%) and lower chloride levels (4%) were found. Urine analysis showed higher levels of urobilinogen (0.6 mg/dL compared with 0.2 mg/dL in controls) and lower pH (6.2 compared with 6.7 in controls) in the high-dose males and higher total urine volume (14.1 mL compared with 5.6 mL in controls) in the high-dose females. Red discoloration of the urine in mid- and high-dose animals was most likely attributed to urinary excretion of the test substance or a metabolite and was considered not to be indicative of renal or systemic toxicity.

In the high-dose males, lower absolute adrenal weight (20%) and higher relative kidney weight (14%), relative liver weight (9%) and thyroid/parathyroid weights (absolute 21%, relative 50%) were observed. In high-dose females, lower adrenal weights (absolute 28%, relative 21%) and higher kidney weights (absolute 14%, relative 24%) and liver weights (absolute 13%, relative 22%) were found. The lower adrenal gland weights in the high-dose males and females correlated with histological findings of atrophy and vacuolization of the adrenal cortex. Higher liver weights in high-dose females corresponded to the microscopic finding of mild hepatocellular hypertrophy.

Minimal to moderate vacuolization of the adrenal cortex (macrovesicular and intracytoplasmic within distended cytoplasm of cells of the zona fasciculata) was noted in the mid-

and high-dose males and in one high-dose female, and atrophy of the adrenal cortex was observed in both sexes at the high dose. In the nasal cavity, degeneration of the olfactory epithelium (high-dose males), degeneration of the respiratory epithelium (both sexes at the high dose), hyperplasia of the respiratory epithelium (males of all treatment groups and mid- and high-dose females), squamous metaplasia of the respiratory epithelium (mid-dose males and both sexes at the high dose), inflammatory exudate and infiltration of lymphocytes in the nasal passages (both sexes at the high dose) and lymphoid hyperplasia of nasal passages (males of all treatment groups and high-dose females) were observed. Mild hepatocellular hypertrophy was seen in high-dose females. The histopathological changes in the nasal cavity were considered to be due to local effects of tioxazafen. Depletion of seminal vesicles in the 284 mg/m^3 group males was secondary to the loss of body weight. Mild to moderate depletion of seminal vesicle contents in the high-dose males was considered secondary to the loss of body weight and decreased feed consumption.

The NOAEC was 15.1 mg/m^3, based on adrenal cortical vacuolization in the males at 49.7 mg/m^3 (Randazzo, 2014b).

2.3 Long-term studies of toxicity and carcinogenicity

Mice

In an 18-month dietary carcinogenicity study, tioxazafen (batch no. GLP-1106-21504-T; purity 97.4–99.8%) was administered to groups of 50 male and 50 female CD-1 (Crl:CD-1(ICR)) mice at 0, 5, 50, 250, 750 (both sexes) or 1750 ppm (males only) (equal to 0, 1, 8, 41, 120 and 282 mg/kg bw per day for males and 0, 1, 10, 50 and 153 mg/kg bw per day for females, respectively). The mice were checked daily for mortality and clinical signs and weekly for palpable masses. Body weights and feed consumption were recorded weekly during the first 3 months and every 2 weeks (body weight) or 4 weeks (feed consumption) thereafter. Blood was sampled for haematological examinations at termination and from all animals killed in extremis. Animals found dead or euthanized before or at the end of the treatment period underwent necropsy. Organs (adrenals, brain, heart, liver, kidneys, thyroids with parathyroids, spleen, ovaries with oviducts, uterus, testes and epididymides) were weighed. A wide range of tissues was examined microscopically from all males at 0, 250, 750 and 1750 ppm and from all females at 0, 50, 250 and 750 ppm. In addition, livers were examined from all males at 5 and 50 ppm and from all females at 5 ppm.

At termination, the number of surviving females was reduced at 750 ppm (28 versus 40 in controls, statistically significant in the log-rank dose–response trend test of the survival rates in females). There were higher incidences of yellow, red or brown material, which was considered most likely to be a coloured metabolite, on one or more body surfaces (urogenital, anogenital and ventral trunk) in males at 750 and 1750 ppm and of yellow material on the same areas in females at 750 ppm. There were no other treatment-related clinical observations. No effects on body weight, feed consumption or incidences of palpable masses were observed. In the 1750 ppm males, increases in absolute (32%) and relative liver weights (29%) were observed. Histopathological examination revealed an increased incidence of foci of cellular alteration (clear, basophilic and eosinophilic combined) in males and a dose-related increased incidence and severity of hepatocellular hypertrophy in the 750 and 1750 ppm group males and 250 and 750 ppm group females. Furthermore, an increased incidence and/or an increased severity of pigmented macrophages in the 750 and 1750 ppm group males and 250 and 750 ppm group females were observed (Table 8). This change was characterized by clusters of periportal macrophages with cytoplasm engorged with pale yellow to deep granular brown pigment and was often accompanied by widely scattered necrotic hepatocytes.

An increase in the incidence of hepatocellular carcinomas was observed in the 1750 ppm group males, and an increased incidence of hepatocellular adenomas was seen in the 750 ppm group females (Table 9).

Table 8. Histopathological non-neoplastic findings in the liver of mice administered tioxazafen for 18 months

Finding	Males						Females				
	0 ppm	5 ppm	50 ppm	250 ppm	750 ppm	1 750 ppm	0 ppm	5 ppm	50 ppm	250 ppm	750 ppm
No. examined	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*	*50*
Focus of cellular alteration, all types[a]	0	0	0	5	1	8	0	0	2	0	0
Hypertrophy, hepatocellular	11	9	24*	15	29**	49**	2	2	3	10	23**
Minimal	4	2	5	5	4	0	1	0	1	1	2
Mild	6	6	15	9	21	14	1	2	2	9	20
Moderate	1	1	4	1	4	35	0	0	0	0	1
Macrophages, pigmented	1	6	2	4	11**	31**	11	13	9	20	12
Minimal	1	6	2	3	2	3	7	7	4	10	4
Mild	0	0	0	1	6	15	4	6	5	8	4
Moderate	0	0	0	0	2	11	0	0	0	2	4
Severe	0	0	0	0	1	2	0	0	0	0	0

ppm: parts per million; *: $P \leq 0.05$; **: $P \leq 0.01$ (Bonferroni corrected one-tailed Fisher's exact test)
[a] Not analysed statistically.
Source: Mertens (2014a)

Table 9. Incidence of neoplastic histopathological findings in mice administered tioxazafen for 18 months

Finding	0 ppm	5 ppm	50 ppm	250 ppm	750 ppm	1 750 ppm	Historical control data, % of total [highest %]
Males							
Liver							
Number examined	*50*	*50*	*50*	*50*	*50*	*50*	
Hepatocellular adenoma, single or multiple	4	2	7	2	4	6	7.8% [9.2%]
Hepatocellular carcinoma	0	1	2	0	2	6	3.3% [4.6%]
Hepatocellular adenoma and/or carcinoma	4	3	7	2	6	9	10.6% [13.9%]
Haemangioma	0	0	0	0	0	0	NA
Haemangiosarcoma	1	0	1	0	2	3	NA
Haemangioma or haemangiosarcoma	1	0	1	0	2	3	NA
Systemic tumour[a]							
Number examined	*50*	*50*	*50*	*50*	*50*	*50*	

Finding	0 ppm	5 ppm	50 ppm	250 ppm	750 ppm	1 750 ppm	Historical control data, % of total [highest %]
Systemic haemangioma	0	–	–	0	0	0	0.6% [1.5%]
Systemic haemangiosarcoma	2	–	–	0	2	6	7.8% [10.8%]
Systemic haemangioma or haemangiosarcoma	2	–	–	0	2	6	8.3% [10.8%]
Histiocytic sarcoma	0	–	–	1	0	0	1.1% [1.1%]
Females							
Liver							
Number examined	*50*	*50*	*50*	*50*	*50*		
Hepatocellular adenoma, single or multiple	0	2	0	2	5**		2.1% [3.3%]
Hepatocellular carcinoma	0	1	0	0	0		0.8% [3.1%]
Hepatocellular adenoma and/or carcinoma	0	2	0	2	5**		2.9% [6.2%]
Haemangioma	2	0	0	1	0		NA
Haemangiosarcoma	1	1	0	3	1		NA
Haemangioma or haemangiosarcoma	3	1	0	4	1		NA
Systemic tumour[b]							
Number examined	*50*	*50*	*50*	*50*	*50*		
Systemic haemangioma	2	–	2	3	0		11.3% [15.0%]
Systemic haemangiosarcoma	2	–	0	4	2		6.3% [9.2%]
Systemic haemangioma or haemangiosarcoma	4	–	2	7	2		17.5% [21.7%]
Histiocytic sarcoma	1	–	0	0	5		6.3% [8.3%]
Lung							
Number examined	*50*	*50*	*50*	*50*	*50*		
Bronchioloalveolar adenoma	0	–	6**	2	3		10.8% [18.0%]
Bronchioloalveolar carcinoma	4	–	1	3	2		6.3% [10.0%]
Bronchioloalveolar adenoma or carcinoma	4	–	7	5	5		16.3% [22.0%]

NA: not available; ppm: parts per million; **: $P \leq 0.01$ (one-tailed Peto's mortality prevalence method)

[a] For the 5 and 50 ppm group males, only livers were examined. Data not presented for comparison with dose groups having full tissue analysis.

[b] For the 5 ppm females, only livers were examined. Data not presented for comparison with dose groups having full tissue analysis.

Source: Mertens (2014a)

The incidence of haemangiosarcoma as a systemic tumour in the 1750 ppm group males (12%, 6/50) was slightly above the limited historical control data range (10.8%, 7/65) from the laboratory. The range of variability in haemangiosarcoma incidence between males and females was comparable in the liver (incidences ranged from 0 to 3 in males and females) and as a systemic tumour (incidences ranged from 0 to 6 in males and from 0 to 4 in females). Additionally, the

variability in the incidence of combined haemangiosarcomas and haemangiomas was also comparable (ranging from 0 to 6 in males with a possible dose–response relationship and from 0 to 7 in females without a dose–response relationship). Evaluation of the possible range of values indicates that the higher incidences in males likely reflected normal variability in response for these common tumour types. Haemangiosarcomas have been associated with increased iron pigments; however, in the current study, there was no increase in iron deposition, as shown by the special staining used. The higher incidences of haemangiosarcomas in males were therefore not considered by the study author to be test substance related.

There were slightly increased incidences of hepatic and systemic haemangiosarcomas in the 250 ppm group females. The incidences of the systemic haemangiosarcomas (8%) and of the systemic haemangiosarcomas combined with haemangiomas (14%) were well within the range of the four control groups included in test laboratory historical control data. Furthermore, the incidences for liver and systemic haemangiosarcomas in the 750 ppm group females were lower than in the 250 ppm group and were either equal to or lower than the incidences in the control group animals. Therefore, the higher incidences of haemangiosarcomas in the liver and as a systemic tumour in the 250 ppm group (second highest dose group) females were considered to be unrelated to test substance exposure.

The observed histiocytic sarcomas in this study were multicentric and involved not only the liver, but also other organs, including the spleen, lung, ovary, uterus, cervix and small intestine. There was an apparent higher incidence of histiocytic sarcoma in the 750 ppm group females that was only slightly higher than the highest incidence (8.3%, 5/60) of the four control groups within the test laboratory historical control data. Furthermore, the difference between the incidence in control and 750 ppm group females was not statistically significant using the one-tailed Bonferroni corrected Fisher’s exact test and the survival-adjusted Peto analysis. The apparent higher incidence of histiocytic sarcomas was therefore not considered to be test substance related by the study author.

In the lungs of females, there was an increased incidence of bronchioloalveolar adenomas at 50, 250 and 750 ppm compared with the control group (Table 9). The zero incidence in the control group was reported to be unusually low for this tumour type, which averages 10.8% in the laboratory historical control database. The difference between the control and 50 ppm group females was statistically significant. Nevertheless, the incidence profile of this common tumour was not considered to be test substance related, as there was no dose–response relationship, the values fell within the test laboratory historical control range and the incidence profile of combined bronchioloalveolar adenomas and carcinomas in females was flat.

The NOAEL for toxicity was 50 ppm (equal to 10 mg/kg bw per day), based on increases in pigmented macrophages with scattered necrotic hepatocytes and centrilobular hepatocellular hypertrophy in females at 250 ppm (equal to 50 mg/kg bw per day). Tioxazafen induced hepatocellular carcinomas in males at 1750 ppm and hepatocellular adenomas in females at 750 ppm. In addition, there was equivocal evidence of increases in the incidence of systemic haemangiosarcomas at 1750 ppm in males and of histiocytic sarcomas at 750 ppm in females (Mertens, 2014a).

Rats

In a 2-year combined toxicity and carcinogenicity study, tioxazafen (batch no. GLP-1106-21504-T; purity 97.4–99.8%) was administered to groups of 62 male and 62 female Sprague Dawley (Crl:CD®(SD)) rats in the diet at 0, 5, 25, 75, 250 or 750 ppm (equal to 0, 0.3, 1.3, 3.9, 13.3 and 39.6 mg/kg bw per day for males and 0, 0.3, 1.6, 4.9, 16.0 and 48.1 mg/kg bw per day for females, respectively). Ten rats of each sex per group were assigned to the chronic toxicity phase of the study and were offered the test diet for up to 1 year. All rats were checked daily for mortality and clinical signs. A detailed physical examination, including for the presence of palpable masses, was performed weekly. Body weights and feed consumption were recorded weekly during the first 3 months and about every 2 weeks thereafter. Blood and urine from the animals in the chronic toxicity phase were

sampled for haematological and clinical chemistry examinations and urine analysis at week 26 and at termination in week 52. Ophthalmic examinations were conducted prior to test substance exposure and during study week 51. Blood smears were prepared from animals euthanized in extremis and from all surviving animals in the carcinogenicity phase at termination. Animals found dead or euthanized before or at the end of the treatment period underwent necropsy. Organs (adrenals, brain, heart, liver, kidneys, thyroids with parathyroids, spleen, ovaries with oviducts, uterus, testes and epididymides) were weighed. A wide range of tissues was examined microscopically from all animals in the chronic toxicity phase that were found dead or killed in extremis and those from the 0 and 750 ppm groups. In addition, masses and gross lesions from all animals in the 5, 25, 75 and 250 ppm groups, adrenals from all males in the 75 and 250 ppm groups, kidneys from all animals in the 75 and 250 ppm groups, and bone with marrow (from femur with joint) from all animals in the 250 ppm group were examined microscopically. For animals in the carcinogenicity phase, histopathological examination was performed on all masses found during the in-life phase and on a wide range of tissues from all animals in the 0, 75, 250 and 750 ppm groups. In addition, thyroids from all males in the 5 and 25 ppm groups and thoracic, thymic and aortic masses from all females in the 5 and 25 ppm groups were examined microscopically at termination.

Mortality was not affected by treatment. Owing to low group survival (28.8%) in the 25 ppm group males, the terminal necropsy for the males was conducted between study days 706 and 711. No treatment-related clinical signs were observed.

During weeks 1 and 2 of treatment, transient, small, but statistically significant, reductions in body weight gain were observed in males and females at 250 and 750 ppm. As the effects were small and not related to dose, they were considered not to be biologically relevant. Terminal body weights of the treatment groups were not affected.

Haematological parameters were not affected. Treatment-related and statistically significantly higher cholesterol levels were seen at 750 ppm in males at 52 weeks (25%) and in females at 26 (32%) and 52 (32%) weeks. The values were within the historical control range.

Urine discoloration was seen in females at 250 ppm and in both sexes at 750 ppm. This discoloration was most likely related to urinary excretion of a metabolite of the test substance and not indicative of renal or systemic toxicity. Ophthalmoscopy showed no treatment-related effects. Macroscopic examination revealed no effects related to treatment. Organ weights were also not affected.

Histological examination of the chronic phase toxicity groups after 1 year of treatment showed cytoplasmic vacuolization in the adrenal cortex in the 750 ppm group males, metaphyseal hyperostosis/increased metaphyseal bone in the femur in both sexes at 750 ppm and foreign material in the kidney in both sexes at 250 and 750 ppm.

The incidences of neoplastic findings are presented in Table 10.

Table 10. Incidence of selected neoplastic findings in rats administered tioxazafen for up to 2 years

Finding	**0 ppm**	**5 ppm**	**25 ppm**	**75 ppm**	**250 ppm**	**750 ppm**	**Historical control data, % of total [highest %]**
Males							
Soft tissue, thoracic cavity[a]							
Number examined	*4*	*1*	*4*	*5*	*9*	*5*	
Hibernoma (all)	4	1	4	4	8	5	–
Benign	3	0	2	1	3	1	2.2% [7.7%]
Benign (X)	0	0	0	0	0	0	–

Finding	0 ppm	5 ppm	25 ppm	75 ppm	250 ppm	750 ppm	Historical control data, % of total [highest %]
Malignant	1	1	2	3	5	3	2.7% [8.3%]
Malignant (X)	0	0	0	0	0	1	–
Soft tissue, abdominal cavity[a]							–
Number examined	*NA*	*NA*	*NA*	*3*	*NA*	*1*	
Hibernoma (all)	–	–	–	1	–	1	–
Benign	–	–	–	1	–	1	0.2% [1.7%]
Thyroid gland							
Number examined	*52*	*52*	*52*	*52*	*51*	*52*	
Follicular cell tumours (all)	2	0	2	3	0	6	–
Adenoma	2	0	2	3	0	5	3.8% [10.8%]
Adenoma (X)	0	0	0	0	0	1	–
Carcinoma	0	0	0	0	0	0	1.1% [3.4%]
Females							
Soft tissue, thoracic cavity[a]							
Number examined	*3*	*3*	*6*	*3*	*3*	*9*	
Hibernoma (all)	2	3	6	3	3	9	–
Benign	1	2	4	0	1	3	1.9% [5.0%]
Benign (X)	0	0	0	0	1	0	–
Malignant	1	1	2	3	1	5	1.4% [11.7%]
Malignant (X)	0	0	0	0	0	1	–
Soft tissue, abdominal cavity[a]							
Number examined	*1*	*NA*	*NA*	*2*	*2*	*2*	
Hibernoma (all)	1	–	–	0	0	0	–
Benign	1	–	–	0	0	0	0% [0%]
Uterus							
Number examined	*52*	*0*	*2*	*52*	*52*	*52*	
Tumours, endometrial stromal (all)	0	NA	0	4	7*	5*	–
Polyp	0	NA	0	3	6*	4	5.5% [10.1%]
Polyp (X)	0	NA	0	0	0	1	–
Sarcoma	0	NA	0	1	1	0	0.2% [1.7%]

NA: not applicable, no tissues evaluated; ppm: parts per million; X: multiple (bilateral) tumours; *: $P \leq 0.05$ (one-tailed Fisher exact test)

[a] Not a protocol-required tissue, but was evaluated grossly in all rats.

Source: Mertens (2014b)

Although the historical control levels for hibernomas were slightly exceeded in the 250 ppm group males (three benign and five malignant tumours out of 52 rats; 15%) and 750 ppm group females (three benign and six malignant tumours out of 52 rats; 17%), the absence of statistical significance at $P \leq 0.05$ for this common tumour type in both sexes and the lack of a dose-related

response in the males suggest that these tumours were incidental and unrelated to tioxazafen administration. In addition, brown fat was not a protocol-specified tissue for evaluation, and these hibernomas were identified only at necropsy as masses for histological evaluation. Consequently, the variable number of tissues evaluated between groups makes strictly numerical comparisons between the groups more challenging.

For the thyroid gland, although the historical control levels were slightly exceeded in the 750 ppm group males (six adenomas out of 52 rats; 12%), the number of tumours seen was not statistically significantly different from that in the concurrent control group. In addition, chemically induced thyroid gland adenomas and carcinomas are most commonly associated with early follicular cell hypertrophy and hyperplasia. The absence of these premalignant lesions in this or previous studies would also suggest that the higher numbers of thyroid gland follicular adenomas seen in the 750 ppm group males were incidental and unrelated to tioxazafen exposure.

In the uterus, small increased incidences of endometrial stromal tumours (polyps) were found at doses of 75 ppm and above. A pairwise comparison (one-tailed Fisher exact test) showed that these reached statistical significance at 250 and 750 ppm. The incidence exceeded the upper range of the historical control data at 250 ppm and was at the upper range at 750 ppm. The Meeting noted that although certain types of uterine polyps can progress to cancer in rare cases, there is no instance of this occurring in this study in the absence of other indications of malignancy (i.e. evidence of preneoplastic changes in the uterus, tumours at other sites).

The NOAEL was 75 ppm (equal to 4.9 mg/kg bw per day), based on an increased incidence of endometrial stromal tumours (polyps) in females at 250 ppm (equal to 16.0 mg/kg bw per day). The NOAEL for carcinogenicity was 750 ppm (equal to 39.6 mg/kg bw per day), the highest dose tested (Mertens, 2014b).

2.4 Genotoxicity

Tioxazafen was tested for genotoxicity in three in vitro assays and one in vivo assay. All tests gave negative results (Table 11).

Table 11. Overview of genotoxicity tests with tioxazafen

End-point	Test system	Concentrations/doses tested	Purity (%)	Results	Reference
In vitro					
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537; *Escherichia coli* WP2*uvr*A	1.6–5 000 µg/plate in DMSO (±S9)	99.2	Negative[a]	Xu (2011a)
Mammalian cell gene mutation	Chinese hamster ovary cell (CHO/HPRT)	5–1 400 µg/mL in DMSO (−S9) 0.5–10 µg/mL in DMSO (+S9)	99.2	Negative[b]	Xu (2011b)
Chromosomal aberration	Human peripheral lymphocytes	Initial test: 4.75–700 µg/mL in DMSO (±S9, 3 hours) Confirmatory test: 4.9–250 µg/mL in DMSO (−S9, 22 hours) 42–500 µg/mL in DMSO (+S9, 3	95.0	Negative[c]	Xu (2011c)

End-point	Test system	Concentrations/doses tested	Purity (%)	Results	Reference
		hours)			
In vivo					
Mouse micronucleus	Male CD-1 mouse, bone marrow	250, 500 and 1 000 mg/kg bw in 0.5% aqueous carboxymethylcellulose by gavage as single doses	99.2	Negative[d]	Xu (2011d)

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

[a] No increase in the numbers of revertant colonies was recorded for any of the strains of bacteria used, either with or without metabolic activation. Precipitation of test substance was observed at concentrations of 160 or 500 μg/plate and above. Batch no. GLP-1009-20900-T.

[b] The average relative survivals ranged from 1.46% to 100% in the presence of the S9 mix and from 29.01% to 100% without the S9 mix. Tioxazafen precipitated at concentrations above 50 μg/mL without the S9 mix. No treatment-related increase in mutant frequencies was observed at any dose with or without the S9 mix. Batch no. GLP-1009-20900-T.

[c] In the initial test, the highest concentrations analysed (168 μg/mL without S9 and 240 μg/mL with S9) exhibited a greater than 50% reduction in mitotic index and a precipitate at the end of the treatment period. In the confirmatory test, the highest concentrations analysed (122 μg/mL without S9 and 175 μg/mL with S9) exhibited a 13–15% reduction in mitotic index and a precipitate at the end of the treatment period. No significant increase in cells with chromosomal aberrations, polyploidy or endoreduplication was observed in the cultures analysed. Batch no. GLP-1009-20900-T.

[d] Male CD-1 mice received a single oral gavage administration of tioxazafen at a dose of 250, 500 or 1000 mg/kg bw. Doses were based on a range-finding test using doses ranging from 200 to 2000 mg/kg bw administered to groups of three males and three females per dose. A concurrent negative control group received the vehicle only, whereas a positive control group was treated with cyclophosphamide. Bone marrow smears were obtained from five males per dose group at 24 and 48 hours after dosing, with the exception that mice in the positive control group were sampled at the 24-hour time point only. No unscheduled deaths and no clinical signs of toxicity were seen in any of the groups. Tioxazafen induced no statistically significant changes in the incidence of micronucleated immature erythrocytes. At 1000 mg/kg bw, tioxazafen induced a 34% decrease in the ratio of polychromatic erythrocytes to normochromatic erythrocytes after 48 hours of incubation. Cyclophosphamide induced a statistically significant increase in the percentage of cells with micronuclei. Batch no. GLP-1009-20900-T.

2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

In a two-generation reproductive toxicity study, groups of 30 male and 30 female Sprague Dawley (Crl:CD(SD)) rats were exposed to tioxazafen (batch no. GLP-1106-21504-T; purity 98.86%) in the diet. The tioxazafen concentrations were adjusted weekly to provide target test substance doses of 0, 5, 20 and 60 mg/kg bw per day for the parental (F_0) and first filial (F_1) generations. F_0 adults were treated over a 10-week premating period and throughout the 3-week mating period, gestation and 21-day lactation of the F_1 pups. On postnatal day (PND) 4, litters were culled to eight pups. Once weaned, one male pup and one female pup per litter were selected to become F_1 parents for the second filial (F_2) generation offspring. The remaining F_1 weanlings were euthanized and subjected to a gross external examination and necropsy. Treatment of the selected F_1 rats continued to PND 95 and during the subsequent 3-week mating, gestation and lactation periods. After the weaning of the F_{2a} generation, treatment of F_1 rats continued through a second 20-day premating period, mating period, gestation and 28-day lactation period of the F_{2b} generation. Clinical examinations were performed daily. Detailed observations were made weekly on all parental rats and on maternal F_0 and F_1 animals on gestation days (GDs) 0, 7, 14 and 20 and PNDs 1, 4, 7, 11, 14 and 21. Body weights and feed consumption of parental rats were recorded weekly. In addition, body weights and feed consumption of females were recorded on GDs 0, 7, 14 and 20 and PNDs 1, 4, 7, 11, 14 and 21. Individual pup weights were recorded on PNDs 1, 4, 7, 14 and 21. Estrous cycle length, fertility parameters, including spermatological examinations, and lengths of gestation were determined. All the litters were examined for number of pups, sex of pups, number of stillbirths, number of live births and gross anomalies. All pups were checked daily for mortality and clinical signs. Each pup received a detailed physical examination on PNDs 1, 4, 7, 14 and 21. The age of balanopreputial separation or vaginal opening was studied in all F_1 weanlings selected for further treatment. The culled pups and pups

euthanized at weaning were macroscopically examined. After weaning of the F_1 and F_2 pups, the respective F_0 and F_1 parents were euthanized and necropsied. The weights of brain, pituitary, liver, kidneys, adrenals, spleen, thyroid, thymus, uterus with oviducts and cervix, seminal vesicles, prostate, epididymides, testes and ovaries from all parental F_0 and F_1 animals were recorded. In F_1 and F_2 weanlings, the weights of the brain, spleen and thymus of one pup of each sex per litter were recorded. A range of organs and tissues of the parental animals was examined histopathologically.

There were no treatment-related effects on survival, clinical observations, gross pathology or microscopic findings in the male or female reproductive tissues for either generation. Final body weights were 8–9% lower than those of controls in F_0 and F_1 males at 60 mg/kg bw per day. Feed consumption was similarly reduced (about 7%) in F_0 and F_1 males in this dose group. Slightly lower (not statistically significantly) maternal body weight gains were noted in the F_0 females at 60 mg/kg bw per day during gestation, whereas no effects were noted in F_1 females.

Foreign material was present in the tubular lumina of the kidney cortex of F_0 and F_1 males and females in the 20 and 60 mg/kg bw per day groups, but these findings were not considered to be an adverse effect because there was no microscopic evidence of renal toxicity. Minimal hyperostosis of the metaphyseal region of the femur was observed in the F_0 and F_1 males in the 60 mg/kg bw per day group. Femoral metaphyseal hyperostosis in the high-dose F_0 and F_1 females occurred at an incidence similar to that of the controls. Minimal to mild cytoplasmic alteration of hepatocytes and corresponding slightly higher relative liver weights observed in the F_0 and F_1 males at 60 mg/kg bw per day had no histopathological correlation and were considered non-adverse. Minimal to mild vacuolization of the zona fasciculata of the adrenal cortex, as observed in the F_0 and F_1 males at 20 and 60 mg/kg bw per day, was considered not unusual for normal rats and not indicative of abnormal adrenal histology. No effects were observed on reproductive performance (mating, fertility, copulation and conception indices, estrous cyclicity, and spermatogenic end-points) or on the offspring (litter size, pup sex ratio, pup survival, pup body weights, pup organ weights, gross pathology and development).

The NOAEL for parental toxicity was 20 mg/kg bw per day, based on reduced body weight gains and hyperostosis in F_0 and F_1 males at 60 mg/kg bw per day.

The NOAEL for offspring toxicity was 60 mg/kg bw per day, the highest dose tested.

The NOAEL for reproductive toxicity was 60 mg/kg bw per day, the highest dose tested (Stump, 2014).

(b) Developmental toxicity

Rats

In a developmental toxicity study, tioxazafen (batch no. GLP-1009-20903-T; purity 99.3%; formulated in a 0.5% aqueous carboxymethylcellulose suspension) was administrated by gavage to 25 pregnant Sprague Dawley (Crl:CD(SD)) rats from GD 6 to GD 19 at a dose of 0, 10, 50 or 200 mg/kg bw per day. The dams were checked daily for mortality and clinical signs. Body weights and feed consumption were recorded on GDs 0 and 6–20 (daily). The fetuses were delivered by caesarean section on GD 20. The liver, kidneys and adrenal glands of the dams were weighed. The uteri, placentae and ovaries were examined, and the numbers of fetuses, early and late resorptions, total implantations and corpora lutea were recorded. Gravid uterine weights were recorded, and net body weights and net body weight changes were calculated. The fetuses were weighed, sexed and examined for external, visceral and skeletal malformations and developmental variations.

No mortality was observed. Hair loss around the rump, hindlimbs, dorsal abdominal area and/or ventral neck, and thoracic areas during GDs 8 through 20 was observed in females at 200 mg/kg bw per day. During the first 3 days of treatment, a reduction in body weight gain was observed in females at 50 mg/kg bw per day (4 g body weight gain on GD 9 versus 14 g in controls), whereas high-dose females showed a body weight loss up to 16 g during GDs 6–9. At 50 and 200 mg/kg bw per day, final body weights at GD 20 were 3% and 9% lower, respectively, than those of control

dams. Feed consumption was reduced throughout the treatment period at 50 mg/kg bw per day (8%) and 200 mg/kg bw per day (23%). No effects on gravid uterine weights or macroscopy were seen. Maternal adrenal weights at the high dose were reduced by 13% compared with controls. No effects on fetal development or survival were found.

The NOAEL for maternal toxicity was 10 mg/kg bw per day, based on reduced feed intake and body weight gain at 50 mg/kg bw per day.

The NOAEL for embryo and fetal toxicity was 200 mg/kg bw per day, the highest dose tested (Stump, 2012a).

Rabbits

In a developmental toxicity study, tioxazafen (batch no. GLP-1009-20903-T; purity 99.3%; formulated in a suspension of 0.5% carboxymethylcellulose in deionized water) was administered daily by gavage to groups of 25 female New Zealand white (Hra:(NZW)SPF) rabbits from GD 7 to GD 28 at a dose of 0, 5, 20 or 100 mg/kg bw per day. The fetuses were delivered by caesarean section on GD 29. The does were checked daily for mortality and clinical signs. Body weights were recorded on GDs 0, 4 and 7–29 (daily). Feed consumption was recorded on GDs 0 and 4–29 (daily). The fetuses were delivered by caesarean section on GD 29. The liver, kidneys and adrenal glands of the does were weighed. The uteri, placentae and ovaries were examined, and the numbers of fetuses, early and late resorptions, total implantations and corpora lutea were recorded. Gravid uterine weights were recorded, and net body weights and net body weight changes were calculated. The fetuses were weighed, sexed and examined for external, visceral and skeletal malformations and developmental variations.

No mortality was observed. Higher incidences of decreased defecation were noted in the 20 and 100 mg/kg bw per day groups. Slightly lower body weight gains were noted during the first days of treatment (days 7–10) at 20 mg/kg bw per day (115 g, not statistically significant) and 100 mg/kg bw per day (52 g, statistically significant) compared with 145 g body weight gain in controls. Compared with controls, over the entire treatment period, net body weight gain was 119 and 145 g lower in does of the 20 and 100 mg/kg bw per day groups, respectively. These reductions were small, but statistically significant. Feed consumption was reduced throughout the treatment period in the high-dose females, with the largest reductions during the first part of the study. Overall, the feed intake was reduced by 12% in this dose group. There was no effect on mean organ weights at any dose. There were no effects on uterine growth, survival or fetal morphology.

The NOAEL for maternal toxicity was 5 mg/kg bw per day, based on reduced body weight gain at 20 mg/kg bw per day.

The NOAEL for embryo and fetal toxicity was 100 mg/kg bw per day, the highest dose tested (Stump, 2012b).

2.6 Special studies

(a) Immunotoxicity

In a 28-day immunotoxicity study, groups of female CD-1 mice (10 per group) were administered tioxazafen (batch no. GLP-1106-21504-T; purity 99.8%) in the diet at a concentration of 0, 100, 300 or 1000 ppm (equal to 0, 26, 80 and 240 mg/kg bw per day, respectively). All animals were immunized with an intravenous injection of sheep red blood cells on study day 23. In addition, all animals in the positive immunosuppressive control group were administered an intraperitoneal injection of cyclophosphamide monohydrate (50 mg/kg bw per day) once daily for 5 consecutive days (study days 23–27). The animals were observed daily for mortality and clinical signs Detailed physical examinations were performed approximately weekly. Body weight and feed consumption were recorded weekly. At termination on day 28, blood samples were collected for clinical chemistry and serum immunoglobulin M (IgM) antibody analysis to assess the T cell–dependent antibody

response to sheep red blood cells. All animals were necropsied, and the liver, spleen and thymus were collected and weighed. The liver was examined microscopically from all animals in the control and high-dose groups.

There were no treatment-related effects on survival, clinical observations, feed consumption or macroscopic findings. There were no test substance–related effects on the T cell–dependent antibody response, as measured by the anti–sheep red blood cell IgM enzyme-linked immunosorbent assay. In high-dose females, increased total bilirubin levels (0.21 mg/dL compared with 0.04 mg/dL in controls), higher absolute and relative liver weights (17–18%) and minimal to mild centrilobular hepatocellular hypertrophy were noted.

The study showed no indication of an immunotoxic effect of tioxazafen at dietary concentrations up to 1000 ppm (equal to 240 mg/kg bw per day). The NOAEL for systemic toxicity was 300 ppm (equal to 80 mg/kg bw per day), based on an increase in bilirubin levels, higher absolute and relative liver weights (17–18%) and minimal to mild centrilobular hepatocellular hypertrophy at 1000 ppm (equal to 240 mg/kg bw per day) (Bultman, 2014).

(b) Mode of action

Three additional studies, for which no test guidelines are applicable, were conducted following completion of the mouse oncogenicity study in order to better understand the toxicological significance and/or human relevance of the mouse tumours, especially the hepatocellular tumours. These additional studies consisted of 1) an immunohistochemical evaluation of the livers from the previous 28-day and 90-day mouse studies for evidence of hepatocellular and/or peroxisomal proliferation, 2) a 14-day mode of action (MOA) study to evaluate several potential MOAs for the increased incidence of liver tumours and 3) a 28-day MOA study to confirm the results of the previous study and evaluate additional alternative MOAs.

Immunohistochemical evaluation of livers from 28-day and 90-day mouse studies

An immunohistochemical study was performed to investigate potential hepatocellular proliferation and/or peroxisomal proliferation in livers from previously conducted mouse studies (Mertens, 2014a). Liver tissues from the 28-day and 90-day feeding studies in mice (Kirkpatrick, 2013a, and Kirkpatrick, 2013b, respectively) were used to evaluate liver cell proliferation (Ki67 antibody staining of liver tissues from the 28- and 90-day studies) and peroxisome proliferation (PMP70 and catalase antibody staining of liver tissues from the 90-day study). Mice were treated with tioxazafen at a dietary concentration of 0, 20, 100, 300 or 1000 ppm in the 28-day study and 0, 10, 50, 200, 600 or 1250 ppm in the 90-day study. Immunohistochemically stained liver sections were evaluated by two-dimensional morphometric analysis.

There were no statistically significant or biologically relevant differences in the cell proliferation rates in treated animals in the 28-day or 90-day study, except for one 1250 ppm female from the 90-day study that was euthanized in extremis on day 3; this female had an exceptionally high mean labelling index, 22 times the mean control value and 24 times higher than those of the other animals in the high-dose group. In the original study (Kirkpatrick, 2013a), the cause of death was listed as “liver necrosis”. The animal was excluded from the summary statistics, as it was a numerical outlier and was treated for only 3 days, in contrast to the other members of the dose group, which were treated for 90 days. It is likely that the increased cellular proliferation observed in that animal reflected a regenerative response to the liver necrosis.

The exceptionally high mean labelling index and associated high standard deviation for females of the 20 ppm group in the 28-day study was due to a single outlier value (labelling index = 4.05). The stained section was examined microscopically, and subjective evaluation revealed a clear increase in the level of Ki67 staining. There was no evidence of inflammation, necrosis, degeneration or other pathological processes that could result in increased cellular proliferation. There was no evidence of artefactual staining or technical flaws. The higher level of Ki67 staining in this animal

was considered to be a biologically based outlier of unknown genesis and significance, and data from this animal were not included in the summary statistics.

The data indicate that there was no treatment-related alteration in cell proliferation rate in mice from the 28-day or 90-day study, and there was no indication of a treatment-related effect on peroxisome proliferation in the livers of mice from the 90-day study (Mertens, 2014c).

Fourteen-day mode of action study

In order to further investigate the increased incidence of liver carcinomas and adenomas in high-dose male and female mice, respectively, in an 18-month study with tioxazafen (Mertens, 2014a), the potential MOA for mouse liver tumour formation was investigated. In addition to traditional clinical pathology and histology evaluations, immunohistochemical, enzyme and gene expression profiling that focused on the potential for tioxazafen to act through a constitutive androstane receptor (CAR)/pregnane X receptor (PXR) or other common MOA for mouse liver tumour formation were assessed. The purpose of this study was to define dose- and time-dependent response relationships with regard to several end-points at various levels of biological organization (tissue/organ level, cellular level and biochemical/enzymatic level) at both tumorigenic (1750 ppm in males and 750 ppm in females) and non-tumorigenic (250 ppm in males and 50 ppm in females) doses. Phenobarbital (500 ppm) administered in drinking-water was used as a positive control for a CAR/PXR MOA.

Groups of six male mice were fed diets containing tioxazafen at either 250 or 1750 ppm (equivalent to 36 and 250 mg/kg bw per day, respectively) and groups of six female mice were fed diets containing tioxazafen at either 50 or 750 ppm (equivalent to 7 and 107 mg/kg bw per day) for 4 and 14 days. Alzet osmotic pumps with 5-bromo-2′-deoxyuridine (BrdU) were implanted into mice about 4 days prior to the scheduled day 4 and day 14 necropsies. The mice were checked daily for mortality and clinical signs. Detailed clinical signs, body weights and feed and water consumption were assessed prior to the start of treatment, once a week during treatment and on the day of termination. At termination, blood was collected for measurement of serum enzyme concentrations indicative of liver injury, and all animals were necropsied. BrdU infusion was used to measure cell proliferation. Livers were weighed, and samples were taken for evaluating histopathology, nuclear receptor translocation (CAR immunohistochemistry), nuclear labelling index (measure of cell proliferation), gene expression changes (*Cyp1a1*, *Cyp2b9*, *Cyp2b10*, *Cyp3a11* and *Cyp4a10*), quantification of glutathione and reduced glutathione in the liver, and liver enzyme activity (ethoxyresorufin *O*-deethylation [EROD], 7-benzyloxyresorufin *O*-debenzylation [BROD] and pentoxyresorufin *O*-depentylation [PROD]).

No mortality or clinical signs were observed. Body weight gains were not markedly affected in low-dose males. Body weight gains in high-dose males were significantly reduced after 4 days (0.7 g compared with 2.6 g in controls) and 14 days (1.4 g compared with 3.0 g in controls). Body weight gain was not affected in low- and high-dose females. Feed consumption was unaffected in the low-dose males, but was lower in the high-dose males (25% and 7% after 4 and 14 days of treatment, respectively). Feed consumption data for the females were compromised by animal spilling, precluding interpretation.

No treatment-related increases in liver weights were noted in the low-dose males or females at either day 4 or day 14. Relative liver weights were increased in high-dose males at day 4 (8%) and day 14 of treatment (26%).

Macroscopy revealed mottled to nutmeg hepatic discoloration in two high-dose males and one high-dose female at day 4. Histopathological examination showed centrilobular hepatocellular hypertrophy, increased mitoses, karyomegaly and mixed infiltration in tioxazafen-treated males and females at high doses and in phenobarbital-treated mice (Table 12). In addition, in tioxazafen-treated males and females at the high dose, single-cell necrosis, fatty change, cytoplasmic eosinophilia (females) and histiocytic infiltration (males) were observed. Fatty change and histiocytic infiltrates were the only microscopic findings that were not present at day 14. In high-dose males at day 4,

marked increases in AST (713%) and ALT values (1593%) were seen. At day 14, ALT and AST values were 3–5 times higher than control values. In high-dose females, the increases in ALT and AST were 3- to 5-fold at day 4, whereas values at day 14 were comparable to control values. Increased mitoses, mixed and histiocytic infiltration and karyomegaly were considered secondary to cytotoxicity. All other tioxazafen-related findings remained present, in some capacity, at day 14, although with lesser incidence and severity than at day 4.

Table 12. Histopathological findings in the liver

	Day 4				Day 14			
Males	**0 ppm**	**PB**	**250 ppm**	**1 750 ppm**	**0 ppm**	**PB**	**250 ppm**	**1 750 ppm**
Hepatocellular hypertrophy								
Minimal	0	0	0	0	0	1/6	0	2/6
Mild	0	3/6	0	2/6	0	2/6	0	4/6
Moderate	0	2/6	0	2/6	0	2/6	0	0
Severe	0	1/6	0	2/6	0	1/6	0	0
Increased mitoses								
Minimal	0	1/6	1/6	2/6	0	0	0	0
Mild	0	2/6	0	1/6	0	1/6	0	0
Moderate	0	0	0	1/6	0	0	0	0
Karyomegaly								
Minimal	0	1/6	0	1/6	0	1/6	0	3/6
Mild	0	2/6	0	2/6	0	2/6	0	2/6
Moderate	0	0	0	0	0	3/6	0	0
Necrosis, single-cell								
Minimal	0	0	0	4/6	0	0	0	2/6
Mild	0	0	0	1/6	0	0	0	3/6
Moderate	0	0	0	1/6	0	3/6	0	0
Necrosis, hepatocellular								
Minimal	0	0	0	0	0	1/6	0	0
Fatty change								
Minimal	0	0	0	1/6	0	0	0	0
Mild	0	0	0	1/6	0	0	0	0
Moderate	0	0	0	2/6	0	0	0	0
Severe	0	0	0	2/6	0	0	0	0
Infiltration, mixed								
Minimal	2/6	1/6	1/6	3/6	2/6	0	1/6	2/6
Mild	0	0	0	0	0	0	2/6	0
Infiltration, histiocytic								
Minimal	0	0	0	1/6	0	0	0	0
Mild	0	0	0	1/6	0	0	0	0

Females	4 days: 0 ppm	PB	50 ppm	750 ppm	14 days: 0 ppm	PB	50 ppm	750 ppm
Hepatocellular hypertrophy								
Minimal	0	1/6	3/6	0	0	2/6	2/6	1/6
Mild	0	2/6	0	4/6	0	4/6	0	2/6
Moderate	0	3/6	0	2/6	0	0	0	1/6
Increased mitoses								
Minimal	3/6	3/6	1/6	2/6	2/6	3/6	2/6	0
Mild	1/6	2/6	0	0	1/6	0	1/6	0
Moderate	0	0	0	1/6	0	0	2/6	0
Karyomegaly								
Minimal	1/6	2/6	0	1/6	0	1/6	0	1/6
Necrosis, single-cell								
Minimal	0	0	0	1/6	0	0	0	1/6
Mild	0	0	0	1/6	0	0	0	0
Fatty change								
Minimal	1/6	0	0	3/6	0	0	0	0
Mild	0	0	0	2/6	0	0	0	0
Moderate	0	0	0	1/6	0	0	0	0
Infiltration, mixed								
Minimal	1/6	3/6	1/6	2/6	1/6	1/6	2/6	1/6
Mild	0	0	0	2/6	0	0	0	0
Eosinophilia, cytoplasm								
Minimal	0	0	0	3/6	0	0	0	1/6
Mild	0	0	0	2/6	0	0	1/6	2/6

PB: phenobarbital; ppm: parts per million
Source: Streicker (2014)

Increased hepatocellular proliferation in males reflected an adverse effect, as regeneration was most likely secondary to chemically induced single-cell necrosis. BrdU labelling was markedly increased in males administered the high dose of tioxazafen and phenobarbital, in particular at day 4 of treatment (Table 13).

In phenobarbital-treated males, BrdU staining was predominantly centrilobular. In tioxazafen-treated males, BrdU staining distribution tended to be periportal and bridging to more random and diffuse.

Tioxazafen was shown to be a weak inducer of cytochrome P450 (CYP) activity related to aryl hydrocarbon receptor (AhR), CAR/PXR or peroxisome proliferator–activated receptor alpha (PPARα) and did not show the same enzyme specificity (magnitude or timing of induction) shown for phenobarbital. In tioxazafen-treated animals, increases in AhR- and PPARα-related genes and enzyme activities did not suggest an MOA related to activation of these receptors. Tioxazafen and phenobarbital did not affect either glutathione or reduced glutathione levels in the liver, evaluated as indicators of oxidative stress. These data demonstrate that tioxazafen at the same levels as used in the

carcinogenicity study of Mertens (2014a) induces cytochrome P450 mRNA levels and enzyme activity in a non-specific pattern, unrelated to known MOAs involving activation of nuclear hormone receptors.

Table 13. BrdU labelling index

Dose	% of cells with positive labelling	
	Day 4	**Day 14**
Males		
0	0.3	0.12
250 ppm	1.37	0.017
1 750 ppm	24.9	1.63
Phenobarbital	10.7	0.83
Females		
0	0.5	1.12
50 ppm	0.44	1.0
750 ppm	1.2	0.63
Phenobarbital	13.2	0.43

BrdU: 5-bromo-2′-deoxyuridine; ppm: parts per million
Source: Streicker (2014)

In conclusion, substantial differences in the hepatocellular toxicity profiles of tioxazafen and phenobarbital indicate that the hepatocarcinogenic MOA for these two chemicals is not the same. In addition, there were substantial differences in the incidence and severity of the hepatocellular effects noted between mice treated with the low dose (non-tumorigenic) and high dose (tumorigenic) of tioxazafen. The effects in the high-dose groups were indicative of substantial hepatic enzyme induction (ALT and AST) and cytotoxicity and support a cytotoxic MOA for mouse liver tumour formation (Streicker, 2014).

Twenty-eight-day mode of action study

An additional study was performed to evaluate the potential MOAs for the slightly higher incidences of hepatocellular tumours and haemangiosarcomas observed in high-dose males and/or females in an 18-month mouse feeding study with tioxazafen (Mertens, 2014a). This work was a follow-up to the 14-day MOA study previously conducted by Streicker (2014).

This study included seven male and two female groups of CD-1 mice. Three groups of males (30–40 animals each) were administered a diet containing tioxazafen at 50, 250 or 1750 ppm (equal to 7, 39 and 263 mg/kg bw per day, respectively). A control group of 40 males was administered the basal diet. Three additional groups of males (10–20 animals each) were administered phenobarbital or ciprofibrate in the diet (at 750 and 125 ppm, respectively) or troglitazone by oral gavage (400 mg/kg bw per day) and used as positive controls. Basal diet and diets containing tioxazafen, phenobarbital or ciprofibrate were offered ad libitum for 7, 14 or 28 consecutive days, and troglitazone was administered once daily for 14 or 28 consecutive days. Ten high-dose and 10 control males were assigned to a 28-day recovery period. Groups of 20 females were administered tioxazafen at a dietary concentration of 0 or 750 ppm (equal to 0 and 166 mg/kg bw per day, respectively) for 7 or 14 days. All animals were implanted with an osmotic pump containing BrdU 7 days prior to necropsy.

All animals were observed daily for clinical signs of toxicity. Body weights and feed consumption were recorded weekly. Blood was collected from all animals at termination at days 7,

14, 28 and 56 for clinical chemistry evaluations. All animals were necropsied, and heart, kidneys, liver and spleen were weighed. Liver and abdominal fat samples were collected from all animals. All livers were examined microscopically following routine staining as well as after special staining for iron and lipofuscin. Fat accumulation in the liver was assessed in all animals except the recovery group. Dual immunohistochemistry staining for BrdU and CD31 was also conducted on all livers to assess hepatocellular and endothelial cell proliferation. Liver samples were evaluated for enzyme and gene expression (mRNA) markers for activation of AhR, CAR, PXR, PPARα and PPARγ and gene expression (mRNA) markers for angiogenesis/hypoxia and inflammation. Fat samples were evaluated for gene expression markers for PPARγ activation.

One high-dose male was found dead on day 3. There were no gross or microscopic findings in this animal, but the death was considered likely to be treatment related, as mortality was also observed in the 28-day and 90-day mouse studies. No treatment-related clinical signs or effects on body weight or feed consumption were observed.

Various parameters indicative of liver toxicity were changed in males at 1750 ppm and in females at 750 ppm at all time points (Table 14).

Liver weights were increased in males at 1750 ppm and in females at 750 ppm at all time points. ALT, AST, sorbitol dehydrogenase (SDH) and total bilirubin were increased in 1750 ppm males. SDH and total bilirubin were also increased in 750 ppm females.

Microscopic examination revealed several treatment-related changes in the liver (Table 15).

In the livers of the 1750 ppm males on days 7 and/or 14, centrilobular necrosis, hepatocellular degeneration with microvesicular and macrovesicular vacuolization (steatosis), single-cell necrosis, inflammation, karyocytomegaly, multinucleated hepatocytes and increased mitoses were observed. Centrilobular hepatocellular hypertrophy was noted at days 14 and 28. Minimal centrilobular hypertrophy and minimal pigmented macrophages were each observed in single 250 ppm group males on day 28, and minimal coagulative necrosis, minimal single-cell necrosis, hepatocyte degeneration and centrilobular hepatocellular hypertrophy were observed in 750 ppm females on days 7 and 14. The signs of cytotoxicity had mostly resolved by day 28, except for the presence of pigmented macrophages containing lipofuscin and a slightly higher incidence of single-cell necrosis. No treatment-related microscopic findings were noted at day 56 (following the 28-day recovery period) except for one 1750 ppm male with minimal pigmented macrophages.

Marked increases in hepatocellular proliferation (BrdU-labelled endothelial cells) were noted on day 7 and continued at lower levels through days 14 and 28 (Table 16). These increases were greater in the periportal region than in the centrilobular region, as is commonly observed with chemically induced cytotoxicity. Increases in endothelial cell proliferation were also observed, but were considered secondary to the hepatocellular toxicity and increased hepatocellular proliferation.

The elevated endothelial cell proliferation in the 1750 ppm group was considered secondary to the prominent centrilobular cytotoxicity observed histologically on day 7, with subsequent regeneration of hepatocytes and endothelial cells. Additionally, the elevated endothelial proliferation was also likely secondary to the increased hepatocellular proliferation, as epithelial cell proliferation, benign or malignant, is associated with increased endothelial cell proliferation. In the recovery group at day 56, values were only slightly higher than the control group values, indicating reversibility. In females, there was a slightly increased percentage of BrdU-labelled hepatocytes within the periportal areas on day 7, but not on day 14, indicating reversibility.

There was no biologically meaningful activation of AhR, CAR, PXR, PPARα or PPARγ, and there was no effect on two markers for angiogenesis or hypoxia. Only small differences from controls were observed for several gene expression and enzyme markers related to possible non-genotoxic carcinogenic MOAs. These differences were deemed biologically irrelevant, particularly when compared with the responses observed with the positive control substances.

Table 14. Body weight, liver weight and selected serum chemistry parameters

(a) Males, days 7 and 14

	Day 7						Day 14					
Parameter	**0 ppm**	**50 ppm**	**250 ppm**	**1 750 ppm**	**PB**	**Cipro**	**0 ppm**	**50 ppm**	**250 ppm**	**1 750 ppm**	**Cipro**	**Trog**
Body weight (g)	36.31	36.26	36.70	36.37	37.05	37.59*	36.77	36.71	37.29	36.87	38.19	35.24*
Absolute liver weight (g)	1.886	1.976	1.987	2.556**	2.613**	3.953**	1.946	1.966	2.143	2.435**	4.448**	2.000
ALT (U/L)	66	82	54	205**	80	149**	70	77	74	82	326**	68
AST (U/L)	80	87	78	173**	80	122*	96	87	91	98	220**	106
SDH (U/L)	21	16	20	>65**	18	80**	36	37	36	90**	259**	33
tBIL (mg/dL)	0.07	0.09	0.11	0.28**	0.00**	0.00**	0.11	0.10	0.10	0.28**	0.01**	0.07*
Glucose (mg/dL)	184	193	193	141**	181	177	158	158	155	152	145	162
Cholesterol (mg/dL)	133	136	152	128	121	80**	125	131	134	155	73**	139
HDL (mg/dL)	120	119	136	107	107*	66**	121	128	131	153	63**	135
Triglycerides (mg/dL)	79	68	61	123**	123**	53	91	79	81	76	51**	74

(b) Males, days 28 and 56

	Day 28					Day 56	
Parameter	**0 ppm**	**50 ppm**	**250 ppm**	**1 750 ppm**	**Trog**	**0 ppm**	**1 750 ppm**
Body weight (g)	37.42	37.39	38.15	37.85	35.22*	39.45	39.73
Absolute liver weight (g)	1.952	1.988	2.106	2.355**	1.952	2.141	2.249
ALT (U/L)	58	92	59	84	49	84	51
AST (U/L)	120	125	109	142	141	105	81
SDH (U/L)	44	44	39	63*	42	39	39
tBIL (mg/dL)	0.07	0.05	0.09	0.23**	0.00**	0.05	0.02

Parameter	Day 28					Day 56	
	0 ppm	50 ppm	250 ppm	1 750 ppm	Trog	0 ppm	1 750 ppm
Glucose (mg/dL)	164	154	155	162	164	168	175
Cholesterol (mg/dL)	142	126	133	152	141	132	126
HDL (mg/dL)	137	121	128	151	138	114	115
Triglycerides (mg/dL)	59	51	58	39**	52	123	87

(c) Females, days 7 and 14

Parameter	Day 7		Day 14	
	0 ppm	750 ppm	0 ppm	750 ppm
Body weight (g)	28.66	29.18	29.34	29.59
Absolute liver weight (g)	1.622	1.851**	1.614	1.789**
ALT (U/L)	33	41	34	46
AST (U/L)	82	77	86	84
SDH (U/L)	13	18**	30	36
tBIL (mg/dL)	0.00	0.09**	0.00	0.12**
Glucose (mg/dL)	158	177*	161	160
Cholesterol (mg/dL)	107	110	88	113**
HDL (mg/dL)	93	100	78	102**
Triglycerides (mg/dL)	59	56	74	77

ALT: alanine aminotransferase; AST: aspartate aminotransferase; Cipro: ciprofibrate; HDL: high-density lipoprotein; PB: phenobarbital; ppm: parts per million; SDH: sorbitol dehydrogenase; tBIL: total bilirubin; Trog: troglitazone; U: units; *: $P \leq 0.05$; **: $P \leq 0.01$
Source: Bultman (2017)

Table 15. Incidence of selected liver histopathology findings

(a) Males

	Day 7						Day 14						Day 28					Day 56	
Finding	**0 ppm**	**50 ppm**	**250 ppm**	**1 750 ppm**	**PB**	**Cipro**	**0 ppm**	**50 ppm**	**250 ppm**	**1 750 ppm**	**Cipro**	**Trog**	**0 ppm**	**50 ppm**	**250 ppm**	**1 750 ppm**	**Trog**	**0 ppm**	**1 750 ppm**
No. of tissues examined	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*10*	*9*	*10*	*9*
Necrosis, centrilobular	0	0	0	9	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Necrosis, single-cell	0	0	0	6	0	10	0	1	4	3	2	0	2	2	1	4	0	1	0
Necrosis, coagulative	0	0	0	0	0	0	0	4	1	1	6	1	0	0	0	0	0	1	2
Hepatocellular degeneration	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0
Multinucleated hepatocytes	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Increased mitoses	0	0	0	7	5	8	0	0	0	2	9	0	0	0	0	0	0	0	0
Inflammation, centrilobular	0	0	0	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Karyocytomegaly	0	0	0	10	1	0	0	0	0	4	0	0	0	0	0	0	0	0	0
Centrilobular hypertrophy	0	0	0	0	0	0	0	0	0	6	0	6	0	0	1	9	4	0	0
Pigmented macrophages	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	7	0	1	1
AFIP positive (lipofuscin)	0	0	0	0	0	0	0	2	0	0	0	0	1	0	0	8	0	0	1
Perls’ positive (iron)	0	0	0	0	0	0	0	2	0	0	0	0	1	1	1	2	0	1	3

(b) Females

Finding	Day 7		Day 14	
	0 ppm	**750 ppm**	**0 ppm**	**750 ppm**
No. of tissues examined	*10*	*10*	*10*	*10*
Necrosis, single-cell	0	1	0	1
Necrosis, coagulative	0	2	0	0
Hepatocellular degeneration	0	0	0	1
Centrilobular hypertrophy	0	0	0	1

AFIP: Armed Forces Institute of Pathology; Cipro: ciprofibrate; PB: phenobarbital; ppm: parts per million; Trog: troglitazone
Source: Bultman (2017)

Table 16. Hepatocellular and endothelial cell proliferation

	Labelling index (%)											
	Day 7			Day 14			Day 28			Day 56		
	Centr	Peri	Endo	Centr	Peri	Endo	Centr	Peri	Endo	Centr	Peri	Endo
Males												
0 ppm	0.3	0.4	13.9	0.2	0.3	12.5	0.4	0.9	18.5	0.3	0.4	23.0
50 ppm	0.5	0.9	16.0	0.3	0.7	16.2	0.3	0.4	22.1	–	–	–
250 ppm	0.5	1.0	20.2	1.5	2.2	14.7	0.1	0.5	23.5	–	–	–
1 750 ppm	14.4**	66.5**	55.8**	3.6**	8.3**	20.8**	1.0	3.4**	26.3*	1.5*	1.0	25.5
PB	33.3**	1.9	30.8**	–	–	–	–	–	–	–	–	–
Cipro	8.6	31.2**	39.5**	17.3**	56.5**	32.7**	–	–	–	–	–	–
Trog	–	–	–	0.1	0.3	11.4	0.1	0.2	22.1*	–	–	–
Females												
0 ppm	6.9	7.5	30.5	6.2	6.5	23.5	–	–	–	–	–	–
750 ppm	9.4	15.0*	41.1**	6.8	7.1	24.7	–	–	–	–	–	–

Centr: centrilobular; Cipro: ciprofibrate; Endo: endothelial; PB: phenobarbital; Peri: periportal; ppm: parts per million; Trog: troglitazone; *: $P \leq 0.05$; **: $P \leq 0.01$
Source: Bultman (2017)

Based on these results, the carcinogenic response observed only at the high doses in the previous 18-month mouse feeding study is considered to be a result of a cytotoxic MOA (Bultman, 2017).

(c) Neurotoxicity

Acute neurotoxicity

In an acute neurotoxicity study, groups of Sprague Dawley (Crl:CD (SD)) rats (12 of each sex per group) were administered a single oral gavage dose of tioxazafen (batch no. GLP-1106-21504-T; purity 99.8%; dissolved in 0.5% carboxymethylcellulose in deionized water) at 0, 250, 750 or 2000 mg/kg bw. The animals were observed daily for mortality and clinical signs. Detailed physical examinations were recorded weekly. Body weights were recorded weekly. FOB and locomotor activity data were recorded for all animals prior to treatment and at the time of peak effect on study days 0 (approximately 4 hours following dose administration, estimated on the basis of ADME data), 7 and 14. At termination on day 15, brain weights and brain dimensions (excluding olfactory bulbs) were recorded. In addition, a neuropathological evaluation of selected tissues from the central and peripheral nervous systems was performed on six animals of each sex in the control and 2000 mg/kg bw groups.

No deaths occurred. In all treatment groups, brown material around the anogenital area, decreased defecation, small faeces and red faeces were observed, primarily during the first week of treatment. At day 7, body weights were lower than control values in males at 750 mg/kg bw (4%) and 2000 mg/kg bw (8%). It is likely that the largest effects on body weight will have occurred during the first few days after dosing. However, body weights were not measured between day 0 and day 7 after treatment.

At 4 hours after treatment, a lower body temperature (1.3–1.6 °C) compared with controls was observed in males and females at 750 and 2000 mg/kg bw. Motor activity counts for the 250, 750

and 2000 mg/kg bw group males and females were lower (29–55%) than the control group values on study day 0. Ambulatory motor activity counts were lower in the first few intervals (24–59%) in the 250, 750 and 2000 mg/kg bw group males and females on study day 0.

There were no treatment-related macroscopic or microscopic findings or effects on brain weights or brain dimensions.

A NOAEL for neurotoxicity could not be identified. The lowest-observed-adverse-effect level (LOAEL) for neurotoxicity was 250 mg/kg bw, the lowest dose tested, based on a transient decrease in motor activity observed 4 hours after treatment in males and females at this dose in the absence of any neuropathological changes (Herberth, 2014a).

Subchronic neurotoxicity

In a subchronic neurotoxicity study, groups of Sprague Dawley (Crl:CD (SD)) rats (12 of each sex per group) were administered tioxazafen (batch no. GLP-1106-21504-T; purity 99.8%) in the diet for 13 weeks at a concentration of 0, 100, 300 or 1000 ppm (equal to 0, 7, 20 and 67 mg/kg bw per day for males and 0, 8, 24 and 75 mg/kg bw per day for females, respectively). The animals were observed daily for mortality and clinical signs. Detailed physical examinations were recorded weekly. Body weights and feed consumption were recorded prior to treatment and on study days 0, 1, 2, 3 and 7, weekly thereafter and on the day of the scheduled euthanasia (study day 91). FOB and locomotor activity data were recorded for all animals prior to treatment and in weeks 3, 7 and 12. At termination, the central and peripheral nervous system tissues were dissected and preserved. In addition, the femur bone (with marrow), liver, kidneys and adrenal glands were collected from all animals. Fixed brain weight and brain dimensions (length [excluding olfactory bulbs] and width) were recorded. Any observable gross changes, abnormal coloration or lesions of the brain and spinal cord were recorded. A detailed microscopic neuropathological examination was performed on six randomly selected animals of each sex in the control and 1000 ppm groups.

No deaths occurred. No treatment-related clinical signs were observed. Final body weight gains were slightly lower than control values (5%) in high-dose males and markedly lower in females at the middle (11%) and high doses (18%). Lower feed consumption was noted for the high-dose males on study day 1–2 (24% compared with controls) and throughout the study for the high-dose females (about 24% during the first days of treatment, 11% over the entire treatment period). Lower feed efficiency was noted for the 1000 ppm group males and females on study day 1–2. Metaphyseal hyperostosis of the femur was noted in high-dose males (3/6 animals versus 1/6 animals in controls) and females (4/6 animals versus 2/6 animals in controls). No effects were noted on FOB parameters, locomotor activity, brain weight, brain measurement or histopathology.

The NOAEL for systemic toxicity was 100 ppm (equal to 8 mg/kg bw per day), based on decreased body weight gain in females at 300 ppm (equal to 24 mg/kg bw per day). There was no evidence of a neurotoxic effect of tioxazafen (Herberth, 2014b).

(d) Studies with metabolites and impurities

MON 102130 (3-phenyl-5-thiophen-3-yl-1,2,4-oxadiazole), a photolytic metabolite of tioxazafen, was tested in an acute toxicity study, a 28-day toxicity study and two genotoxicity studies. The molecular structure of MON 102130 is provided in Fig. 6.

Acute toxicity

Results of an acute toxicity study with MON 102130 are shown in Table 17.

Fig. 6. Molecular structure of MON 102130

Table 17. Results of acute toxicity study with MON 102130

Species	Strain	Sex	Route	Metabolite	Purity (%)	LD_{50} (mg/kg bw)	Reference
Rat	Sprague Dawley	F	Oral	MON 102130[a]	97.84	>5 000	Lowe (2014)[b]

bw: body weight; F: female; GLP: good laboratory practice; LD_{50}: median lethal dose; MON 102130: 3-phenyl-5-thiophen-3-yl-1,2,4-oxadiazole

[a] Five female rats were given a single oral dose (gavage) of MON 102130 administered as a 20% weight per weight dilution in corn oil at a dose of 5000 mg/kg bw. Two animals died within 4 days of test substance administration. Prior to death, the animals were hypoactive and exhibited hunched posture, irregular respiration and reduced faecal volume. The surviving animals were hypoactive and exhibited hunched posture, irregular respiration, reduced faecal volume, soft faeces and/or anogenital staining, but recovered by day 7. Gross necropsy of the decedents revealed distention of the stomach and intestines. No gross abnormalities were noted for the euthanized animals when necropsied at the conclusion of the 14-day observation period. Batch no. GLP-1308-22843-T.

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

Short-term study of toxicity

In a 28-day dietary toxicity study, MON 102130 (batch no. GLP-1311-23002-T; purity 98.7%) was administered to groups of six male and six female Sprague Dawley (Crl:CD(SD)) rats at 0, 200, 1000 or 3000 ppm (equal to 0, 15, 72 and 207 mg/kg bw per day for males and 0, 16, 77 and 211 mg/kg bw per day for females, respectively). The animals were checked daily for mortality and clinical signs of toxicity. Body weights and feed consumption were measured weekly throughout the study. The rats were subjected to ophthalmoscopy prior to treatment and during week 4 of treatment. Blood was sampled on the day of necropsy for haematological and clinical biochemistry examinations. All the rats were macroscopically examined, and weights of liver, spleen, kidneys, heart, brain, pituitary, thymus, thyroid with parathyroids, uterus, ovaries with oviducts, testes, prostate with seminal vesicles, epididymides and adrenals were determined. An extensive range of organs and tissues from all control and high-dose rats and liver, kidneys and all gross lesions from rats in the other treatment groups were microscopically examined.

No deaths, clinical signs or ophthalmic changes were observed. Treatment-related lower body weight gains were noted from study days 0 to 7 in the 1000 and 3000 ppm males (33 and 3 g body weight gain, respectively, compared with 45 g in controls) and the 1000 and 3000 ppm females (4 g body weight gain and 3 g body weight loss, respectively, compared with 14 g body weight gain in controls). The body weight differences were statistically significant in the 1000 ppm group females and 3000 ppm group males and females. Lower feed consumption correlated with the lower body weights noted in the 1000 and 3000 ppm group males and females during the first week of test diet administration (study days 0–7). In the 3000 ppm group, observations included lower mean red blood cell mass (red blood cell count, haemoglobin and haematocrit), higher mean absolute lymphocyte, reticulocyte and large unstained cell counts and higher mean red cell and haemoglobin distribution width. Higher mean haemoglobin distribution width was noted in the 1000 ppm group, and lower mean haemoglobin and haematocrit values were noted in the 1000 ppm group (Tables 18 and 19).

In the 1000 and 3000 ppm group males, higher albumin (5% and 13%, respectively), globulin (8% and 8%, respectively), total protein (8% and 11%, respectively) and cholesterol values (60% and 118%, respectively) were observed. In the 1000 and 3000 ppm group females, higher albumin (10% and 15%, respectively), globulin (7% and 19%, respectively), total protein (10% and 15%, respectively), bilirubin (233% and 300%, respectively) and cholesterol values (72% and 193%,

respectively) were observed. Bilirubin levels were also increased in high-dose males. In males and females at 3000 ppm, higher SDH values (100% and 88%, respectively) were observed.

Table 18. Haematology parameters in males

Parameter	0 ppm	200 ppm	1 000 ppm	3 000 ppm
WBC (×10^3/µL)	10.12	8.92	9.77	8.77
RBC (×10^6/µL)	9.02	8.77	8.54	8.35
HGB (g/dL)	16.8	15.9	15.3**	14.7**
HCT (%)	50.6	48.2	46.7*	45.5**
MCH (pg)	18.7	18.2	18.0	17.6**
MCHC (g/dL)	33.3	33.1	32.8	32.3**
PT (s)	15.9	15.9	15.1*	14.5**
Reticulocytes, absolute (×10^3/µL)	139.6	132.0	162.5	189.9*

HCT: haematocrit; HGB: haemoglobin; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; ppm: parts per million; PT: prothrombin time; RBC: red blood cells; WBC: white blood cells; *: $P < 0.05$: **: $P < 0.01$
Source: Kappeler (2014)

Table 19. Haematology parameters in females

Parameter	0 ppm	200 ppm	1 000 ppm	3 000 ppm
WBC (×10^3/µL)	6.51	5.78	5.83	9.20
RBC (×10^6/µL)	8.56	8.48	8.28	7.88**
HGB (g/dL)	16.0	15.8	15.1*	14.3**
HCT (%)	47.9	46.9	45.2*	43.0**
Lymphocytes, absolute (×10^3/µL)	5.23	4.87	4.94	8.07*
LUC, absolute (×10^3/µL)	0.04	0.03	0.04	0.10**
RDW (%)	11.3	11.3	11.7	12.5*
HDW (g/dL)	2.51	2.83*	2.81*	3.03**

HCT: haematocrit; HDW: haemoglobin distribution width; HGB: haemoglobin; LUC: large unstained cell counts; ppm: parts per million; RBC: red blood cells; RDW: red cell distribution width; WBC: white blood cells; *: $P < 0.05$: **: $P < 0.01$
Source: Kappeler (2014)

Necropsy showed swelling of the liver in the 3000 ppm males. In males at 1000 and 3000 ppm, increases in absolute (31% and 57%, respectively) and relative liver weights (37% and 75%, respectively) and absolute (20% and 19%, respectively) and relative spleen weights (25% and 32%, respectively) were observed. In females at 1000 and 3000 ppm, increases in absolute (26% and 56%, respectively) and relative liver weights (37% and 75%, respectively) and absolute (10% and 17%, respectively) and relative kidney weights (14% and 27%, respectively) were observed. In addition, in high-dose males, higher relative kidney weights (14%) were observed, and in high-dose females, higher absolute (34%) and relative spleen weights (44%) were observed.

Minimal to mild centrilobular hepatocyte hypertrophy was noted in the 1000 and 3000 ppm group males and females. Minimal to mild subacute inflammation of the kidney was noted only in the

3000 ppm group, but this was within the historical control range and was considered not to be treatment related.

The NOAEL was 200 ppm (equal to 15 mg/kg bw per day), based on decreased body weight, body weight gain and feed consumption, alterations in haematological and clinical chemistry parameters, organ weight changes and histopathological changes in the liver observed in males and females at 1000 ppm (equal to 72 mg/kg bw per day) (Kappeler, 2014).

Genotoxicity

MON 102130 was tested for genotoxicity in a bacterial mutation test and an in vivo micronucleus test. Both tests gave negative results (Table 20).

Table 20. Overview of genotoxicity tests with MON 102130

End-point	Test system	Concentrations/doses tested	Purity (%)	Results	Reference
In vitro					
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A	15–5 000 µg/plate in DMSO (±S9; TA100, TA1535 and TA1537; *E. coli* WP2*uvr*A) 0.15–5 000 µg/plate in DMSO (±S9, T98)	97.84	Negative[a]	Wagner (2014)
In vivo					
Mouse micronucleus	CD-1 mouse, bone marrow	50, 100 and 200 mg/kg bw in male mice, 75, 150 and 300 mg/kg bw in female mice by gavage as single doses (vehicle was corn oil) 200, 500, 1 000 and 2 000 mg/kg bw in male mice by gavage as single doses (vehicle was 0.5% aqueous carboxymethylcellulose)	97.84	Negative[b]	Kulkarni (2014)

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

[a] MON 102130 (batch no. GLP-1308-22843-T) was evaluated in the initial mutagenicity assay, in all five tester strains, at doses of 1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 µg/plate with and without S9. Precipitate was observed beginning at 150 µg/plate. No background lawn toxicity was observed; however, reductions in revertant counts were observed beginning at 1500 or 5000 µg/plate in some of the tests. Owing to an unacceptable vehicle control value, tester strain WP2*uvr*A in the absence of S9 activation was not evaluated for mutagenicity. In a retest with tester strain WP2*uvr*A in the absence of S9 activation, no positive mutagenic response was observed. No toxicity was observed. In a confirmatory mutagenicity assay, no positive mutagenic responses were observed in the absence or presence of S9 activation or with the tester strains. Precipitate was observed beginning at 150 or 500 µg/plate. No background lawn toxicity was observed; however, reductions in revertant counts were observed beginning at 500 or 5000 µg/plate in some of the tests. Owing to excessive toxicity (beginning at 500 µg/plate), tester strain TA98 in the presence of S9 activation was not evaluated for mutagenicity. In a retest with tester strain TA98 with doses of 0.15, 0.50, 1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 µg/plate, no positive mutagenic response was observed in the presence of S9 activation. Precipitate was observed beginning at 500 µg/plate. No background lawn toxicity was observed; however, a reduction in revertant counts was observed at 5000 µg/plate.

[b] In one test, CD-1 mice received a single oral gavage administration of MON 102130 (batch no. GLP-1308-22843-T) dissolved in corn oil at a dose of 50, 100 or 200 mg/kg bw in males or 75, 150 or 300 mg/kg bw in females. In a second test, CD-1 male mice received a single oral gavage administration of MON 102130 dissolved in aqueous carboxymethylcellulose at a dose of 200, 500, 1000 or 2000 mg/kg bw. As no differences in the clinical signs of toxicity were observed between the sexes, only male mice were used for the additional definitive assay. Doses were based on a range-finding test. A concurrent negative control group received the vehicle only, whereas a positive control group was treated with cyclophosphamide. Bone marrow smears were obtained from five animals per dose group at 24 and 48 hours after dosing. In the range-finding study using corn oil as vehicle, mortality was observed at doses of 300 mg/kg bw and

above. In the definitive tests, no unscheduled deaths were seen in any of the groups. In the mice treated with MON 102130 using corn oil as vehicle, clinical signs included piloerection and diarrhoea observed at 200 mg/kg bw in male mice and piloerection, lethargy, diarrhoea and prostration observed at 300 mg/kg bw in female mice. In the mice treated with MON 102130 using aqueous carboxymethylcellulose as vehicle, clinical signs included piloerection observed at 500, 1000 and 2000 mg/kg bw. MON 102130 induced no statistically significant changes in the incidence of micronucleated immature erythrocytes in either test. No changes in the polychromatic erythrocyte/normochromatic erythrocyte ratio were observed. Cyclophosphamide induced a statistically significant increase in the percentage of cells with micronuclei.

3. Observations in humans

The sponsor (Monsanto, personal communication, 2018) informed JMPR that skin rashes were observed in a limited number of individuals who were potentially exposed to tioxazafen in a laboratory setting or during large-scale manufacture of tioxazafen and commercial-scale seed treating where tioxazafen formulations were applied to seed. Evaluation by an occupational medicine team concluded that in the majority of cases, the dermal symptoms observed were consistent with irritant contact dermatitis. The basis for these conclusions is that direct exposure to skin appeared to be the primary cause of symptoms. These observations in humans are consistent with the results of in vivo irritancy and sensitization testing, which indicated that the tioxazafen formulation is slightly irritating and is not a sensitizer.

Comments

Biochemical aspects

Following the administration of a single oral [^{14}C]tioxazafen dose of 3 or 100 mg/kg bw to rats, absorption was rapid, with a peak concentration in plasma after 2 or 4 hours, respectively. Excretion was rapid, with more than 95% of the radioactivity excreted within 48 hours. Urinary excretion was 24–35%, and faecal excretion was 45–69%. In bile duct–cannulated rats administered a [^{14}C]tioxazafen dose of 100 mg/kg bw, approximately 32–60% of the radiolabel was recovered in the bile, 21–45% in the urine and 3.3–11% in the faeces over 48 hours post-dosing, indicating that at least 89% of the administered dose was absorbed. There were no major differences in excretion or metabolism due to dose, sex or dosing regimen. Tissue distribution was widespread, but levels in tissues were low. Highest levels were found in adrenals, kidneys, liver and thyroid (Thomas, 2014).

Tioxazafen was extensively metabolized to approximately 30 components in rats. No parent compound was found in urine, faeces or bile. Major routes of metabolism of tioxazafen in rats were oxidation (hydroxylation) of the thiophene ring, followed by conjugation primarily with glucuronic acid, and reductive cleavage and subsequent hydrolysis of the oxadiazole ring. The major metabolites were benzamidine, 5-hydroxy-tioxazafen glucuronide and 2-thenoylglycine. Benzamidine was the only metabolite that was recovered in urine at more than 10% of the administered dose (Thomas, 2014).

Toxicological data

The acute oral LD_{50} in rats was greater than 5000 mg/kg bw (Durando, 2011a), the acute dermal LD_{50} was greater than 5000 mg/kg bw (Durando, 2011b) and the acute inhalation LC_{50} was greater than 5.2 mg/L (Durando, 2011c). Tioxazafen was not irritating to the skin of rabbits (Durando, 2011d) and was mildly irritating to the eyes of rabbits (Durando, 2011e). Tioxazafen was not skin sensitizing in a maximization test in guinea-pigs (Durando, 2011f).

In repeated-dose oral toxicity studies with tioxazafen in mice, rats and dogs, a number of effects were observed, most notably reduced body weight gain, increased liver weight, hepatocellular hypertrophy, increased levels of bilirubin and cholesterol, haematological changes, histopathological changes in the adrenals and hyperostosis (bone thickening).

In a 28-day range-finding study in mice using dietary tioxazafen concentrations of 0, 20, 100, 300, 1000 and 3000 ppm (equal to 0, 4, 19, 58, 184 and 437 mg/kg bw per day for males and 0, 5, 25,

70, 219 and 399 mg/kg bw per day for females, respectively), the NOAEL was 300 ppm (equal to 58 mg/kg bw per day), based on increased bilirubin, liver weights and hepatocellular hypertrophy in both sexes, increased cholesterol and GGT levels in females, and termination of one female in extremis at 1000 ppm (equal to 184 mg/kg bw per day). All animals in the 3000 ppm group died or were terminated early (Kirkpatrick, 2013a).

In a 90-day study in mice using dietary tioxazafen concentrations of 0, 10, 50, 200, 600 and 1250 ppm (equal to 0, 2.1, 10.3, 42, 125 and 260 mg/kg bw per day for males and 0, 2.6, 13.8, 54, 174 and 319 mg/kg bw per day for females, respectively), the NOAEL was 600 ppm (equal to 125 mg/kg bw per day), based on increased bilirubin and cholesterol levels in females, increased liver weights and hepatocellular hypertrophy in both sexes, and termination of one female in extremis at 1250 ppm (equal to 260 mg/kg bw per day) (Kirkpatrick, 2013b).

In a 28-day study in rats using dietary tioxazafen concentrations of 0, 50, 200, 1000, 3000 and 10 000 ppm (equal to 0, 4, 15, 76, 201 and 628 mg/kg bw per day for males and 0, 5, 18, 89, 221 and 760 mg/kg bw per day for females, respectively), the NOAEL was 200 ppm (equal to 15 mg/kg bw per day), based on decreases in body weight gain and histopathological changes in the liver in males and decreased feed consumption and feed efficiency, hyperostosis in the femur and increased adipose tissue of the sternal bone marrow in both sexes at 1000 ppm (equal to 76 mg/kg bw per day) (Kirkpatrick, 2013c).

In a 90-day study in rats using dietary tioxazafen concentrations of 0, 10, 50, 250, 750 and 1500 ppm (equal to 0, 1, 3, 16, 47 and 91 mg/kg bw per day for males and 0, 1, 4, 19, 55 and 113 mg/kg bw per day for females, respectively), the NOAEL was 250 ppm (equal to 16 mg/kg bw per day), based on a reduction in body weight gain in females and metaphyseal hyperostosis in both sexes at 750 ppm (equal to 47 mg/kg bw per day) (Kirkpatrick, 2013d).

In a 13-week oral toxicity study in dogs administered tioxazafen by gelatine capsule at a dose of 0, 1, 3, 10, 40 or 120 mg/kg bw per day, the NOAEL was 40 mg/kg bw per day, based on an increase in lung weights in both sexes and one female mortality at 120 mg/kg bw per day (Cada, 2013).

In an 18-month carcinogenicity study in mice using dietary concentrations of 0, 5, 50, 250, 750 (both sexes) and 1750 ppm (males only) (equal to 0, 1, 8, 41, 120 and 282 mg/kg bw per day for males and 0, 1, 10, 50 and 153 mg/kg bw per day for females, respectively), the NOAEL for toxicity was 50 ppm (equal to 10 mg/kg bw per day), based on increases in pigmented macrophages with scattered necrotic hepatocytes and centrilobular hepatocellular hypertrophy in females at 250 ppm (equal to 50 mg/kg bw per day). The NOAEL for carcinogenicity was 250 ppm (equal to 50 mg/kg bw per day), based on an increased incidence of hepatocellular adenomas in females at 750 ppm (equal to 153 mg/kg bw per day). The incidence of hepatocellular carcinomas, but not of adenomas, was increased in males at 1750 ppm, and there was equivocal evidence of increases in the incidence of systemic haemangiosarcomas at 1750 ppm in males and of histiocytic sarcomas at 750 ppm in females (Mertens, 2014a).

Three studies were performed to investigate the MOA for the observed tumours in the carcinogenicity study in mice (Mertens, 2014c; Streicker, 2014; Bultman, 2017). Mice were administered tioxazafen in their diet for 4–90 days at doses ranging from 20 to 1750 ppm in males and from 10 to 750 ppm in females. Tumorigenic doses of tioxazafen induced increased liver weights and serum ALT, AST, SDH and total bilirubin levels in mice. Histopathology showed hepatocellular degeneration, centrilobular necrosis, inflammation, fatty changes, increased mitoses and histiocytic infiltration. Tioxazafen also induced marked increases in hepatocellular proliferation (BrdU labelling), in particular in the periportal region, as is commonly observed with chemically induced cytotoxicity. Observed increases in endothelial cell proliferation were considered secondary to the hepatocellular toxicity and increased hepatocellular proliferation. The effects were predominantly observed in males at 1750 ppm and, to a lesser extent, in females at 750 ppm. There was no biologically meaningful activation of AhR, CAR, PXR, PPARα or PPARγ, indicating that the tumour induction was unrelated to MOAs involving activation of these nuclear hormone receptors. No effects on two markers for angiogenesis or hypoxia were observed. Based on these results, the hepatocellular

carcinogenicity observed at the high doses in the 18-month mouse feeding study was considered to be a result of a cytotoxic MOA. This MOA is relevant to humans, but exhibits a threshold, because tumours would not occur in the absence of hepatotoxicity.

In a 2-year combined toxicity and carcinogenicity study in rats using dietary tioxazafen concentrations of 0, 5, 25, 75, 250 and 750 ppm (equal to 0, 0.3, 1.3, 3.9, 13.3 and 39.6 mg/kg bw per day for males and 0, 0.3, 1.6, 4.9, 16.0 and 48.1 mg/kg bw per day for females, respectively), the NOAEL for toxicity was 75 ppm (equal to 4.9 mg/kg bw per day), based on an increased incidence of endometrial stromal polyps in females at 250 ppm (equal to 16.0 mg/kg bw per day). This lesion is a common, benign, non-cancerous finding in female rodents. Although certain types of uterine polyps can progress to cancer in rare cases, there is no instance of this occurring in the absence of other indications of malignancy (i.e. evidence of preneoplastic changes in the uterus, tumours at other sites). There was no increase in any tumour type in rats that could be attributed to treatment with tioxazafen. The NOAEL for carcinogenicity was 750 ppm (equal to 39.6 mg/kg bw per day), the highest dose tested (Mertens, 2014b).

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

Tioxazafen was tested for genotoxicity in an adequate range of in vitro and in vivo assays. No evidence of genotoxicity was found (Xu, 2011a,b,c,d).

The Meeting concluded that tioxazafen is unlikely to be genotoxic.

In view of the lack of genotoxicity, the absence of carcinogenicity in rats, the fact that hepatocellular adenomas and carcinomas were increased in mice by a cytotoxic MOA and the fact that there was an equivocal increase in the incidence of systemic haemangiosarcomas in male mice and of histiocytic sarcomas in female mice only at the highest dose tested, the Meeting concluded that tioxazafen is unlikely to pose a carcinogenic risk to humans from the diet.

In a two-generation reproductive toxicity study in rats administered tioxazafen in the diet at a dose of 0, 5, 20 or 60 mg/kg bw per day (concentrations were adjusted weekly to provide target test substance doses), the NOAEL for parental toxicity was 20 mg/kg bw per day, based on reduced body weight gains and hyperostosis in F_0 and F_1 males at 60 mg/kg bw per day. The NOAEL for offspring toxicity was 60 mg/kg bw per day, the highest dose tested. The NOAEL for reproductive toxicity was 60 mg/kg bw per day, the highest dose tested (Stump, 2014).

In a developmental toxicity study of tioxazafen in rats using gavage doses of 0, 10, 50 and 200 mg/kg bw per day from GDs 6 to 19, the NOAEL for maternal toxicity was 10 mg/kg bw per day, based on reduced feed intake and body weight gain at 50 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 200 mg/kg bw per day, the highest dose tested (Stump, 2012a).

In a developmental toxicity study in rabbits administered tioxazafen by gavage at a dose of 0, 5, 20 or 100 mg/kg bw per day from GDs 7 to 28, the NOAEL for maternal toxicity was 5 mg/kg bw per day, based on reduced body weight gain at 20 mg/kg bw per day. The NOAEL for embryo and fetal toxicity was 100 mg/kg bw per day, the highest dose tested (Stump, 2012b).

The Meeting concluded that tioxazafen is not teratogenic.

In an acute neurotoxicity study in which rats were administered tioxazafen by gavage at a dose of 0, 250, 750 or 2000 mg/kg bw and then observed for 14 days, no NOAEL could be identified. The LOAEL for neurotoxicity was 250 mg/kg bw, the lowest dose tested, based on a transient decrease in motor activity observed 4 hours after treatment in males and females at this dose in the absence of any neuropathological changes (Herberth, 2014a).

In a 13-week neurotoxicity study in rats using dietary tioxazafen concentrations of 0, 100, 300 and 1000 ppm (equal to 0, 7, 20 and 67 mg/kg bw per day for males and 0, 8, 24 and 75 mg/kg bw per day for females, respectively), the NOAEL for systemic toxicity was 100 ppm (equal to 8 mg/kg bw per day), based on decreased body weight gain in females at 300 ppm (equal to 24 mg/kg bw per day). There was no evidence of a neurotoxic effect of tioxazafen, and the NOAEL for neurotoxicity was 1000 ppm (equal to 67 mg/kg bw per day), the highest dose tested (Herberth, 2014b).

Although there were no indications of neuropathological effects of tioxazafen, the Meeting concluded that tioxazafen may cause transient, acute neurobehavioural effects at high doses.

In a 28-day immunotoxicity study in female mice using dietary tioxazafen concentrations of 0, 100, 300 and 1000 ppm (equal to 0, 26, 80 and 240 mg/kg bw per day, respectively), no signs of an immunotoxic effect were observed. The NOAEL for systemic toxicity was 300 ppm (equal to 80 mg/kg bw per day), based on an increase in bilirubin levels, higher absolute and relative liver weights (17–18%) and minimal to mild centrilobular hepatocellular hypertrophy at 1000 ppm (equal to 240 mg/kg bw per day) (Bultman, 2014).

The Meeting concluded that tioxazafen is not immunotoxic.

Toxicological data on metabolites and/or degradates

The major residues in crops and livestock were tioxazafen and benzamidine. No specific toxicity studies on benzamidine were available. However, this metabolite occurs in rat urine at up to about 12.6% of the administered dose.

The Meeting concluded that the toxicity of benzamidine would be covered by that of tioxazafen.

Toxicological data on MON 102130 (3-phenyl-5-thiophen-3-yl-1,2,4-oxadiazole), a photolytic metabolite of tioxazafen, were available. The acute oral LD_{50} of MON 102130 was greater than 5000 mg/kg bw (Lowe, 2014). In a 28-day study in rats using dietary MON 102130 concentrations of 0, 200, 1000 and 3000 ppm (equal to 0, 15, 72 and 207 mg/kg bw per day for males and 0, 16, 77 and 211 mg/kg bw per day for females, respectively), the NOAEL was 200 ppm (equal to 15 mg/kg bw per day), based on decreased body weight, body weight gain and feed consumption, alterations in haematological and clinical chemistry parameters, organ weight changes and histopathological changes in the liver in both sexes at 1000 ppm (equal to 72 mg/kg bw per day) (Kappeler, 2014).

MON 102130 was negative in a bacterial reverse mutation assay (Wagner, 2014) and in an in vivo micronucleus test in mice (Kulkarni, 2014).

The Meeting concluded that MON 102130 is of similar potency to tioxazafen.

Human data

Skin rashes were observed in a limited number of individuals who were potentially exposed to tioxazafen (Monsanto, personal communication, 2018).

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

Toxicological evaluation

The Meeting established an acceptable daily intake (ADI) of 0–0.05 mg/kg bw for tioxazafen on the basis of a NOAEL of 4.9 mg/kg bw per day in a 2-year rat study, based on a small increase in the incidence of endometrial stromal polyps in females at 16.0 mg/kg bw per day. A safety factor of 100 was used. The upper bound of the ADI gives a margin of about 3000 relative to the LOAEL for the observed tumours in mice. The ADI is supported by a NOAEL of 5 mg/kg bw per day, based on reduced maternal body weight gain observed at 20 mg/kg bw per day, in a developmental toxicity study in rabbits, and a NOAEL of 8 mg/kg bw per day, based on decreased body weight gain in females at 24 mg/kg bw per day, in a 13-week neurotoxicity study in rats.

The Meeting established an acute reference dose (ARfD) of 0.5 mg/kg bw for tioxazafen on the basis of a LOAEL of 250 mg/kg bw, based on a reduction in locomotor activity in an acute neurotoxicity study in rats. A safety factor of 500 was used. An additional factor of 5 was applied for the use of a LOAEL instead of a NOAEL. The Meeting noted that no neurobehavioural signs were observed in any of the repeated-dose studies at bolus doses up to 120 mg/kg bw per day.

The ADI and ARfD can be applied to benzamidine.

Levels relevant to risk assessment of tioxazafen

Species	Study	Effect	NOAEL	LOAEL
Mouse	Eighteen-month study of carcinogenicity[a]	Toxicity	50 ppm, equal to 10 mg/kg bw per day	250 ppm, equal to 50 mg/kg bw per day
		Carcinogenicity	250 ppm, equal to 50 mg/kg bw per day	750 ppm, equal to 153 mg/kg bw per day
Rat	Two-year study of toxicity and carcinogenicity[a]	Toxicity	75 ppm, equal to 4.9 mg/kg bw per day	250 ppm, equal to 16.0 mg/kg bw per day
		Carcinogenicity	750 ppm, equal to 39.6 mg/kg bw per day[b]	–
	Two-generation study of reproductive toxicity[a]	Reproductive toxicity	60 mg/kg bw per day[b]	–
		Parental toxicity	20 mg/kg bw per day	60 mg/kg bw per day
		Offspring toxicity	60 mg/kg bw per day[b]	–
	Developmental toxicity study[c]	Maternal toxicity	10 mg/kg bw per day	50 mg/kg bw per day
		Embryo and fetal toxicity	200 mg/kg bw per day[b]	–
	Acute neurotoxicity study[c]	Neurotoxicity	–	250 mg/kg bw[d]
	Thirteen-week neurotoxicity study[a]	Toxicity	100 ppm, equal to 8 mg/kg bw per day	300 ppm, equal to 24 mg/kg bw per day
		Neurotoxicity	1 000 ppm, equal to 67 mg/kg bw per day[b]	–
Rabbit	Developmental toxicity study[c]	Maternal toxicity	5 mg/kg bw per day	20 mg/kg bw per day
		Embryo and fetal toxicity	100 mg/kg bw per day[b]	–
Dog	Thirteen-week study of toxicity[e]	Toxicity	40 mg/kg bw per day	120 mg/kg bw per day

[a] Dietary administration.
[b] Highest dose tested.
[c] Gavage administration.
[d] Lowest dose tested.
[e] Capsule administration.

Acceptable daily intake (ADI) (applies to tioxazafen and benzamidine, expressed as tioxazafen)

0–0.05 mg/kg bw

Acute reference dose (ARfD) (applies to tioxazafen and benzamidine, expressed as tioxazafen)

0.5 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 tioxazafen

Absorption, distribution, excretion and metabolism in mammals	
Rate and extent of oral absorption	Rapid (T_{max} 2–4 hours) and almost complete (89–97%) in rats
Dermal absorption	7.45%, 1.59% and 1.65% at 4.5, 45 and 450 g/L, respectively (in vivo, rat) 0.52–4.5% (in vitro, human) 8–33% (in vitro, rat)
Distribution	Widely distributed, highest concentrations found in adrenals, kidney, liver and thyroid
Potential for accumulation	None
Rate and extent of excretion	Rapid; 95% in 48 hours
Metabolism in animals	Extensively metabolized, major metabolites are benzamidine, 5-hydroxy-tioxazafen glucuronide and 2-thenoylglycine
Toxicologically significant compounds in animals and plants	Tioxazafen
Acute toxicity	
Rat, LD_{50}, oral	>5 000 mg/kg bw
Rat, LD_{50}, dermal	>5 000 mg/kg bw
Rat, LC_{50}, inhalation	>5.2 mg/L
Rabbit, dermal irritation	Not irritating
Rabbit, ocular irritation	Mildly irritating
Guinea-pig, dermal sensitization	Not sensitizing (Buehler maximization test)
Short-term studies of toxicity	
Target/critical effect	Body weight gain, liver, haematological effects, adrenals, hyperostosis
Lowest relevant oral NOAEL	15 mg/kg bw per day (rat)
Lowest relevant dermal NOAEL	100 mg/kg bw per day (rat)
Lowest relevant inhalation NOAEC	15.3 mg/m^3 (rat)
Long-term studies of toxicity and carcinogenicity	
Target/critical effect	Uterus, liver

Lowest relevant NOAEL	4.9 mg/kg bw per day (rat)
Carcinogenicity	Carcinogenic in mice, but not in rats[a]
Genotoxicity	
	No evidence of genotoxicity[a]
Reproductive toxicity	
Target/critical effect	No reproductive effects
Lowest relevant parental NOAEL	20 mg/kg bw per day (rat)
Lowest relevant offspring NOAEL	60 mg/kg bw per day, highest dose tested (rat)
Lowest relevant reproductive NOAEL	60 mg/kg bw per day, highest dose tested (rat)
Developmental toxicity	
Target/critical effect	No developmental toxicity
Lowest relevant maternal NOAEL	5 mg/kg bw per day (rabbit)
Lowest relevant embryo/fetal NOAEL	100 mg/kg bw per day, highest dose tested (rabbit)
Neurotoxicity	
Acute neurotoxicity NOAEL	250 mg/kg bw, lowest dose tested (rat)
Subchronic neurotoxicity NOAEL	67 mg/kg bw per day, highest dose tested (rat)
Developmental neurotoxicity NOAEL	No data
Other toxicology studies	
Immunotoxicity (28-day study) NOAEL	240 mg/kg bw per day, highest dose tested (mouse)
Studies on toxicologically relevant metabolites	
MON 102130 (photolytic metabolite of tioxazafen)	LD_{50} > 5 000 mg/kg bw 28-day oral toxicity NOAEL 15 mg/kg bw per day No evidence of genotoxicity
Human data	
	Skin rashes were observed in a limited number of individuals who were potentially exposed to tioxazafen

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

Summary

	Value	Study	Safety factor
ADI	0–0.05 mg/kg bw[a]	Two-year toxicity study in rats	100
ARfD	0.5 mg/kg bw[a]	Acute neurotoxicity study in rats	500

[a] Applies to tioxazafen and benzamidine, expressed as tioxazafen.

References

Bultman J (2014). A 28-day oral (dietary) immunotoxicity study of MON 102100 in female CD-1 mice. Unpublished report no. MSL0025785. Monsanto Company, Chesterfield, MO, USA. Monsanto Study No. WI-2013-0570. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

Bultman J (2017). A 28-day mode of action study with dietary MON 102100 in CD-1 mice. Unpublished report no. MSL0028534. Monsanto Company, Chesterfield, MO, USA. Monsanto Study No. WI-2016-0498. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

Cada A (2013). A 90-day toxicity study in the beagle dog with MON 102100. Unpublished report no. MSL0024886. Monsanto Company, Chesterfield, MO, USA. Monsanto Study No. XEN-2011-0288. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

Craig S, Prisk L (2014). The in vitro percutaneous absorption of MON 102100 in the concentrate formulation and two in-use dilutions through rat and human skin. Unpublished report no. MSL0026060. Monsanto Company, Chesterfield, MO, USA. Monsanto Study No. CRO-2014-0077. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

Durando J (2011a). Acute oral toxicity up and down procedure in rats. Unpublished report no. MSL0023799. Monsanto Company, Chesterfield, MO, USA. Monsanto Study No. EPS-2011-0330. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

Durando J (2011b). MON 102100: acute dermal toxicity study in rats. Unpublished report no. MSL0023800. Monsanto Company, Chesterfield, MO, USA. Monsanto Study No. EPS-2011-0331. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

Durando J (2011c). MON 102100: acute inhalation toxicity study in rats. Unpublished report no. MSL0023801. Monsanto Company, Chesterfield, MO, USA. Monsanto Study No. EPS-2011-0332. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

Durando J (2011d). MON 102100: primary skin irritation study in rabbits. Unpublished report no. MSL0023803. Monsanto Company, Chesterfield, MO, USA. Monsanto Study No. EPS-2011-0334. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

Durando J (2011e). MON 102100: primary eye irritation study in rabbits. Unpublished report no. MSL0023802. Monsanto Company, Chesterfield, MO, USA. Monsanto Study No. EPS-2011-0333. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

Durando J (2011f). MON 102100: dermal sensitization study in guinea pigs (Buehler method). Unpublished report no. MSL0023804. Monsanto Company, Chesterfield, MO, USA. Monsanto Study No. EPS-2011-0335. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

Godsey JL (2015). Pharmacokinetic evaluation of [^{14}C]MON 102100 following oral (gavage) administration to CD-1 mice. Unpublished report no. MSL0026369. Monsanto Company, Chesterfield, MO, USA. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

Herberth MT (2014a). An oral (gavage) acute neurotoxicity study of MON 102100 in rats. Unpublished report no. MSL0025784. Monsanto Company, Chesterfield, MO, USA. Monsanto Study No. WI-2012-0409. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

Herberth MT (2014b). A 90-day dietary neurotoxicity study of MON 102100 in rat. Unpublished report no. MSL0025497. Monsanto Company, Chesterfield, MO, USA. Monsanto Study No. WIL-2013-0108. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

Hutton E (2014). The in vivo percutaneous absorption of radiolabelled MON102100 in the concentrate formulation and two in-use dilutions in the rat. Unpublished report no. MSL0026061. Monsanto Company, Chesterfield, MO, USA. Monsanto Study No. CRO-2014-0076. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

Kappeler KV (2014). A 28-day oral (dietary) toxicity study of MON 102130 in Sprague Dawley rats. Unpublished report no. MSL0025786. Monsanto Company, Chesterfield, MO, USA. Monsanto Study No. WI-2013-0571. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

Kirkpatrick JB (2012). A 28-day oral (capsule) study of MON 102100 in beagle dogs. Unpublished report no. MSL0024635. Monsanto Company, Chesterfield, MO, USA. Monsanto Study No. WI-10-363. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

Kirkpatrick JB (2013a). A 28-day oral (diet) study of MON 102100 in mice. Unpublished report no. MSL0024821. Monsanto Company, Chesterfield, MO, USA. Monsanto Study No. WI-10-259. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

Kirkpatrick JB (2013b). A 90-day oral (diet) study of MON 102100 in mice. Unpublished report no. MSL0024822. Monsanto Company, Chesterfield, MO, USA. Monsanto Study No. WI-10-321. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

Kirkpatrick JB (2013c). A 28-day oral (diet) study of MON 102100 in rats. Unpublished report no. MSL0024823. Monsanto Company, Chesterfield, MO, USA. Monsanto Study No. WI-10-258. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

Kirkpatrick JB (2013d). A 90-day oral (diet) study of MON 102100 in rats. Unpublished report no. MSL0024824. Monsanto Company, Chesterfield, MO, USA. Monsanto Study No. WI-10-320. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

Kirkpatrick JB (2013e). A four-week dermal toxicity study of MON 102100 in Sprague Dawley rats. Unpublished report no. MSL0025782. Monsanto Company, Chesterfield, MO, USA. Monsanto Study No. WI-2012-0408. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

Kulkarni R (2014). In vivo micronucleus assay in mice. Monsanto Study No. BRL-2013-0597. Unpublished report no. MSL0025323. Monsanto Company, Chesterfield, MO, USA. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

Lowe C (2014). MON 102130: acute oral toxicity up and down procedure in rats. Unpublished report no. MSL0025321. Monsanto Company, Chesterfield, MO, USA. Monsanto Study No. EPS-2013-0599. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

Mertens JJWM (2014a). An 18-month oral (diet) carcinogenicity study of MON 102100 in CD-1 mice. Unpublished report no. MSL0025728. Monsanto Company, Chesterfield, MO, USA. Monsanto Study No. WI-2011-0342. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

Mertens JJWM (2014b). A 24-month oral (diet) combined chronic toxicity/carcinogenicity study of MON 102100 in Sprague Dawley rats. Unpublished report no. MSL0025727. Monsanto Company, Chesterfield, MO, USA. Monsanto Study No. WI-2011-0213. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

Mertens JJWM (2014c). A mode of action immunohistochemical study of liver effects of MON 102100 in CD-1 mice. Unpublished report no. MSL0025729. Monsanto Company, Chesterfield, MO, USA. Monsanto Study No. WI-2013-0481. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

Randazzo J (2014a). A 4-week nose-only inhalation toxicity study of MON 102100 in Sprague Dawley rats. Unpublished report no. MSL0025670. Monsanto Company, Chesterfield, MO, USA. Monsanto Study No. WIL-2013-0669. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

Randazzo J (2014b). A 13-week nose-only inhalation toxicity study of MON 102100 in Sprague Dawley rats. Unpublished report no. MSL0025671. Monsanto Company, Chesterfield, MO, USA. Monsanto Study No. WIL-2013-0670. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

Streicker M (2014). In vivo mouse liver tumor CAR/PXR mode-of-action study with MON 102100. Unpublished report no. MSL0025302. Monsanto Company, Chesterfield, MO, USA. Monsanto Study No. ILS-2014-0041. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

Stump DG (2012a). An oral (gavage) prenatal developmental toxicity study of MON 102100 in rats. Unpublished report no. MSL0024825. Monsanto Company, Chesterfield, MO, USA. Monsanto Study No. WI-10-409. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

Stump DG (2012b). An oral (gavage) prenatal developmental toxicity study of MON 102100 in rabbits. Unpublished report no. MSL0024534. Monsanto Company, Chesterfield, MO, USA. Monsanto Study No. WI-10-446. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

Stump DG (2014). A dietary two-generation reproductive toxicity study of MON 102100 in rats. Unpublished report no. MSL0025783. Monsanto Company, Chesterfield, MO, USA. Monsanto Study No. WI-2011-0356. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

Thomas J (2014). The absorption, distribution, metabolism and excretion of [^{14}C]-MON 102100 following oral and intravenous administration to rats. Unpublished report no. MSL0025828. Monsanto Company, Chesterfield, MO, USA. Monsanto Study No. WI-2011-0307. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

Wagner VO (2014). Bacterial reverse mutation assay. Unpublished report no. MSL0025322. Monsanto Company, Chesterfield, MO, USA. Monsanto Study No. BRL-2013-0596. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

Xu Y (2011a). Bacterial reverse mutation assay with a confirmatory assay with MON 102100. Unpublished report no. MSL0023414. Monsanto Company, Chesterfield, MO, USA. Monsanto Study No. CV-10-445. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

Xu Y (2011b). CHO/HPRT forward mutation assay with duplicate cultures and a confirmatory assay with MON 102100. Unpublished report no. MSL0023415. Monsanto Company, Chesterfield, MO, USA. Monsanto Study No. CV-10-444. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

Xu Y (2011c). Chromosome aberrations in cultured human peripheral blood lymphocytes treated with MON 102100. Unpublished report no. MSL0023518. Monsanto Company, Chesterfield, MO, USA. Monsanto Study No. CV-10-436. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

Xu Y (2011d). In vivo mouse bone marrow micronucleus assay with MON 102100. Unpublished report no. MSL0023433. Monsanto Company, Chesterfield, MO, USA. Monsanto Study No. CV-10-435. Submitted to WHO by Monsanto Company, Chesterfield, MO, USA.

ANNEX 1

Reports and other documents resulting from previous Joint Meetings of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group on Pesticide Residues

1. Principles governing consumer safety in relation to pesticide residues. Report of a meeting of a WHO Expert Committee on Pesticide Residues held jointly with the FAO Panel of Experts on the Use of Pesticides in Agriculture. FAO Plant Production and Protection Division Report, No. PL/1961/11; WHO Technical Report Series, No. 240, 1962.
2. Evaluation of the toxicity of pesticide residues in food. Report of a Joint Meeting of the FAO Committee on Pesticides in Agriculture and the WHO Expert Committee on Pesticide Residues. FAO Meeting Report, No. PL/1963/13; WHO/Food Add./23, 1964.
3. Evaluation of the toxicity of pesticide residues in food. Report of the Second Joint Meeting of the FAO Committee on Pesticides in Agriculture and the WHO Expert Committee on Pesticide Residues. FAO Meeting Report, No. PL/1965/10; WHO/Food Add./26.65, 1965.
4. Evaluation of the toxicity of pesticide residues in food. FAO Meeting Report, No. PL/1965/10/1; WHO/Food Add./27.65, 1965.
5. Evaluation of the hazards to consumers resulting from the use of fumigants in the protection of food. FAO Meeting Report, No. PL/1965/10/2; WHO/Food Add./28.65, 1965.
6. Pesticide residues in food. Joint report of the FAO Working Party on Pesticide Residues and the WHO Expert Committee on Pesticide Residues. FAO Agricultural Studies, No. 73; WHO Technical Report Series, No. 370, 1967.
7. Evaluation of some pesticide residues in food. FAO/PL:CP/15; WHO/Food Add./67.32, 1967.
8. Pesticide residues. Report of the 1967 Joint Meeting of the FAO Working Party and the WHO Expert Committee. FAO Meeting Report, No. PL:1967/M/11; WHO Technical Report Series, No. 391, 1968.
9. 1967 Evaluations of some pesticide residues in food. FAO/PL:1967/M/11/1; WHO/Food Add./68.30, 1968.
10. Pesticide residues in food. Report of the 1968 Joint Meeting of the FAO Working Party of Experts on Pesticide Residues and the WHO Expert Committee on Pesticide Residues. FAO Agricultural Studies, No. 78; WHO Technical Report Series, No. 417, 1968.
11. 1968 Evaluations of some pesticide residues in food. FAO/PL:1968/M/9/1; WHO/Food Add./69.35, 1969.
12. Pesticide residues in food. Report of the 1969 Joint Meeting of the FAO Working Party of Experts on Pesticide Residues and the WHO Expert Group on Pesticide Residues. FAO Agricultural Studies, No. 84; WHO Technical Report Series, No. 458, 1970.
13. 1969 Evaluations of some pesticide residues in food. FAO/PL:1969/M/17/1; WHO/Food Add./70.38, 1970.
14. Pesticide residues in food. Report of the 1970 Joint Meeting of the FAO Working Party of Experts on Pesticide Residues and the WHO Expert Committee on Pesticide Residues. FAO Agricultural Studies, No. 87; WHO Technical Report Series, No. 4574, 1971.
15. 1970 Evaluations of some pesticide residues in food. AGP:1970/M/12/1; WHO/Food Add./71.42, 1971.

16. Pesticide residues in food. Report of the 1971 Joint Meeting of the FAO Working Party of Experts on Pesticide Residues and the WHO Expert Committee on Pesticide Residues. FAO Agricultural Studies, No. 88; WHO Technical Report Series, No. 502, 1972.

17. 1971 Evaluations of some pesticide residues in food. AGP:1971/M/9/1; WHO Pesticide Residue Series, No. 1, 1972.

18. Pesticide residues in food. Report of the 1972 Joint Meeting of the FAO Working Party of Experts on Pesticide Residues and the WHO Expert Committee on Pesticide Residues. FAO Agricultural Studies, No. 90; WHO Technical Report Series, No. 525, 1973.

19. 1972 Evaluations of some pesticide residues in food. AGP:1972/M/9/1; WHO Pesticide Residue Series, No. 2, 1973.

20. Pesticide residues in food. Report of the 1973 Joint Meeting of the FAO Working Party of Experts on Pesticide Residues and the WHO Expert Committee on Pesticide Residues. FAO Agricultural Studies, No. 92; WHO Technical Report Series, No. 545, 1974.

21. 1973 Evaluations of some pesticide residues in food. FAO/AGP/1973/M/9/1; WHO Pesticide Residue Series, No. 3, 1974.

22. Pesticide residues in food. Report of the 1974 Joint Meeting of the FAO Working Party of Experts on Pesticide Residues and the WHO Expert Committee on Pesticide Residues. FAO Agricultural Studies, No. 97; WHO Technical Report Series, No. 574, 1975.

23. 1974 Evaluations of some pesticide residues in food. FAO/AGP/1974/M/11; WHO Pesticide Residue Series, No. 4, 1975.

24. Pesticide residues in food. Report of the 1975 Joint Meeting of the FAO Working Party of Experts on Pesticide Residues and the WHO Expert Committee on Pesticide Residues. FAO Plant Production and Protection Series, No. 1; WHO Technical Report Series, No. 592, 1976.

25. 1975 Evaluations of some pesticide residues in food. AGP:1975/M/13; WHO Pesticide Residue Series, No. 5, 1976.

26. Pesticide residues in food. Report of the 1976 Joint Meeting of the FAO Panel of Experts on Pesticide Residues and the Environment and the WHO Expert Group on Pesticide Residues. FAO Food and Nutrition Series, No. 9; FAO Plant Production and Protection Series, No. 8; WHO Technical Report Series, No. 612, 1977.

27. 1976 Evaluations of some pesticide residues in food. AGP:1976/M/14, 1977.

28. Pesticide residues in food – 1977. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues and the Environment and the WHO Expert Group on Pesticide Residues. FAO Plant Production and Protection Paper 10 Rev, 1978.

29. Pesticide residues in food: 1977 evaluations. FAO Plant Production and Protection Paper 10 Suppl., 1978.

30. Pesticide residues in food – 1978. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues and the Environment and the WHO Expert Group on Pesticide Residues. FAO Plant Production and Protection Paper 15, 1979.

31. Pesticide residues in food: 1978 evaluations. FAO Plant Production and Protection Paper 15 Suppl., 1979.

32. Pesticide residues in food – 1979. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Expert Group on Pesticide Residues. FAO Plant Production and Protection Paper 20, 1980.

33. Pesticide residues in food: 1979 evaluations. FAO Plant Production and Protection Paper 20 Suppl., 1980.

34. Pesticide residues in food – 1980. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Expert Group on Pesticide Residues. FAO Plant Production and Protection Paper 26, 1981.

35. Pesticide residues in food: 1980 evaluations. FAO Plant Production and Protection Paper 26 Suppl., 1981.

36. Pesticide residues in food – 1981. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Expert Group on Pesticide Residues. FAO Plant Production and Protection Paper 37, 1982.

37. Pesticide residues in food: 1981 evaluations. FAO Plant Production and Protection Paper 42, 1982.

38. Pesticide residues in food – 1982. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Expert Group on Pesticide Residues. FAO Plant Production and Protection Paper 46, 1982.

39. Pesticide residues in food: 1982 evaluations. FAO Plant Production and Protection Paper 49, 1983.

40. Pesticide residues in food – 1983. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Expert Group on Pesticide Residues. FAO Plant Production and Protection Paper 56, 1985.

41. Pesticide residues in food: 1983 evaluations. FAO Plant Production and Protection Paper 61, 1985.

42. Pesticide residues in food – 1984. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Expert Group on Pesticide Residues. FAO Plant Production and Protection Paper 62, 1985.

43. Pesticide residues in food – 1984 evaluations. FAO Plant Production and Protection Paper 67, 1985.

44. Pesticide residues in food – 1985. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and a WHO Expert Group on Pesticide Residues. FAO Plant Production and Protection Paper 68, 1986.

45. Pesticide residues in food – 1985 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 72/1, 1986.

46. Pesticide residues in food – 1985 evaluations. Part II. Toxicology. FAO Plant Production and Protection Paper 72/2, 1986.

47. Pesticide residues in food – 1986. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and a WHO Expert Group on Pesticide Residues. FAO Plant Production and Protection Paper 77, 1986.

48. Pesticide residues in food – 1986 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 78, 1986.

49. Pesticide residues in food – 1986 evaluations. Part II. Toxicology. FAO Plant Production and Protection Paper 78/2, 1987.

50. Pesticide residues in food – 1987. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and a WHO Expert Group on Pesticide Residues. FAO Plant Production and Protection Paper 84, 1987.

51. Pesticide residues in food – 1987 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 86/1, 1988.

52. Pesticide residues in food – 1987 evaluations. Part II. Toxicology. FAO Plant Production and Protection Paper 86/2, 1988.

53. Pesticide residues in food – 1988. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and a WHO Expert Group on Pesticide Residues. FAO Plant Production and Protection Paper 92, 1988.

54. Pesticide residues in food – 1988 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 93/1, 1988.

55. Pesticide residues in food – 1988 evaluations. Part II. Toxicology. FAO Plant Production and Protection Paper 93/2, 1989.

56. Pesticide residues in food – 1989. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and a WHO Expert Group on Pesticide Residues. FAO Plant Production and Protection Paper 99, 1989.

57. Pesticide residues in food – 1989 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 100, 1990.

58. Pesticide residues in food – 1989 evaluations. Part II. Toxicology. FAO Plant Production and Protection Paper 100/2, 1990.

59. Pesticide residues in food – 1990. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and a WHO Expert Group on Pesticide Residues. FAO Plant Production and Protection Paper 102, Rome, 1990.

60. Pesticide residues in food – 1990 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 103/1, Rome, 1990.

61. Pesticide residues in food – 1990 evaluations. Part II. Toxicology. World Health Organization, WHO/PCS/91.47, Geneva, 1991.

62. Pesticide residues in food – 1991. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and a WHO Expert Group on Pesticide Residues. FAO Plant Production and Protection Paper 111, Rome, 1991.

63. Pesticide residues in food – 1991 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 113/1, Rome, 1991.

64. Pesticide residues in food – 1991 evaluations. Part II. Toxicology. World Health Organization, WHO/PCS/92.52, Geneva, 1992.

65. Pesticide residues in food – 1992. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and a WHO Expert Group on Pesticide Residues. FAO Plant Production and Protection Paper 116, Rome, 1993.

66. Pesticide residues in food – 1992 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 118, Rome, 1993.

67. Pesticide residues in food – 1992 evaluations. Part II. Toxicology. World Health Organization, WHO/PCS/93.34, Geneva, 1993.

68. Pesticide residues in food – 1993. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and a WHO Expert Group on Pesticide Residues. FAO Plant Production and Protection Paper 122, Rome, 1994.

69. Pesticide residues in food – 1993 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 124, Rome, 1994.

70. Pesticide residues in food – 1993 evaluations. Part II. Toxicology. World Health Organization, WHO/PCS/94.4, Geneva, 1994.

71. Pesticide residues in food – 1994. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and a WHO Expert Group on Pesticide Residues. FAO Plant Production and Protection Paper 127, Rome, 1995.

72. Pesticide residues in food – 1994 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 131/1 and 131/2 (2 volumes), Rome, 1995.

73. Pesticide residues in food – 1994 evaluations. Part II. Toxicology. World Health Organization, WHO/PCS/95.2, Geneva, 1995.

74. Pesticide residues in food – 1995. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group. FAO Plant Production and Protection Paper 133, Rome, 1996.

75. Pesticide residues in food – 1995 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 137, 1996.

76. Pesticide residues in food – 1995 evaluations. Part II. Toxicological and environmental. World Health Organization, WHO/PCS/96.48, Geneva, 1996.

77. Pesticide residues in food – 1996. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group. FAO Plant Production and Protection Paper 140, 1997.

78. Pesticide residues in food – 1996 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 142, 1997.

79. Pesticide residues in food – 1996 evaluations. Part II. Toxicological. World Health Organization, WHO/PCS/97.1, Geneva, 1997.

80. Pesticide residues in food – 1997. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group. FAO Plant Production and Protection Paper 145, 1998.

81. Pesticide residues in food – 1997 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 146, 1998.

82. Pesticide residues in food – 1997 evaluations. Part II. Toxicological and environmental. World Health Organization, WHO/PCS/98.6, Geneva, 1998.

83. Pesticide residues in food – 1998. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group. FAO Plant Production and Protection Paper 148, 1999.

84. Pesticide residues in food – 1998 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 152/1 and 152/2 (two volumes).

85. Pesticide residues in food – 1998 evaluations. Part II. Toxicological and environmental. World Health Organization, WHO/PCS/99.18, Geneva, 1999.

86. Pesticide residues in food – 1999. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group. FAO Plant Production and Protection Paper 153, 1999.

87. Pesticide residues in food – 1999 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 157, 2000.

88. Pesticide residues in food – 1999 evaluations. Part II. Toxicological. World Health Organization, WHO/PCS/00.4, Geneva, 2000.

89. Pesticide residues in food – 2000. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group. FAO Plant Production and Protection Paper 163, 2001.

90. Pesticide residues in food – 2000 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 165, 2001.

91. Pesticide residues in food – 2000 evaluations. Part II. Toxicological. World Health Organization, WHO/PCS/01.3, 2001.

92. Pesticide residues in food – 2001. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group. FAO Plant Production and Protection Paper 167, 2001.

93. Pesticide residues in food – 2001 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 171, 2002.

94. Pesticide residues in food – 2001 evaluations. Part II. Toxicological. World Health Organization, WHO/PCS/02.1, 2002.

95. Pesticide residues in food – 2002. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group. FAO Plant Production and Protection Paper 172, 2002.

96. Pesticide residues in food – 2002 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 175/1 and 175/2 (two volumes).

97. Pesticide residues in food – 2002 evaluations. Part II. Toxicological. World Health Organization, WHO/PCS, 2003.

98. Pesticide residues in food – 2003. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group. FAO Plant Production and Protection Paper 176, 2004.

99. Pesticide residues in food – 2003 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 177, 2004.

100. Pesticide residues in food – 2003 evaluations. Part II. Toxicological. World Health Organization, WHO/PCS, 2004.

101. Pesticide residues in food – 2004. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group. FAO Plant Production and Protection Paper 178, 2004.

102. Pesticide residues in food – 2004 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 182, 2005.

103. Pesticide residues in food – 2004 evaluations. Part II. Toxicological. World Health Organization, WHO/PCS, 2005.

104. Pesticide residues in food – 2005. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group. FAO Plant Production and Protection Paper 183, 2005.

105. Pesticide residues in food – 2005 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 184, 2006.

106. Pesticide residues in food – 2005 evaluations. Part II. Toxicological. World Health Organization, WHO/PCS/07.1, 2006.

107. Pesticide residues in food – 2006. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group. FAO Plant Production and Protection Paper 187, 2007.

108. Pesticide residues in food – 2006 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 189/1 and 189/2 (two volumes), 2007.

109. Pesticide residues in food – 2006 evaluations. Part II. Toxicological. World Health Organization, 2008.

110. Pesticide residues in food – 2007. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group. FAO Plant Production and Protection Paper 191, 2008.

111. Pesticide residues in food – 2007 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 192, 2008.

112. Pesticide residues in food – 2007 evaluations. Part II. Toxicological. World Health Organization, 2009.

113. Pesticide residues in food – 2008. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group. FAO Plant Production and Protection Paper 193, 2009.

114. Pesticide residues in food – 2008 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 194, 2009.

115. Pesticide residues in food – 2008 evaluations. Part II. Toxicological. World Health Organization, 2010.

116. Pesticide residues in food – 2009. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group. FAO Plant Production and Protection Paper 196, 2010.

117. Pesticide residues in food – 2009 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 198, 2010.

118. Pesticide residues in food – 2009 evaluations. Part II. Toxicological. World Health Organization, 2011.

119. Pesticide residues in food – 2010. Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group. FAO Plant Production and Protection Paper 200, 2011.

120. Pesticide residues in food – 2010 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 206, 2011.

121. Pesticide residues in food – 2010 evaluations. Part II. Toxicological. World Health Organization, 2011.

122. Pesticide residues in food – 2011. Report of the 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. FAO Plant Production and Protection Paper 211, 2012.

123. Pesticide residues in food – 2011 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 206, 2012.

124. Pesticide residues in food – 2011 evaluations. Part II. Toxicological. World Health Organization, 2012.

125. Pesticide residues in food – 2012. Report of the 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. FAO Plant Production and Protection Paper 215, 2013.

126. Pesticide residues in food – 2012 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 216, 2013.

127. Pesticide residues in food – 2012 evaluations. Part II. Toxicological. World Health Organization, 2013.

128. Pesticide residues in food – 2013. Report of the 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. FAO Plant Production and Protection Paper 219, 2014.

129. Pesticide residues in food – 2013 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 220, 2014.

130. Pesticide residues in food – 2013 evaluations. Part II. Toxicological. World Health Organization, 2014.

131. Pesticide residues in food – 2014. Report of the 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. FAO Plant Production and Protection Paper 221, 2015.

132. Pesticide residues in food – 2014 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 222, 2015.

133. Pesticide residues in food – 2014 evaluations. Part II. Toxicological. World Health Organization, 2015.

134. Pesticide residues in food – 2015. Report of the 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. FAO Plant Production and Protection Paper 223, 2015.

135. Pesticide residues in food – 2015 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 226, 2016.

136. Pesticide residues in food – 2015 evaluations. Part II. Toxicological. World Health Organization, 2016.

137. Pesticide residues in food – 2016. Report of a Special Session of the 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. FAO Plant Production and Protection Paper 227, 2016.

138. Pesticide residues in food – 2016 evaluations (Special Session). Toxicological. World Health Organization, 2017.

139. Pesticide residues in food – 2016. Report of the 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. FAO Plant Production and Protection Paper 229, 2015.

140. Pesticide residues in food – 2016 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 231, 2017.

141. Pesticide residues in food – 2016 evaluations. Part II. Toxicological. World Health Organization, 2017.

142. Pesticides residues in food – 2017. Report of the 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. FAO Plant Production and Protection Paper 232, 2017.

143. Pesticide residues in food – 2017 evaluations. Part I. Residues. FAO Plant Production and Protection Paper 233, 2018.

144. Pesticide residues in food – 2017 evaluations. Part II. Toxicological. World Health Organization, 2018.

This volume contains toxicological monographs that were prepared by the 2018 Joint FAO/WHO Meeting on Pesticide Residues (JMPR), which met in Berlin on 18–27 September 2018.

The monographs in this volume summarize the safety data on 14 pesticides that could leave residues in food commodities. These pesticides are chlorfenapyr, ethiprole, fenpicoxamid, fluxapyroxad, imazalil, kresoxim-methyl, lambda-cyhalothrin, mandestrobin, mandipropamid, norflurazon, pydiflumetofen, pyraclostrobin, pyriofenone and tioxazafen. 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.

ISBN 978 92 4 165533 0