Annex I to the CLH report

Proposal for Harmonised Classification and Labelling

Based on Regulation (EC) No 1272/2008 (CLP Regulation), Annex VI, Part 2

Substance Name: methyl N-(isopropoxycarbonyl)-L-valyl-(3*RS*)-3-(4-chlorophenyl)-β-alaninate; valifenalate

EC Number: -

CAS Number: 283159-90-0

Index Number: -

Contact details for dossier submitter:

National Public Health Center, Directorate of Chemical Safety and Competent Authorities (on behalf of the Hungarian MSCA)

Albert Flórián út 2-6.

H-1097

clh.dossier@nnk.gov.hu

Version number: 2 **Date: November 2019**

CONTENTS

1 PHYSICAL HAZARDS 6

1.1 EXPLOSIVES 6
1.1.1 Explosive properties 6
1.2 FLAMMABLE GASES (INCLUDING CHEMICALLY UNSTABLE GASES) 6
1.3 OXIDISING GASES 6
1.4 GASES UNDER PRESSURE 6
1.5 FLAMMABLE LIQUID 6
1.6 FLAMMABLE SOLIDS 7
1.6.1 Determination of Flammability (Solids) 7
1.7 SELF-REACTIVE SUBSTANCES 8
1.8 PYROPHORIC LIQUIDS 8
1.9 PYROPHORIC SOLID 8
1.10 SELF-HEATING SUBSTANCES 8
1.10.1 Relative Self-Ignition Temperature (Solids) 8
1.11 SUBSTANCES WHICH IN CONTACT WITH WATER EMIT FLAMMABLE GASES 9
1.12 OXIDISING LIQUIDS 9
1.13 OXIDISING SOLIDS 9
1.13.1 Oxidising Properties 9
1.14 ORGANIC PEROXIDES 9
1.15 CORROSIVE TO METALS 9

2 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION) 9

2.1 SINGLE ORAL DOSE ADMINISTRATION IN RATS: PRELIMINARY INVESTIGATION – IR5885 9
2.2 SINGLE ORAL DOSE ADMINISTRATION IN RATS – IR5885 15
2.3 SINGLE AND REPEATED ORAL DOSE ADMINISTRATION IN RATS – IR5885 23
2.4 SINGLE AND REPEATED ORAL ADMINISTRATION IN RATS – IR5885 43

3 HEALTH HAZARDS 66

3.1 ACUTE TOXICITY - ORAL ROUTE 66
3.1.1 Animal data 66
3.1.1.1 Acute Oral toxicity to rat – IR5885 66
3.1.2 Human data 68
3.1.3 Other data 68
3.2 ACUTE TOXICITY - DERMAL ROUTE 68
3.2.1 Animal data 68
3.2.1.1 Acute percutaneous toxicity to rat – IR5885 68
3.3 ACUTE TOXICITY - INHALATION ROUTE 70
3.3.1 Animal data 70
3.3.1.1 Acute inhalation toxicity to rat – IR5885 70
3.3.2 Human data 72
3.3.3 Other data 72
3.4 SKIN CORROSION/IRRITATION 72
3.4.1 Animal data 72
3.4.1.1 Skin irritation study on rabbits – IR5885 72
3.4.2 Human data 74
3.4.3 Other data 74
3.5 SERIOUS EYE DAMAGE/EYE IRRITATION 74
3.5.1 Animal data 74
3.5.1.1 Eye irritation study on rabbits – IR5885 74
3.5.2 Human data 76
3.5.3 Other data 76
3.6 RESPIRATORY SENSITISATION 76

3.6.1 Human data ... *76*
3.6.2 Other data ... *76*
3.7 SKIN SENSITISATION ... 76
3.7.1 Animal data ... *76*
3.7.1.1 Dermal sensitisation study on guinea pigs – IR5885 ... 76
3.7.2 Human data ... *78*
3.7.3 Other data ... *78*
3.8 GERM CELL MUTAGENICITY ... 79
3.8.1 In vitro data ... *79*
3.8.1.1 Bacterial assay for gene mutation – IR5885 ... 79
3.8.1.2 Test for clastogenity in mammalian cells – IR5885 ... 82
3.8.1.3 Test for gene mutation in mammalian cells – IR5885 ... 87
3.8.2 Animal data In vivo genotoxicity testing (somatic cells) ... *93*
3.8.2.1 Metaphase analysis in rodent bone marrow, or micronucleus test in rodents – IR5885 ... 93
3.8.3 Human data ... *96*
3.8.4 Other data ... *96*
3.9 CARCINOGENICITY ... 97
3.9.1 Animal data ... *97*
3.9.1.1 Combined long-term and carcinogenicity study – IR5885 ... 97
3.9.1.2 Carcinogenicity study by dietary administration to mouse – IR5885 ... 107
3.9.2 Human data ... *115*
3.9.3 In vitro data (e.g. in vitro germ cell and somatic cell mutagenicity studies, cell transformation assays, gap junction intercellular communication tests) ... *115*
3.9.4 Other data (e.g. studies on mechanism of action) ... *115*
3.9.4.1 Mechanistic Study in the Male CD-1 Mouse -IR5885 ... 115
3.9.4.2 Bridging study to compare the response to Valifenalate in male CD-1 and C57BL/6 mice ... 128
3.9.4.3 Dietary study with Valifenalate in male C57BL/6 and PPARα knockout mice study ... 133
3.9.4.4 Valifenalate - enzyme and DNA-synthesis induction in cultured male wild-type CD-1 mouse hepatocytes ... 144
3.10 REPRODUCTIVE TOXICITY ... 150
3.10.1 Animal data ... *150*
3.10.1.1 Two-generation reproductive toxicity in rat – IR5885 ... 150
3.10.2 Human data ... *167*
3.10.3 Other data (e.g. studies on mechanism of action) ... *167*
3.10.3.1 Teratogenicity test by the oral route in the rat - IR5885 ... 167
3.10.3.2 Teratogenicity test by the oral route in the rabbit – IR5885 ... 170
3.11 SPECIFIC TARGET ORGAN TOXICITY – SINGLE EXPOSURE ... 174
3.11.1 Animal data ... *174*
3.11.1.1 Acute oral neurotoxicity study in rats – Valifenalate technical ... 175
3.11.2 Human data ... *184*
3.11.3 Other data ... *185*
3.12 SPECIFIC TARGET ORGAN TOXICITY – REPEATED EXPOSURE ... 185
3.12.1 Animal data ... *185*
3.12.1.1 Oral 28-day study on rat – IR5885 ... 185
3.12.1.2 Oral 90-day study on rats – IR5885 ... 189
3.12.1.3 Combined long-term and carcinogenicity study – IR5885 ... 196
3.12.1.4 IR5885 4-Week Dermal Toxicity Study in Rats ... 205
3.12.1.5 Oral 28-day study on mouse – IR5885 ... 209
3.12.1.6 Oral 90-day study on mouse – IR5885 ... 214
3.12.1.7 Oral 28-day study on dog – IR5885 ... 219
3.12.1.8 Oral 90 toxicity study (dog)– IR5885 ... 225
3.12.1.9 Oral 1 year study on dog– IR5885 ... 233
3.12.2 Human data ... *247*
3.12.3 Other data ... *247*
3.13 ASPIRATION HAZARD ... 247
3.13.1 Human data ... *248*
3.13.2 Other data ... *248*

4 ENVIRONMENTAL HAZARDS ... 249

4.1 DEGRADATION ... 249
4.1.1 Ready biodegradability ... *249*
4.1.1.1 Effects on the activity of sludge micro-organisms ... 249
4.1.1.2 Ready biodegradability of the active substance – IR5885 ... 251
4.1.2 BOD_5/COD ... *255*

4.1.3 Hydroysis *255*
4.1.3.1 Hydrolytic degradation – IR5885 255
4.1.4 Aquatic simulation tests *263*
4.1.4.1 Water / sediment study – IR5885 263
4.1.5 Other degradability studies *274*
4.1.6 Environmental fate and other relevant information *274*
4.1.6.1 Adsorption and desorption - five soils – IR5885 274
4.2 BIOACCUMULATION 286
4.2.1 Bioaccumulation test on fish *286*
4.2.1.1 Flow-through fish test in Rainbow trout 286
4.2.2 Bioaccumulation test with other organisms *298*
4.3 ACUTE AQUATIC TOXICITY 298
4.3.1 Short-term toxicity to fish *298*
4.3.1.1 Acute toxicity - Rainbow Trout – IR5885 298
4.3.1.2 Acute toxicity to Zebra fish (Brachydanio rerio) 301
4.3.1.3 Acute toxicity to Sheepshead Minnow (Cyprinodon variegatus) 304
4.3.1.4 Acute toxicity to Bluegill sunfish (Lepomis macrochirus) 307
4.3.2 Short-term toxicity to aquatic invertebrates *309*
4.3.2.1 Acute toxicity to aquatic invertebrates - Daphnia magna 309
4.3.2.2 Acute toxicity test with the saltwater mysid (*Americamysis bahia*) 312
4.3.2.3 96-hour shell deposition test in Eastern Oyster (*Crassostrea virginica*) – IR5885 315
4.3.2.4 Whote sediment acute toxicity to a Marine Amphipod *(Leptocheirus plumulosus*) 318
4.3.3 Algal growth inhibition tests *323*
4.3.3.1 Effects on green algae (*Scenedesmus subspicatus)* 323
4.3.3.2 96-hour growth inhibition test in Marine Diatom, *Skeletonema costatum*) – 326
4.3.3.3 Effects on freshwater diatom (*Navicula pelliculosa)* 331
4.3.3.4 Effects on Cyanobacteria (*Anabaena flos-aquae)* 335
4.3.3.5 Effects on Duckweed - *Lemna gibba* 339
4.3.3.6 Effects on Midge Larvae- (*Chironomus dilutus*) 344
4.4 CHRONIC TOXICITY 351
4.4.1 Fish early-life stage (FELS) toxicity test *351*
4.4.1.1 Early life-stage toxicity test with the Fathead Minnow (Pimephales promelas) – IR5885 351
4.4.2 Fish short-term toxicity test on embryo and sac-fry stages *355*
4.4.3 Aquatic Toxicity – Fish, juvenile growth test *355*
4.4.3.1 Chronic Prolonged toxicity test on juvenile fish – Rainbow Trout – IR5885 355
4.4.4 Chronic toxicity to aquatic invertebrates *360*
4.4.4.1 Chronic toxicity to *Daphnia magna* – IR5885 360
4.4.5 Chronic toxicity to algae or aquatic plants *364*
4.5 CHRONIC TOXICITY TO OTHER AQUATIC ORGANISMS 364
4.5.1.1 Effects on sediment dwelling organisms *(Chironomus riparius)* - IR5839 ..... **Error! Bookmark not defined.**

SUPPORT ON HOW TO COMPILE ANNEX I TO THE CLH REPORT

Annex I to the CLH report may be compiled from DARs, CARs and/or other sources. Non-confidential DAR/CAR can be annexed as such provided that it has sufficient level of details on the studies. The DS is encouraged to remove any irrelevant parts of the DAR/CAR. The DS must ensure that Annex I can be published during PC, i.e. it does not contain any confidential information.

For support, below is an example on how each study could be presented individually under its own subchapter including the study reference, detailed study summary and results. The format of the detailed study summary of an individual study is flexible as long as the summary is clearly reported and under a correct hazard class. Detailed support can be found below under each subchapter. If DAR/CAR is annexed to the CLH report as Annex I, it must be indicated clearly in the evaluation part of the report where in Annex I the relevant study can be found. If read-across to structurally or mechanistically similar substance is used please provide a justification for using data from this substance and, if known, present the calculations to convert dose/concentration levels from the test substance to the substance for which CLH is proposed. Please provide also a justification for providing non-testing data by any other approaches such as quantitative structure-activity relationships (QSARs) or grouping methods. Support on grouping of substances and read-across can be found in the following links:

http://echa.europa.eu/documents/10162/13632/information_requirements_r6_en.pdf

http://echa.europa.eu/documents/10162/13655/pg_report_qsars_en.pdf

http://echa.europa.eu/documents/10162/13655/pg_report_readacross_en.pdf

http://www.qsartoolbox.org/

http://www.oecd.org/chemicalsafety/risk-assessment/groupingofchemicalschemicalcategoriesandread-across.htm

http://echa.europa.eu/en/view-article/-/journal_content/title/assessing-read-across-how-echa-does-it

1 PHYSICAL HAZARDS

1.1 Explosives

1.1.1 Explosive properties

Report:	**See annex conf. 43**

Guidelines: Method A14 of Commission Directive 92/69/EEC

Deviation: none

GLP: Yes (certified laboratory)

Detailed study summary and results:

The test material has been predicted to be non-explosive.

Material and methods

Sponsor's identification	IR 5885 (TECHNICAL)
Description	white powder
Batch number	T025/02
Date received	18 March 2003
Storage conditions	room temperature, in the dark

***Explosive Properies* :** The structure was examined for groups that would infer that the material could possess explosive properties.

Results

There are no chemical groups that would infer explosive properties, therefore the result has been predicted negative.

Conclusion: The test material has been predicted to be non-explosive.

1.2 Flammable gases (including chemically unstable gases)

Not applicable as valifenalate is not a gas

1.3 Oxidising gases

Not applicable as valifenalate is not a gas

1.4 Gases under pressure

Not applicable as valifenalate is not a gas

1.5 Flammable liquid

Not applicable as valifenalate is not a gas

1.6 Flammable solids

1.6.1 Determination of Flammability (Solids)

Report:	**See annex conf. 42**

Guidelines: Method A10 Council Directive 91/414/EEC, OPPTS 830.6315.

Deviation: none

GLP: Yes (certified laboratory)

Material and methods

Sponsor's identification	IR 5885 (TECHNICAL)
Description	white powder
Batch number	T025/02
Date received	18 March 2003
Storage conditions	room temperature, in the dark

Method*:* The flammability (solids) was determined by measuring the burning rate of test material prepared as a pile of set dimensions.

Preliminary screening test

A mould (250 x 20 x 10 mm) was loosely filled with test material (tested as received). A noncombustible, non-porous board was placed onto the mould which was then inverted. The mould was removed and an air-rich Bunsen burner flame applied to one end of the pile until ignition occurred

Moisture content

The moisture content was determined gravimetrically.

An aliquot (approximately 1 g) of test material was weighed (in duplicate; A and B) into loss bottles. The samples were dried to constant weight at approximately 105°C.

Calculation

The moisture content was calculated as : Moisture Content (%) $= \frac{b - c}{b - a} \times 100$

Where: a = mass of loss bottle (g),
b = mass of loss bottle and test material (g)
c = mass of loss bottle and test material after drying (g)

Results

Preliminary screening test

The pile melted then ignited but self-extinguished 2 seconds after the Bunsen flame was removed without propagating combustion.

The result of the preliminary screening test obviated the need to perform the main test.

Moisture content

	Determination A	Determination B
a) Mass of loss bottle (g)	19.0387	18.5901
b) Mass of loss bottle and test material (g)	20.0350	19.5902
c) Mass of loss bottle and test material after drying (g)	20.0316	19.5863
Moisture content (% w/w)	0.341	0.390
Mean moisture content (% w/w)	0.366	

Conclusion: The test material has been determined to be not highly flammable as it did not propagate combustion over the 200 mm of the preliminary screening test.

1.7 Self-reactive substances

Not evaluated. Valifenalate is not an thermally unstable substance and is not liable to undergo a strongly exothermic decomposition even without participation of oxygen (air). Valifenalate is not explosive, an organic peroxide nor is it oxidising.

1.8 Pyrophoric liquids

Not applicable as valifenalate is not a liquid

1.9 Pyrophoric solid

Not evaluated. Valifenalate, even in small quantities, is not liable to ignite within five minutes after coming into contact with air.

1.10 Self-heating substances

1.10.1 Relative Self-Ignition Temperature (Solids)

Report:	**See annex conf. 44**
Guidelines:	Method A16, Council Directive 91/414/EEC.
Deviation:	none
GLP:	Yes (certified laboratory)

Material and methods

Sponsor's identification	IR 5885 (TECHNICAL)
Description	white powder
Batch number	T025/02
Date received	18 March 2003
Storage conditions	room temperature, in the dark

Method: The test material was heated in an oven and the relative self ignition temperature determined.

Procedure: An aliquot of the test material was suspended in a stainless steel mesh cube (approximately 20 x 20 x 20 mm, mesh size 0.047 mm) in an oven. A thermocouple was placed in the centre of the sample and another in the oven. The oven temperature was programmed to increase from ambient to 163°C (which was approximately 10°C higher than the melting temperature, determined as part of Safepharm project number 1700/034) at a rate of 0.5°C/min. The temperature/time curves relating to the condition in the centre of the sample and the oven were recorded on a two channel chart recorder.

Results

Observations after the test : The cube contained a piece of white solid. A pool of liquid was underneath the cube.

Conclusion : The test material has been determined not to have a relative self-ignition temperature below its melting temperature.

1.11 Substances which in contact with water emit flammable gases

Not evaluated. Valifenalate does not interact with water to become spontaneously flammable nor does it give off flammable gases in dangerous quantities.

1.12 Oxidising liquids

Not applicable as valifenalate is not a liquid

1.13 Oxidising solids

1.13.1 Oxidising Properties

Report:	**See annex conf. 45**
Guidelines:	Method A17, Council Directive 92/69/EEC.
Deviation:	none
GLP:	Yes (certified laboratory)

Material and methods

Sponsor's identification	IR 5885 (TECHNICAL)
Description	white powder
Batch number	T025/02
Date received	18 March 2003
Storage conditions	room temperature, in the dark

Methods The structure was examined for groups that would infer that the material could possess oxidising properties.

Results

There are no chemical groups that would imply oxidising properties, therefore the result has been predicted negative.

Conclusion: The test material has been predicted to be non-oxidising.

1.14 Organic peroxides

Not Applicable as valifenalate is not a peroxide

1.15 Corrosive to metals

Not evaluated

2 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

2.1 Single oral dose administration in rats: preliminary investigation – IR5885

Reference Number: KII- Section 3, Volume 1, Annex IIA, point 5.1.1/01

Report:	See annex conf. 8

Guidelines: US EPA Pesticide Assessment Guidelines, OPPTS 870.7485 Commission Directive 87/302/EEC, Part B: Methods for the determination of toxicity.

Deviation: none

GLP: Yes (certified laboratory)

Executive summary:

The study was designed to provide preliminary information on the excretion, plasma kinetics and tissue distribution of total radioactivity following oral administration of [^{14}C]-IR5885 to rats.

Two male and 2 female rats each received a single oral administration of [^{14}C]-IR5885 at a target dose level of 250 mg.kg^{-1}. Urine, faeces, expired air and cage wash were collected at selected time points up to 168 hours post dose. The animals were humanely killed by CO_2 narcosis at the end of the collection period and selected tissues retained. The levels of total radioactivity were determined in each sample collected.

A further two male and female rats each received a single oral administration of [^{14}C]-IR5885 at a target dose level of 250 mg.kg^{-1}. Blood samples were collected at intervals up to 72 hours and the levels of radioactivity present determined. A portion of the blood collected at 72 hours was centrifuged to obtain plasma for radioactivity analysis.

The major route of excretion of total radioactivity was *via* faeces accounting for a mean of *ca* 89% (males) and *ca* 72% (females) of the administered dose. Urine excretion accounted for a mean of *ca* 10% for males and *ca* 26% for females. Excretion occurred rapidly with up to 95% of the dose recovered by 24 hours and the dose was quantitatively recovered by the end of the collection period (>99%).

Radioactivity in expired air accounted for ≤ 0.01% of the administered dose, indicating metabolic stability of the site of labelling.

Concentrations of total radioactivity in tissues at 168 hours were below the limit of reliable measurement, consistent with the observed complete excretion of the dose by that time.

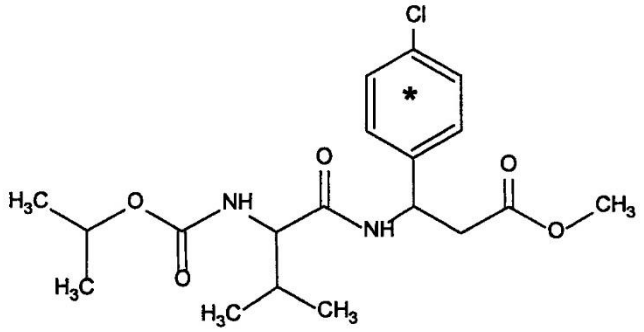
Peak mean concentrations of total radioactivity in whole blood were observed at 1-2 hours post dose, with values of 18.6 and 10.3 µg equiv.mL^{-1} measured at 2 hours and 1 hour for males and females, respectively. Mean concentrations of total radioactivity decreased to 8.3 and 4.2 µg equiv.mL^{-1} at 6 hours for males and females respectively and were at or near the limit of reliable measurement by 24 hours post dose.

<u>Materials and Methods:</u>

Labelled Test Material

[^{14}C-U-phenyl]-IR5885 Batch 192

Specific Activity: 5.089 MBq/mg^{-1}



*denotes ^{14}C position

Radiochemical purity (HPLC): >97%

Unlabelled Test Material

IR5885

Batch No. FCT/T/180-00

Chemical purity (HPLC) 98.9 %

Test animals

Species: Rat (Sprague Dawley, 4 males and 4 females)

Age: 7-8 weeks

Weight at dosing: 200-300 g

Source: Charles River (UK)

Acclimation period: at least 5 days

Diet: standard laboratory diet of known formulation (SDS Rat and Mouse Maintenance Diet No. 1, Batch No. 1784, Special Diets Services, 1 Stepfield, Witham, Essex) RM1 (E) SQC, *ad libitum*

Water: Tap water *ad libitum*

Housing: During pretrial holding period, the animals were housed up to 2 per cage in polypropylene and stainless steel cages with raised wire mesh floors. Following dose administration animals dosed for excretion kinetics (Phase 1) were housed singly in all-glass metabolism cages specially designed for the separate, quantitative collection of urine, faeces and expired air. Animals dosed for blood kinetics (Phase 2) were housed singly in polypropylene and stainless steel cages with raised wire mesh floors.

Environmental conditions -

Temperature: 21 – 22° C

Humidity: 42 – 45 %

Photoperiod: 12-hour cycle (7.00 a.m. – 7 p.m.)

<u>Study Design and Methods</u>

In life dates: January 15 to March 5, 2002

Dose Administration and Determination

The dose was administered by gastric gavage at a target dose volume of 10 mL.kg^{-1}. The actual dose received by each animal was determined with reference to the weight of dose administered and specificity of [^{14}C]-IR-5885 in the formulated dose. The actual dose received by each animal is documented in Table 5.1.1-01.

Table 2.1-01 Individual Dosing Animal Summary

Animal	Weight (kg)	Dose Received		
		mg	MBq	mg.kg^{-1}
001M	0.293	67.80	0.7	231.41
002M	0.278	66.12	0.7	237.83
003F	0.233	55.25	0.6	237.11
004F	0.246	58.54	0.6	237.99
005M	0.286	67.83	0.7	237.15

006M	0.292	70.90	0.7	242.82
007F	0.229	56.37	0.6	246.16
008F	0.231	55.57	0.6	240.54

Collection of Biological Samples

- **Excretion Kinetics and Tissue Residues**

Two male and 2 female rats each received a single oral administration of [^{14}C]-IR-5885 at a target dose level of 250 mg kg^{-1} and were placed singly in all-glass metabolism cages.

Urine was quantitatively collected at the following periods: 8, 12, 24, 48, 72, 96, 120, 144 and 168 hours post dose. Faeces were quantitatively collected at the time points: 24, 48, 72, 96, 120, 144 and 168 hours post dose.

At the end of the collection period, the rats were humanely killed by CO_2 narcosis and the following tissues retained separately:

Bone	Ovaries (as appropriate)
Brain	Residual carcass
Intestinal wall	Spleen
Kidney	Stomach wall
Liver	Subcutaneous fat
Lung	Testes (as appropriate)
Muscle	Whole blood

The levels of radioactivity were determined in each sample collected.

- **Blood Kinetics**

Two male and 2 female rats each received a single oral administration of [^{14}C]-IR-5885 at a target dose level of 250 mg.kg^{-1} and were placed singly in polypropylene and stainless steel cages with raised wire mesh grids.

Blood samples (*ca* 0.3 ml) were collected into heparinised tubes by venesection of a tail vein at the following target times: 0.25, 0.5, 1, 2, 4, 6, 8, 24, 48 and 72 hours post dose.

At 72 hours plasma was also separated by centrifugation and the blood cells discarded. The levels of total radioactivity were determined in each sample of blood collected and in plasma at 72 hours post dose.

Results and Discussion

Excretion Kinetics Following Oral Administration

Following oral administration, the predominant route of excretion was *via* the faeces (mean of 88.8% and 72.0% of the administered dose in males and females, respectively). Excretion of total radioactivity *via* urine accounted for a mean of 10.5% and 26.3% of the administered dose in male and females, respectively. Radioactivity was excreted relatively quickly with up to 95% of the dose recovered by 24 hours post dose.

Only a small proportion (≤0.01%) of the administered dose was recovered in expired air, indicating metabolic stability of the site of radiolabelling.

The recovery of total radioactivity was quantitative at the end of the collection period (168 hours post dose), ranging from 99-101% of the administered dose (Table 2.1-02). Retention of radioactivity in tissues at 168 hours following administration of [^{14}C]-IR-5885 was minimal and all the values obtained were below the limit of reliable measurement (Table 2.1-03).

Table 2.1-02 Cumulative Excretion of Total Radioactivity Following Single Oral Administration of [^{14}C]-IR5885 to Male and Female Rats. Target Dose Level: 250 mg.kg^{-1}

Results expressed as % administered dose

Sample	Timepoint	001M	002M	Mean (Male)	003F	004F	Mean (Female)
Urine	8 h	11.20	5.77	8.48	19.93	17.36	18.65
	12 h	12.30	6.49	9.39	23.38	19.72	21.55

Sample	Timepoint	001M	002M	Mean (Male)	003F	004F	Mean (Female)
	24 h	13.11	7.38	10.24	26.44	23.76	25.10
	48 h	13.28	7.55	10.41	27.58	24.54	26.06
	72 h	13.30	7.60	10.45	27.70	24.61	26.16
	96 h	13.32	7.61	10.46	27.76	24.66	26.21
	120 h	13.32	*7.61	°10.47	27.77	24.67	26.22
	144 h	13.33	*7.62	°10.47	27.80	24.68	26.24
	168 h	13.33	*7.62	°10.48	27.83	24.69	26.26
Faeces	24 h	71.95	86.81	79.38	64.96	62.39	63.67
	48 h	82.94	92.32	87.63	71.59	71.37	71.48
	72 h	84.77	92.52	88.65	71.84	71.96	71.90
	96 h	85.00	92.54	88.77	71.90	72.02	71.96
	120 h	85.02	*92.55	°88.79	*71.92	72.03	°71.97
	144 h	*85.03	*92.56	°88.79	71.93	*72.03	°71.98
	168 h	*85.04	*92.56	°88.80	*71.94	72.04	°71.99
Cage Wash	24 h	0.47	0.80	0.63	1.34	1.99	1.66
	48 h	0.56	*0.83	°0.70	1.52	2.48	2.00
	72 h	*0.60	*0.88	°0.74	*1.54	2.60	°2.07
	96 h	*0.62	*0.89	°0.76	*1.60	2.70	°2.15
	120 h	*0.64	*0.90	°0.77	*1.64	*2.72	°2.18
	144 h	*0.64	*0.90	°0.77	*1.64	*2.72	°2.18
	168 h	*0.66	*0.91	°0.79	1.70	*2.74	°2.22
Exp Air-1	4 h	*0.00	*0.00	°0.00	*0.00	*0.00	°0.00
	8 h	*0.00	*0.00	°0.00	*0.00	*0.00	°0.00
	24 h	*0.01	*0.01	°0.01	*0.01	*0.01	°0.01
Exp Air-2	4 h	*0.00	*0.00	°0.00	*0.00	*0.00	°0.00
	8 h	*0.00	*0.00	°0.00	*0.00	*0.00	°0.00
	24 h	*0.00	*0.00	°0.00	*0.01	*0.01	°0.01
Recovery	4 h	*0.00	*0.00	°0.00	*0.00	*0.00	°0.00
	8 h	*11.21	*5.77	°8.49	*19.94	*17.37	°18.65
	12 h	12.31	6.49	9.40	23.38	19.73	21.56
	24 h	*85.54	*95.00	°90.27	*92.76	*88.15	°90.45
	48 h	96.79	*100.71	°98.75	100.70	98.41	99.55
	72 h	*98.69	*101.02	°99.85	*101.10	99.20	°100.15
	96 h	*98.95	*101.06	°100.00	*101.27	99.38	°100.33
	120 h	*99.00	*101.09	°100.05	*101.35	*99.43	°100.39
	144 h	*99.01	*101.10	°100.06	*101.38	*99.44	°100.41
	168 h	*99.05	*101.11	°100.08	*101.49	*99.50	°100.49

*=Results calculated from data less than 30 d.p.m. above background
°=Mean includes results calculated from data less than 30 d.p.m above background

Table 2.1-03 Concentration of Total Radioactivity in Tissues at 168 h Following Single Oral Administration of [^{14}C]-IR5885 to Male and Female Rats. Target Dose Level: 250 mg.kg^{-1}

Results expressed µg equiv.g^{-1}

Sample	001M	002M	Male (Mean)	003F	004F	Female (Mean)
Bone Mineral	*0.0	*0.0	°0.0	*0.1	*0.4	°0.3
Brain	*0.0	*0.0	°0.0	*0.0	*0.1	°0.1
Fat-Subcutaneous	*0.1	*0.1	°0.1	*0.0	*0.0	°0.0
Kidneys	*0.1	*0.1	°0.1	*0.1	*0.0	°0.1
Liver	*0.1	*0.1	°0.1	*0.2	*0.1	°0.1
Lungs	*0.0	*0.0	°0.0	*0.0	*0.0	°0.0
Muscle	*0.0	*0.0	°0.0	*0.1	*0.0	°0.0
Spleen	*0.1	*0.1	°0.1	*0.0	*0.0	°0.0
Testes/Ovaries	*0.0	*0.1	°0.1	*0.1	*0.2	°0.1
Stomach wall	*0.0	*0.0	°0.0	*0.0	*0.0	°0.0

Intestine wall	*0.0	*0.0	°0.0	*0.0	*0.1	°0.1
Carcass	*0.1	*0.1	°0.1	*0.2	*0.3	°0.2

Whole Blood Kinetics Following Oral Administration

Following administration to male animals, the mean peak concentration of total radioactivity was measured at 2 hours post dose with a mean value of 18.6 µg equiv.mL^{-1}. Mean concentrations fell to 8.3 µg equiv.mL^{-1} at 6 hours and were below the limit of reliable measurement by 24 hours post dose.

Following administration to female animals the concentrations of total radioactivity were similar to those observed in male animals. The peak concentration of total radioactivity was observed at 1 hour with a mean value of 10.3 µg equiv.mL^{-1}. The mean concentration of radioactivity then decreased to 4.2 µg equiv.mL^{-1} at 6 hours and was below the limit of reliable measurement by 24 hours post dose.

The concentrations of total radioactivity in plasma at 72 hours post dose were at or near the limit of reliable measurement. There was, however, an indication that concentrations in plasma were higher than those in whole blood at the equivalent time point, suggesting that the radioactivity in blood maybe mainly associated with the plasma fraction (Table 2.1-04).

Table 2.1-04 Concentration of Total Radioactivity in Whole Blood Following Oral Administration of [^{14}C]-IR5885 to Rats. Target Dose Level: 250 mg.kg^{-1} . Results expressed as µg equiv.g^{-1}

Sample	Timepoint	005M	006M	Mean	007F	008F	Mean
Wh Blood	0.25 h	3.3	7.2	5.3	4.4	6.0	5.2
	0.50 h	7.2	10.5	8.8	8.3	8.6	8.5
	1 h	12.0	15.3	13.7	12.1	8.6	10.3
	2 h	20.1	17.2	18.6	8.4	4.9	6.6
	4 h	10.9	17.4	14.1	3.6	4.7	4.1
	6 h	10.7	5.8	8.3	6.1	2.3	4.2
	8 h	3.5	1.0	2.3	7.4	*0.5	°3.9
	24 h	0.9	*1.5	°1.2	*0.3	*0.5	°0.4
	48 h	*0.0	*0.0	°0.0	*0.0	*0.1	°0.1
	72 h	*0.0	*0.2	°0.1	*0.0	0.2	°0.1
Plasma	72 h	*0.0	0.3	°0.2	*0.1	0.4	°0.3

*=Results calculated from data less than 30 d.p.m. above background
°=Mean includes results calculated from data less than 30 d.p.m above background

Conclusions

Following oral administration of [^{14}C]-IR5885 to rats at a target dose level of 250 mg.kg^{-1}, the major route of excretion was *via* faeces, accounting for 85-93% and 72% of the administered dose for male and female rats, respectively. High faecal excretion indicates either relatively low absorption of the oral dose and/or biliary elimination of absorbed radiolabelled components (test item or related metabolites). The dose was quantitatively recovered over the collection period.

Retention of total radioactivity in tissues was minimal, consistent with the observed near complete excretion of radioactivity by the end of the collection period.

A sex difference in the disposition of this test item in the male and female rat was observed. Urinary excretion in the female rats was higher (25-28% dose) compared to male animals (8-13% dose). Whole blood concentrations of radioactivity were higher in male animals than in female animals (mean Cmax of 18.6 µg equiv.mL^{-1} in males compared to 10.3 µg equiv.mL^{-1} in females). However it should be noted that these comparisons were made from a small data set (n = 2 of each sex) and may not be reflected in a larger study design.

2.2 Single oral dose administration in rats – IR5885

Reference Number:	KII- Section 3, Volume 1, Annex IIA, point 5.1.1/02
Report:	See annex conf. 36
Guidelines:	Council Directive 91/414/EEC, Part A, Annex II, Section 5 amended by Commission Directive 94/79/EC, Annex I, Section 5, and U.S.EPA Health Effects Test Guidelines, OPPTS 870.7485
Deviation:	none
GLP:	Yes (certified laboratory)

Executive summary:

Profiling of metabolites of [^{14}C-U-phenyl] IR5885 in urine and faeces of male and female rats after single oral administration (250 mg/kg bw) was carried out on samples generated during the in-life portions of excretion and tissue distribution study Inveresk Research Project No.172721 performed by Inveresk Research, Tranent EH33 2NE – Scotland (Dossier reference Annex IIA point 5.1.1/01).

Radioactivity was mainly excreted in the faeces (within 48 hours: 87.63% and 71.48% of administered dose - AD- in male and female, respectively) with an appreciable amount excreted via the urine (within 24 hours: 10.24% and 25.10% AD in male and female, respectively) as shown in Inveresk report.

The radioactivity was negligible in all analyzed tissues at 168 hours after dosing.

The urine samples of the same sex up to 24 hours were pooled per time interval and analyzed for radioactivity content by LSC. Aliquots of pooled urine samples were analyzed directly by HPLC and TLC for radioactivity distribution.

The faeces samples of the same sex up to 48 hours were pooled per time interval and extracted twice with acetone and then once with acetone-water. The extracts were analyzed for radioactivity content by LSC and the profile of the metabolites was obtained by HPLC and TLC. The dried faeces residues were oxidized to determine the unextractable radioactivity content by LSC.

98.98% of found radioactivity was extracted by the solvent mixture and only 1.02% was found in the faeces residue.

Chromatographic analyses established that IR5885 was extensively metabolized and five compounds were pointed out: R2, R3, R4, R5, and R6.

Study results showed that:

- the metabolic profile was the same in male and female rats, although the amounts of some compounds were slightly different in the two sexes;
- two of the compounds were identified in urine and faeces;
- unchanged IR5885 (R1) was only found in faeces and amounted to about 41% AD both for male and female rats;
- IR5885 acid (R2) was found both in faeces and urine and reached about 50% AD in the excreta;
- none of the other metabolites reached 4% AD in the excreta.

The HPLC analysis showed that the diastereoisomeric ratio (S,R/S,S) of unchanged IR5885 (R1) and of IR5885 acid (R2) did not change remarkably both in faeces and in urine.

<u>Materials and Methods</u>

Labelled Test Material

[^{14}C-U-phenyl]-IR5885	Lot # 192
Specific Activity:	5.089 MBq/µg
	137.536 µCi/µg
	305331 dpm/µg
Radiochemical purity:	>97% (TLC and HPLC)
Diastereoisomeric ratio	S,R/S,S (by HPLC):0.97

Unlabelled Test Material

IR5885	
Batch No.	FCF/T/180-00
Chemical purity (HPLC)	98.9 %

Source of the biological samples

The urine and faeces used as sources of metabolites in this study were generated during the in-life portions of the Inveresk Research Project No.172721: "A preliminary investigation of the disposition of [^{14}C] IR5885 in the rat following single oral administration".

In the Inveresk study [^{14}C-U-phenyl] IR5885 was orally administered to 4 Sprague-Dawley rats (2 males and 2 females) at a single dose level of 250 mg/kg body weight. Approximately 20 µCi of radiolabelled test material were administered to each animal.

All rats were humanely terminated at 168 hours after dose administration.

Urine was collected from all animals for the periods 0-8, 8-12, 12-24, and then at 24 h intervals to 168 h post dose. Faeces were collected at 24 h intervals to 168 h post dose and homogenized with H_2O (ratio 1/3 w/v).

Tissues were collected at sacrifice of animals at 168 h after dose administration.

The samples were stored frozen.

Identification of samples

Each sample from Inveresk Research was unequivocally identified by a label reporting:

Inveresk Project No.; Urine or Faeces;, animal No. and sex; Collection interval.

<u>Study Design and Methods</u>

Table 2.2-01 Experimental Design (Inveresk Research Project No.172721)

Total IR5885 dose (mg/kg)	250
Nominal Radioactive dose (µCi/kg)	100
Rat No. (sex)	1M, 2M (♂) 3F, 4F (♀)
Urine collection interval (h)	**0-8, 8-12, 12-24**, 24-48, 48-72, 72-96, 96-120, 120-144, 144-168
Faeces collection interval (h)	**0-24, 24-48**, 48-72, 72-96, 96-120, 120-144, 144-168

Transport and storage of samples

The urine and faeces were sent from Inveresk Research to Isagro Ricerca on March 6, 2002 packed in a suitable amount of dry ice so that arrived frozen.

The samples were successively stored at –20°C until the analyses were performed.

Analysis of samples

Radioactivity content of urine and faeces samples

The radioactivity content and the original weight of urine and faeces samples were shown in the Inveresk Research Project No.172721.

Analysis of urine

The urine from each animal of the same sex and at the same time interval (0-8, 8-12, and 12-24 hours) were thawed and pooled by combining 30% of the original volume of the individual samples (Table 2.2-02).

Table 2.2-02 Urine sample identifcation

MU8 and FU8	Urine from Male and Female rats, time 0-8 h
MU12 and FU12	Urine from Male and Female rats, time 8-12 h
MU24 and FU24	Urine from Male and Female rats, time 12-24 h

0.05-mL duplicate aliquots of each pooled urine sample were analyzed for radioactivity content by LSC.

The profile of the radiolabelled urinary compounds was obtained by TLC and HPLC.

Analysis of faeces

The faeces from each animal of the same sex and at the same time interval (0-24 and 24-48 hours) were thawed and pooled by combining 30% of the original weight of the individual samples (Table 2.2-03).

Table 2.2-03 Faecal sample identification

MF24 and FF24	Faeces from Male and Female rats, time 0-24 h
MF48 and FF48	Faeces from Male and Female rats, time 24-48 h

Pooled faecal samples were extracted twice by shaking at 300 strokes/min for 45 minutes with acetone and once with acetone-H_2O 1-1 (ratio faeces/solvent 1/5 w/v).

After each extraction, the residue was separated by centrifugation (15 minutes at 7500 rcf), the supernatants **[Extract I]**, **[Extract II]** and **[Extract III]** were separately collected in volumetric flasks and made up to the mark (100 mL) with acetone.

1-mL duplicate aliquots of all the extracts were analyzed for radioactivity by LSC.

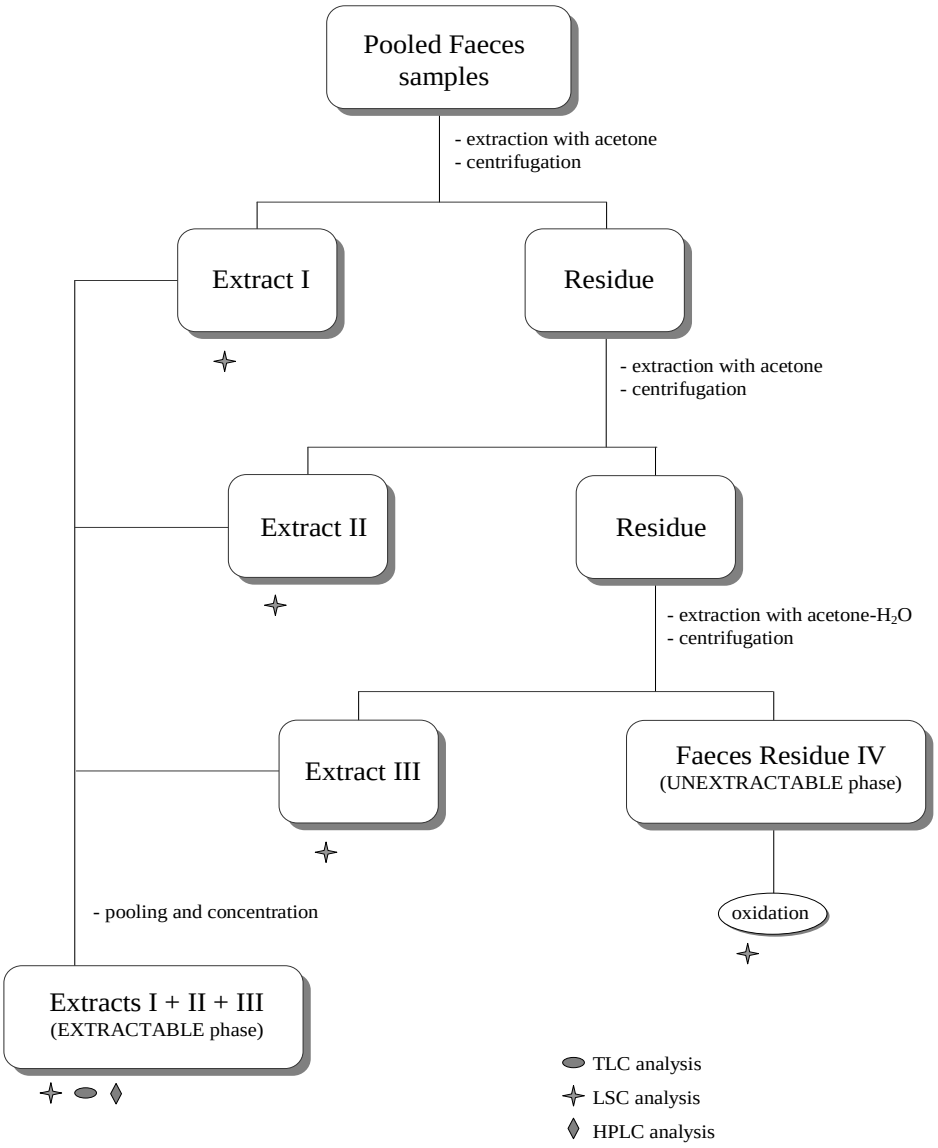
The profile of the radiolabelled faecal compounds was obtained by HPLC and TLC after concentration of a mixture of Extracts I+II+III for each pooled sample.

The **[Faeces Residues IV]** were allowed to dry to constant weight at room temperature and four aliquots (approximately 0.15 g) were then oxidized by means of a biological oxidizer. The radioactivity was determined by LSC.

The complete procedure of extraction/analysis is shown in **Scheme 1**.

Results and Discussion

SCHEME 1. Procedure of extraction and analysis of Faeces.



Inveresk excretion results

The study No.172721 established that excretion after a single oral administration (250 mg/kg bw) occurred almost completely within 24 hours for urine and 48 hours for faeces: total excretion amounted to 97.87% and 96.58% AD in male and female, respectively (Table 2.2-04).

The urinary excretion was 10.24% and 25.10% and faecal excretion was 87.63% and 71.48% in male and female, respectively.

Table 2.2-04 Radioactivity found in Urine within 24 hours, in Faeces within 48 hours and analyzed radioactivity.

Results are expressed as percentage of ^{14}C-IR5885 equivalents referred to administered dose.
The data are derived from Inveresk Project No.172721

Rat	Urine			Faeces		Total excreted analyzed
	0-8 h	8-12 h	12-24 h	0-24 h	24-48 h	
1M	11.20	1.10	0.81	71.95	10.99	
2M	5.77	0.72	0.89	86.81	5.51	
Mean value	**8.48**	**0.91**	**0.85**	**79.38**	**8.25**	
(1M, 2M)	(MU8)	(MU12)	(MU24)	(MF24)	(MF48)	
Total analyzed	10.24			87.63		97.87
3F	19.93	3.45	3.06	64.96	6.63	
4F	17.36	2.36	4.04	62.39	8.98	
Mean value	**18.65**	**2.90**	**3.55**	**63.67**	**7.81**	
(3F, 4F)	(FU8)	(FU12)	(FU24)	(FF24)	(FF48)	
Total analyzed	25.10			71.48		96.57
Mean value total analyzed	17.67			79.55		97.22

Extractable radioactivity in faeces

The radioactivity in faeces was almost completely extracted by acetone and acetone-H_2O; the values referred to radioactivity found in faeces extracts ranged from 98.43% to 99.49% for Extractable phase (Extracts I+II+III) and from 0.51% to 1.57% for Unextractable phase (Faeces Residue IV).

All the values are shown in Table 2.2-05.

The radioactivity referred to AD in the Extractable phase amounted to 78.97% (MF24), 8.15% (MF48), 63.16% (FF24), and 7.69% (FF48), while in the Unextractable phase amounted to 0.41% (MF24), 0.10% (MF48), 0.51% (FF24), and 0.12% (FF48).

All the values are shown in Table 2.2-06.

Table 2.2-05 Radioactivity distribution in Faeces (Scheme 1).
Data are expressed as percentage referred to found radioactivity.

Phase	Male		Female		Mean value
	MF24	**MF48**	**FF24**	**FF48**	**±SD**
EXTRACTABLE	99.49	98.79	99.20	98.43	98.98
(Extracts I + II + III)					0.46
UNEXTRACTABLE	0.51	1.21	0.80	1.57	1.02
(Faeces Residue IV)					0.46
Total	100.00	100.00	100.00	100.00	100.00

Table 2.2-06 Radioactivity distribution in Faeces (see Scheme 1).
Data are expressed as percentage referred to administered dose.

Phase	Male		Female	
	MF24	MF48	FF24	FF48
EXTRACTABLE (Extracts I + II + III)	78.97	8.15	63.16	7.69
UNEXTRACTABLE (Faeces Residue IV)	0.41	0.10	0.51	0.12
Total	79.38	8.25	63.67	7.81

Profiling of ^{14}C-compounds in urine and faeces

Urine

HPLC and TLC analyses of the pooled urine samples showed four/five degradation compounds (Table 2.2-07): R2, R3, R4, R5, and R6. No traces of parent IR5885 were found.

The main degradation compound was R2 (6.29% and 21.83% AD in male and female, respectively). The amounts of the other compounds were always lower than 3% AD both in male and female.

Table 2.2-07 Proportion of compounds in Urine within 24 hours.
Data are expressed as percentage of ^{14}C-IR5885 equivalents referred to administered dose.

Male

Compound	Sample			
	MU8	MU12	MU24	total
R1 (IR5885)	nd	nd	nd	nd
R2	5.49	0.48	0.32	6.29
R3	0.36	0.04	0.04	0.44
R4	2.22	0.07	0.12	2.41
R5	nd	0.05	0.05	0.11
R6	0.41	0.27	0.32	0.99

nd = not detected

Female

Compound	Sample			
	FU8	FU12	FU24	total
R1 (IR5885)	nd	nd	nd	nd
R2	15.75	2.71	3.37	21.83
R3	0.29	0.04	0.05	0.38
R4	2.02	0.05	0.04	2.10
R5	nd	0.02	0.02	0.04
R6	0.60	0.09	0.07	0.75

nd = not detected

Faeces extracts

HPLC and TLC analyses of the pooled faeces extracts showed two degradation compounds (R2, and R3) and, for collection interval 0-24 h, also the parent IR5885 (Table 5.1.1-12).
IR5885 (compound R1) amounted to 40.76% and 41.33% AD in male and female rats, respectively. The main degradation compound was R2 (43.44% and 28.10% AD). The amounts of R3 compound were always lower than 3% and 2% AD, in male and female rats, respectivey.

Table 2.2-08 Proportion of compounds in Faeces extracts within 48 hours.
Data are expressed as percentage of ^{14}C-IR5885 equivalents referred to administered dose.

Male

Compound	Sample		
	MF24	**MF48**	**total**
R1 (IR5885)	40.76	nd	40.76
R2	35.69	7.75	43.44
R3	2.52	0.40	2.92
R4	nd	nd	nd
R5	nd	nd	nd
R6	nd	nd	nd

nd = not detected

Female

Compound	Sample		
	FF24	**FF48**	**total**
R1 (IR5885)	41.33	nd	41.33
R2	20.67	7.42	28.10
R3	1.16	0.27	1.43
R4	nd	nd	nd
R5	nd	nd	nd
R6	nd	nd	nd

nd = not detected

Total extraction

Chromatographic analyses of both urine and faeces showed that IR5885 was extensively metabolized and five compounds were pointed out: R2, R3, R4, R5, and R6 (Table 5.1.1-13).
IR5885 (compound R1) was only found in faeces and amounted to 40.76% and 41.33% AD in male and female rats, respectively.
The main degradation compound was R2 (49.73% and 49.93% AD) and was found both in faeces and urine. The amounts of the other four compounds were always lower than 4% and 3% AD in male and female, respectively.

Table 2.2-09 Proportion of compounds excreted in both Urine and Faeces.
Data are expressed as percentage of ^{14}C-IR5885 equivalents referred to administered dose.

Compound	Sample	
	Male	Female
R1 (IR5885)	40.76	41.33
R2	49.73	49.93
R3	3.36	1.81
R4	2.41	2.10
R5	0.11	0.04
R6	0.99	0.75

Comparison between urinary and faecal compounds

ComComparison between urinary and faecal compounds was performed by co-chromatographic analyses of representative urine and faecal extracts. Urinary compounds in male rats corresponded to urinary compounds

in female rats. Faecal compounds in male rats corresponded to faecal compounds in female rats. Faecal compounds in female rats are present also into urinary profile of female rats.

Identification of IR5885 and its degradation compounds

Identification of the ^{14}C-compounds was performed by co-chromatographic analyses (both TLC and HPLC) of representative samples with authentic reference standards.

Compound R1 (unchanged IR5885)

The radiochromatograms of sample MF24 with authentic ^{14}C-IR5885 reference standard show that the compound R1 had the same chromatographic properties of IR5885.

On the basis of co-HPLC and co-TLC analyses, the compound R1 was identified as unchanged IR5885: RS-β-Alanine, N-[(1-methylethoxy)carbonyl]-L-valyl-3-(4-chlorophenyl) methyl ester.

Compound R2

The radiochromatograms of samples FU8 and FF24 with authentic ^{14}C-IR5885 acid reference standard show that the compound R2 had the same chromatographic properties of IR5885 acid.

On the basis of co-HPLC and co-TLC analyses, the compound R2 was identified as: RS-β-alanine, N-[(1-methylethoxy)carbonyl]-L-valyl-3-(4-chlorophenyl) acid.

Diastereoisomeric composition

Diastereoisomeric ratio (S,R/S,S) of unchanged IR5885 (R1) and of IR5885 acid (R2) did not change remarkably both in faeces and in urine.

Conclusions

Study results showed that IR5885 (compound R1) was extensively metabolized following a single oral administration to rats and 5 degradation compounds were pointed out: R2, R3, R4, R5 and R6.

The metabolic profiles were very similar in male and female rats at the different sampling collection times. Differences between urinary and faecal route were noted only for R1 (unchanged IR5885) absent in urine.

The radioactivity was mainly excreted *via* the faeces (within 48 hours: 87.63% AD and 71.48% AD in male and female rats, respectively). Unchanged IR5885 was only present in faeces and its amount was 40.76% AD and 41.33% AD. The main degradation compound found in faeces was R2 (43.44% AD and 28.10% AD). Another compound (R3) was also found in faeces, but it did not reach 3% AD and 2% AD.

The radioactivity excreted *via* the urine within 24 hours was 10.24% AD and 25.10% AD.

No traces of unchanged IR5885 was present in urine both in male and female.

The main degradation compound found in urine was R2 (6.29% AD and 21.83% AD).

Four compounds were also found in urine, but none of them amounted to values higher than 3% AD.

The identification of compound R2 (IR5885 acid) was carried out by co-TLC and co-HPLC with ^{14}C-reference standards.

The HPLC analysis showed that the diastereoisomeric ratio (*S,R*/*S,S*) of unchanged IR5885 (R1) and of IR5885 acid (R2) did not change remarkably both in faeces and in urine.

2.3 Single and repeated oral dose administration in rats – IR5885

Reference Number: KII- Section 3, Volume 1, Annex IIA, point 5.1.3/01

Report:	See annex conf. 22

Guidelines: US EPA Health Effects Test Guidelines, OPPTS 870.7485
Commission Directive 94/79/EEC, Annex I, Section 5
Council Directive 91/414/EEC, Part A, Annex II, Section 5

Deviation: none

GLP: Yes (certified laboratory)

Executive summary:

This study was designed to examine the excretion and blood kinetics and tissue distribution of total radioactivity following oral administration of [^{14}C]-IR5885 to male and female rats.

The study was conducted in 10 phases. These are outlined in the table below:

Table 2.3-01

Phase	Dose Regime	Dose Level of [^{14}C]-IR-5885	Number of Animals	Experiment
1	Single Oral	100 mg.kg^{-1}	4 ♂, 4 ♀	Excretion Kinetics
2	Single Oral	1000 mg.kg^{-1}	4 ♂, 4 ♀	Excretion Kinetics
3	Multiple Oral*	100 mg.kg^{-1}.day^{-1}	4 ♂, 4 ♀	Excretion Kinetics
4	Single Oral	100 mg.kg^{-1}	4 ♂, 4 ♀	Blood Kinetics
5	Single Oral	1000 mg.kg^{-1}	4 ♂, 4 ♀	Blood Kinetics
6	Multiple Oral*	100 mg.kg^{-1}.day^{-1}	4 ♂, 4 ♀	Blood Kinetics
7	Single Oral	100 mg.kg^{-1}	4 ♂, 4 ♀	Tissue Distribution
8	Single Oral	1000 mg.kg^{-1}	4 ♂, 4 ♀	Tissue Distribution
9	Multiple Oral*	100 mg.kg^{-1}.day^{-1}	4 ♂, 4 ♀	Tissue Distribution
10	Single Oral	100 mg.kg^{-1}	4 ♂, 4 ♀	Biliary Elimination

* = Dosed once daily for 14 consecutive days with cold IR5885 followed by one dose of [^{14}C]-IR5885 (target dose 100 mg.kg^{-1}) on Day 15.

Following dose administration for Phases 1 and 2 and final dose administration for Phase 3, urine, faeces and cagewash were collected at various timepoints until sacrifice. At post mortem the gastrointestinal tract (plus contents) and carcass were retained.

Following dose administration for Phases 4 and 5 and final dose administration for Phase 6, whole blood samples were collected at various timepoints.

The male animals from Phase 7 were sacrificed at 2 hours post dose and the females at 1 hour post dose. The animals from Phases 8 and 9 were sacrificed at 2 hours and 1 hours post dose, respectively. The following organs, tissues and body fluids were removed at necropsy: Bone, Lung, Brain, Muscle, Brown fat, Residual carcass, Gastrointestinal tract, Spleen, Heart, Subcutaneous fat, Kidney, Whole blood and Liver.

Following dose administration for Phase 10, urine, faeces, bile and cagewash were collected at various timepoints until sacrifice. At post mortem the gastrointestinal tract (plus contents) and carcass were retained.

All samples from each phase were analysed for total radioactive residues (TRR). Urine, bile and cagewash were analysed directly by liquid scintillation counting (LSC). All other sample matrices were analysed for TRR by combustion and subsequent LSC.

At the higher dose level (1000 $mg.kg^{-1}$) the excretion pattern following single oral administration was consistent with the single low dose level. Approximately 90% of the administered dose was recovered by 48 hours post dose with the majority of the administered radioactivity being recovered in faeces with mean recoveries of 76.22% (males) and 65.35% (females) of dose at 72 hours post dose. Urine contained mean values of 15.87% and 27.04% of the dose at 72 hours post dose in male and female animals, respectively.

The excretion of radioactivity was not markedly different after the administration of repeated low doses of non-radiolabelled IR5885 followed by a single radiolabelled dose. Again the greater proportion of the administered dose was excreted in faeces with mean recoveries of 82.95% (males) and 57.70% (females) of dose at 72 hours post dose. Urine contained mean values of 8.86% and 33.65% of dose in male and female animals, respectively.

The maximum concentration reached in whole blood following a single oral administration at the high dose level (1000 $mg.kg^{-1}$) was at 2 hours post dose for both male and female animals, 30.24 µg $equiv.g^{-1}$ and 19.86 respectively. In comparison to the low dose level this increase is far less than the 10-fold difference in dose levels.

The pharmacokinetics of total radioactivity in whole blood were not markedly different in male and female animals given repeated low doses of non-radiolabelled IR5885 followed by a single radiolabelled dose compared to animals given a single oral administration. Maximum concentrations were reached 1 hour after dosing and the respective mean peak concentrations were 9.14 and 8.20 µg $equiv.g^{-1}$ for male and female animals, respectively.

Following a single low dose of [^{14}C]-IR5885 to male and female rats the tissue containing the highest concentration of radioactivity at C_{max} was the gastrointestinal tract. The only other tissues to contain a concentration greater than that associated with whole blood were liver and kidney. This pattern of distribution was also observed at the higher dose level and in animals which received repeated administrations of IR5885.

At 72 hours post dose the carcass, that included all the organs and the tissues, showed clearance of radioactivity as it contained amounts always lower than 0.22%.

<u>Materials and Methods</u>

Labelled Test Material

[^{14}C]-IR5885	Lot # 199
Description:	solid
Specific Activity:	4.435 MBq/mg^{-1}
Radiochemical purity:	>97% (TLC and HPLC)

Unlabelled Test Material

IR5885	
Batch No.	FCT/T/180-00
Description:	white, crystalline solid
Chemical purity (HPLC)	98.9 %

Test animals

Species:	Rat (male and female Sprague Dawley rats)
Age:	*ca* 6-12 weeks
Weight at dosing:	range from 170 g to 284 g

Source: Charles River (UK)

Diet: A complete diet of known formulation (SDS Rat and Mouse Maintenance Diet No. 1, Special Diets Services, 1 Stepfield, Witham, Essex) RM3 (E) SQC, *ad libitum*

Water: water *ad libitum*

Housing: During pretrial holding periods, the animals were housed in pairs, where possible, in polypropylene and stainless steel cages with raised wire mesh floors. Following dose administration, the rats used for the excretion studies were housed singly in all-glass metabolism cages specially designed for the separate, quantitative collection of urine, faeces and expired air. In all other phases of the study, the rats were housed singly in polypropylene and stainless steel cages with wire mesh floors.

Temperature: 19 – 25° C

Humidity: 37 – 71 %

Photoperiod: 12-hour cycle (7.00 a.m. – 7 p.m.)

Study Design and Methods

In life dates: May 27 to August 16, 2002

Dose Administration and Determination

The study was conducted in 10 phases (see Table 2.3-01). The dose was administered to each animal by stainless steel gastric gavage attached to a syringe at a volume of *ca* 10 mL.kg^{-1}. The actual dose administered to each animal was determined with reference to the dose concentration, the weight of dose administered and the specific activity of the dose formulation. The individual animal doses are summarised in Table 2.3-02.

Table 2.3-02 Actual Dose of [^{14}C]-IR5885 Administered Orally to Rats

Phase	Animal No.	Animal Weight (g)	Administered Dose			
			Total g	Total µCi	mg Compound	mg.kg^{-1}
1	001M	301	2.986	22.54	29.88	99.26
	002M	305	3.043	22.97	30.45	99.83
	003M	260	2.591	19.55	25.93	99.71
	004M	266	2.624	19.80	26.26	98.71
	005F	254	2.580	19.47	25.82	101.64
	006F	266	2.544	19.20	25.46	95.70
	007F	273	2.765	20.87	27.67	101.34
	008F	279	2.810	21.21	28.12	100.78
2	009M	342	3.345	26.60	333.53	975.22
	010M	332	3.281	26.09	327.19	985.53
	011M	323	3.174	25.24	316.54	980.01
	012M	319	3.146	25.02	313.74	983.52
	013F	221	2.176	17.30	216.94	981.64
	014F	213	2.092	16.64	208.63	979.47
	015F	232	2.244	17.84	223.72	964.33
	016F	211	2.104	16.74	209.85	994.56

Phase	Animal No.	Animal Weight (g)	Administered Dose			
			Total g	Total µCi	mg Compound	mg.kg^{-1}
3	081M*	397	3.964	14.54	39.65	99.87
	018M	432	4.330	31.68	43.34	100.32
	019M	310	3.144	23.00	31.47	101.52
	020M	357	3.550	25.98	35.54	99.54
	021F	282	2.840	20.78	28.43	100.80
	022F	275	2.755	20.15	27.57	100.26
	023F	296	2.977	21.78	29.80	100.67
	024F	262	2.623	19.19	26.26	100.21
4	025M	314	3.178	23.98	31.80	101.27
	026M	315	3.163	23.87	31.65	100.47
	027M	322	3.239	24.45	32.41	100.65
	028M	288	2.868	21.65	28.70	99.64
	029F	283	2.838	21.42	28.40	100.34
	030F	264	2.626	19.82	26.28	99.53
	031F	267	2.682	20.24	26.84	100.51
	032F	252	2.503	18.89	25.05	99.38
5	033M	370	3.603	20.98	374.76	1012.87
	034M	260	2.575	15.00	267.85	1030.20
	035M	266	2.577	15.01	268.03	1007.63
	036M	265	2.548	14.84	265.04	1000.16
	037F	222	2.252	13.11	234.18	1054.88
	038F	226	2.231	12.99	232.02	1026.64
	039F	226	2.241	13.05	233.12	1031.52
	040F	237	2.390	12.72	239.10	1008.84
6	041M	324	3.230	23.64	32.34	99.80
	042M	323	3.205	23.45	32.08	99.33
	043M	375	3.772	27.60	37.76	100.70
	044M	335	3.315	24.26	33.19	99.06
	045F	260	2.603	19.04	26.05	100.21
	046F	273	2.766	20.24	27.68	101.41
	047F	253	2.546	18.63	25.49	100.75
	048F	258	2.566	18.78	25.69	99.57
7	049M	335	3.313	12.16	33.14	98.92
	050M	285	2.786	10.22	27.87	97.77
	051M	292	2.920	10.71	29.21	100.02
	052M	299	2.976	10.92	29.77	99.55
	053F	264	2.602	9.55	26.03	98.58
	054F	249	2.480	9.10	24.81	99.62
	055F	221	2.203	8.08	22.03	99.70
	056F	273	2.700	9.91	27.01	98.92
8	057M	341	3.401	18.10	340.24	997.76
	058M	309	3.113	16.57	311.42	1007.85
	059M	332	3.314	17.64	331.53	998.59
	060M	339	3.311	17.62	331.23	977.09
	061F	241	2.418	12.87	241.90	1003.72
	062F	242	2.419	12.87	242.00	999.99
	063F	256	2.554	13.59	255.50	998.06
	064F	238	2.411	12.83	241.20	1013.43

Phase	Animal No.	Animal Weight (g)	Administered Dose			
			Total g	Total µCi	mg Compound	$mg.kg^{-1}$
9	065M	431	4.334	15.90	43.35	100.58
	066M	420	4.191	15.38	41.92	99.81
	067M	397	3.886	14.26	38.87	97.90
	068M	399	4.009	14.71	40.10	100.50
	069F	271	2.726	10.00	27.27	100.61
	070F	240	2.411	8.85	24.11	100.48
	071F	236	2.350	8.62	23.50	99.60
	072F	283	2.838	10.41	28.39	100.30
10	073M	351	3.496	25.58	35.00	99.70
	074M	313	3.130	22.90	31.33	100.09
	075M	311	3.153	23.07	31.56	101.47
	076M	295	2.949	21.58	29.52	100.07
	077F	232	2.334	17.61	23.35	100.65
	078F	243	2.413	18.21	24.14	99.34
	079F	220	2.236	16.88	22.38	101.71
	080F	244	2.462	18.58	24.63	100.94

* Animal 081M replaced Animal 017M

Biological Sample Collection

- **Excretion Collection (Phases 1-3)**

Following dose administration for Phases 1 and 2 and final dose administration for Phase 3, urine, faeces, expired air and cagewash were collected as described below:

Urine was quantitatively collected (into containers cooled by solid CO_2) at 0-6, 6-12, 12-24 and at 24 hours intervals to 72 hours post dose.

Faeces were quantitatively collected (into containers cooled by solid CO_2) 0-12, 12-24 and at 24 hours intervals to 72 hours post dose.

Expired air was collected, from each animal, into an absorbing solution of ethanolamine: ethanediol (3:7 v/v) for the first 24 hours post dose.

Cages were washed at the time of each faeces collection and the wash retained.

The rats were humanely killed by CO_2 narcosis at the end of the collection period and the gastrointestinal tract and residual carcass retained separately.

- **Blood Collection (Phases 4-6)**

Following dose administration for Phases 4 and 5 and final dose administration for Phase 6, blood samples were collected into heparinised tubes by venesection of a tail vein. Samples were collected at the following timepoints: 0.1, 0.2, 0.4, 1, 2, 4, 6, 8, 24, 48 and 72 hours post dose.

- **Tissue Collection (Phases 7-9)**

For Phase 7, the male and female animals were killed, by CO_2 narcosis, at 2 hours and 1 hour post dose respectively. Phase 8 animals were killed at 2 hours post dose and Phase 9 animals at 1 hour post dose. The following organs, tissues and body fluids were removed at necropsy:

Bone Lung
Brain Muscle
Brown fat Residual carcass
Gastrointestinal tract Spleen
Heart Subcutaneous fat
Kidney Whole blood
Liver

The organs, tissues and body fluids were weighed prior to analysis.

- **Bile and Excretion Collection (Phases 10)**

Bile and urine were collected (into containers cooled by solid CO_2) at 0-6, 6-12, 12-24 and at 24-48 hours post dose.

Faeces were quantitatively collected (into containers cooled by solid CO_2) 0-12, 12-24 and at 24-48 hours post dose.

Cages were washed at the time of each faeces collection and the wash retained.

At the end of the collection period, the animals were killed, by CO_2 narcosis, and the carcass and gastrointestinal tract separately retained.

Results and Discussion

Excretion Kinetics (Phases 1-3)

Phase 1

At 72 hours after administration of [^{14}C]-IR5885 at the lower dose level (100 mg.kg^{-1}), the overall mean recovery of radioactivity was 97.64% and 98.38% in the male and female animals, respectively. For both male and female animals the main route of elimination of total radioactivity was *via* the faeces, which contained a mean of 86.82% and 50.71% of the dose, respectively. Excretion in the urine accounted for 9.50% and 41.55% of the dose, respectively. The excretion of total radioactivity in urine and faeces was very rapid, with the majority of the radioactivity being excreted within *ca* 24 hours of dose administration and excretion was complete by *ca* 48 hours post dose.

Expired air, collected during the first 24 hours of the collection period, accounted for only 0.06% and 0.05% of the administered dose in male and female animals, respectively.

Cagewash samples accounted for a mean of 1.21% (male) and 6.04% (female). At 72 hours post dose, low levels of radioactivity were detected in the gastrointestinal tract and carcass, with mean values in the male animals of 0.02% and 0.03% of the dose, respectively, and mean values in the female animals of 0.01% and 0.02% of the dose, respectively (Tables 2.3-03 and 2.3-04).

Table 2.3-03 Cumulative Excretion of Total Radioactivity Following a Single Oral Administration of [^{14}C]-IR5885 to Male Rats (Phase 1). Target Dose Level: 100 mg.kg^{-1}

Results expressed as % of the administered dose

Sample	Timepoint	1M	2M	3M	4M	Mean	SD
Urine	6 h	9.36	6.24	5.57	2.57	5.93	2.79
	12 h	11.03	7.23	8.41	5.96	8.16	2.16
	24 h	11.91	8.56	9.49	6.87	9.21	2.10
	48 h	12.05	8.85	9.72	7.25	9.47	2.00
	72 h	12.07	8.90	9.77	7.27	9.50	2.00
Faeces	12 h	37.29	0.27	0.21	0.19	9.49	18.53
	24 h	75.68	66.85	68.84	83.78	73.79	7.66
	48 h	81.06	88.98	85.82	89.05	86.23	3.76
	72 h	81.33	90.55	86.22	89.17	86.82	4.08
Cage Wash	12 h	0.51	0.38	0.52	1.25	0.66	0.39
	24 h	1.05	1.35	0.62	1.50	1.13	0.39
	48 h	*1.09	1.40	*0.67	*1.54	º1.17	º0.39
	72 h	*1.09	1.46	0.72	*1.57	º1.21	º0.39
Expired Air-1	24 h	0.05	0.04	0.05	0.05	0.05	0.01
Expired Air-2	24 h	*0.00	*0.01	*0.00	*0.01	º0.01	º0.01
G.I. Tract + Contents	72 h	0.02	0.05	0.02	*0.01	º0.02	º0.02
Carcass#	72 h	*0.08	*0.02	*0.02	*0.00	º0.03	º0.03
Total		94.64	101.02	96.80	98.08	97.64	2.67

*=Results calculated from data less than 30 d.p.m. above background
°=Mean includes results calculated from data less than 30 d.p.m above background
= Includes all organs and tissues

Table 2.3-04 Cumulative Excretion of Total Radioactivity Following a Single Oral Administration of [^{14}C]-IR5885 to Female Rats (Phase 1). Target Dose Level: 100 mg.kg^{-1}

Results expressed as % of the administered dose

Sample	Timepoint	5F	6F	7F	8F	Mean	SD
Urine	6 h	33.50	26.08	33.82	26.89	30.07	4.16
	12 h	41.18	33.69	41.96	35.08	37.98	4.20
	24 h	43.67	35.74	44.84	38.09	40.59	4.38
	48 h	44.49	36.69	45.23	39.43	41.46	4.09
	72 h	44.54	36.85	45.32	39.50	41.55	4.06
Faeces	12 h	*0.00	11.56	18.26	15.38	º11.30	º8.02
	24 h	45.82	44.98	47.11	49.06	46.74	1.77
	48 h	46.73	51.01	50.35	53.83	50.48	2.92
	72 h	46.83	51.37	50.51	54.13	50.71	3.01
Cage Wash	12 h	4.51	8.62	3.35	2.92	4.85	2.60
	24 h	4.81	9.04	3.86	3.82	5.38	2.48
	48 h	6.39	9.23	4.02	4.01	5.91	2.48
	72 h	6.49	9.40	4.12	4.17	6.04	2.49
Expired Air-1	24 h	0.04	0.04	0.04	0.04	0.04	0.00
Expired Air-2	24 h	*0.01	*0.02	*0.00	*0.01	º0.01	º0.00
G.I. Tract + Contents	72 h	0.01	0.02	0.01	0.01	0.01	0.01
Carcass#	72 h	*0.01	*0.02	*0.01	*0.02	º0.02	º0.01
Total		97.93	97.72	100.01	97.87	98.38	1.09

*=Results calculated from data less than 30 d.p.m. above background

°=Mean includes results calculated from data less than 30 d.p.m above background
= Includes all organs and tissues

Phase 2

Following administration of [^{14}C]-IR5885 at the higher dose level (1000 mg.kg^{-1}) the excretion of radioactivity for the male and female animals resulted in a mean recovery of 94.41% and 94.07% of the administered dose, respectively. The main route of elimination of total radioactivity was *via* the faeces for both male and female animals, with mean values of 76.22% and 65.35% of the dose, respectively. Excretion in the urine accounted for 15.87% and 27.04% of the dose, respectively. The excretion of total radioactivity was very rapid, with the majority of the radioactivity being excreted within *ca* 24 hours of dose administration and excretion was complete by *ca* 48 hours post dose.

Expired air, collected during the first 24 hours of the collection period, accounted for only 0.04% and 0.06% of the administered dose in male and female animals, respectively.

Cagewash samples accounted for a mean of 1.06% (male) and 1.59% (female). At 72 hours post dose, very small amounts of radioactivity were detected in the gastrointestinal tract and carcass. In the male animals, the mean values were 1.11 % and 0.11% of the dose, respectively. In the female animals, mean values were 0.04% and <0.01%, respectively (Tables 2.3-05 and 2.3-06).

Table 2.3-05 Cumulative Excretion of Total Radioactivity Following a Single Oral Administration of [^{14}C]-IR5885 to Male Rats (Phase 2. Target Dose Level: 1000 mg.kg^{-1}

Results expressed as % of the administered dose

Sample	Timepoint	9M	10M	11M	12M	Mean	SD
Urine	6 h	6.95	7.98	7.02	3.82	6.44	1.81
	12 h	12.39	15.37	10.90	8.94	11.90	2.71
	24 h	15.30	16.53	12.88	12.94	14.42	1.81
	48 h	16.32	16.78	13.64	15.86	15.65	1.39
	72 h	16.63	16.81	13.76	16.30	15.87	1.43
Faeces	12 h	34.27	30.04	28.86	13.16	26.58	9.24
	24 h	58.33	67.68	67.73	43.99	59.43	11.20
	48 h	64.75	75.94	78.36	65.46	71.13	7.03
	72 h	73.30	76.75	82.03	72.79	76.22	4.26
Cage Wash	12 h	0.81	0.51	0.45	0.28	0.51	0.22
	24 h	1.07	0.63	1.10	0.36	0.79	0.36
	48 h	1.13	*0.65	1.60	0.58	º0.99	º0.48
	72 h	1.24	*0.67	1.71	*0.61	º1.06	º0.52
Expired Air-1	24 h	0.05	0.04	0.03	0.02	0.04	0.01
Expired Air-2	24 h	*0.00	*0.00	*0.01	*0.00	º0.00	º0.00
G.I. Tract + Contents	72 h	1.52	0.03	0.48	2.40	1.11	1.06
Carcass#	72 h	*0.25	*0.02	*0.03	*0.13	º0.11	º0.11
Total		92.99	94.33	98.05	92.25	94.41	2.58

*=Results calculated from data less than 30 d.p.m. above background
°=Mean includes results calculated from data less than 30 d.p.m above background
= Includes all organs and tissues

Table 2.3-06 Cumulative Excretion of Total Radioactivity Following a Single Oral Administration of [^{14}C]-IR5885 to Female Rats (Phase 2). Target Dose Level: 1000 mg.kg^{-1}

Results expressed as % of the administered dose

Sample	Timepoint	13F	14F	15F	16F	Mean	SD
Urine	6 h	6.92	9.43	13.32	10.21	9.97	2.64
	12 h	20.53	19.99	21.96	18.14	20.16	1.58
	24 h	25.32	24.88	26.85	22.02	24.77	2.02
	48 h	27.48	26.49	29.78	23.90	26.91	2.43
	72 h	27.51	26.57	29.86	24.20	27.04	2.34
Faeces	12 h	1.57	0.25	0.31	0.55	0.67	0.62
	24 h	57.36	53.70	50.99	40.63	50.67	7.19
	48 h	65.15	63.96	64.08	64.88	64.52	0.58
	72 h	65.28	64.41	64.45	67.23	65.35	1.32
Cage Wash	12 h	0.66	1.55	0.45	2.08	1.18	0.76
	24 h	0.75	1.82	0.57	2.47	1.40	0.90
	48 h	0.83	1.93	0.76	2.70	1.55	0.93
	72 h	*0.84	*1.95	0.85	*2.74	º1.59	º0.93
Expired Air-1	24 h	0.04	0.04	0.05	0.05	0.05	0.00
Expired Air-2	24 h	*0.00	*0.01	*0.01	*0.00	º0.01	º0.00
G.I. Tract + Contents	72 h	0.01	0.02	0.03	0.10	0.04	0.04
Carcass#	72 h	*0.00	*0.00	*0.00	*0.00	º0.00	º0.00
Total		93.69	92.99	95.25	94.33	94.07	0.96

*=Results calculated from data less than 30 d.p.m. above background
°=Mean includes results calculated from data less than 30 d.p.m above background
= Includes all organs and tissues

Phase 3

The recovery of total radioactivity following an oral administration of [^{14}C]-IR5885 to rats at a target dose level of 100 mg.kg^{-1} after multiple oral administrations of non-radiolabelled IR5885 was similar to that obtained following the single administration of [^{14}C]-IR5885 at 100 mg.kg^{-1} (Phase 1). By the end of the 72 hours collection period, the overall mean recovery in male and female animals was 95.66% and 95.79%, respectively. Mean values indicated that the prominent route of elimination was *via* faeces, with means of 82.95% (males) and 57.70% (females) of the dose excreted by this route. Urinary excretion resulted in mean recoveries of 8.86% and 33.65% of the administered dose in males and females, respectively. Excretion of the dose was rapid with the majority of the administered radioactivity recovered within 24 hours post final dose. Excretion was complete by *ca* 48 hours post last dose.

Expired air, collected during the first 24 hours of the collection period, accounted for only 0.06% and 0.11% of the administered dose in male and female animals, respectively.

At 72 hours post final dose, cagewash samples accounted for a mean of 1.67% (male) and 4.06% (female). Very small amounts of radioactivity were detected in the gastrointestinal tract and carcass. In the male animals, the mean values were 1.91 % and 0.22% of the dose, respectively. In the female animals, mean values were 0.08% and 0.19%, respectively (Tables 2.3-07 and 2.3-08)

Table 2.3-07 Cumulative Excretion of Total Radioactivity Following 14 Daily Oral Administrations of IR5885 Followed by a Single Oral Administration of [^{14}C]-IR5885 to Male Rats (Phase 3).

Target Dose Level: 100 mg.kg^{-1}.day^{-1}. Results expressed as % of the administered dose.

Sample	Timepoint	18M	19M	20M	81M	Mean	SD
Urine	6 h	4.56	5.85	5.42	3.76	4.90	0.93
	12 h	7.23	7.26	7.89	6.33	7.18	0.64
	24 h	8.06	7.92	9.14	7.60	8.18	0.67
	48 h	8.75	8.06	9.70	8.42	8.73	0.70
	72 h	8.96	8.09	9.75	8.65	8.86	0.69
Faeces	12 h	0.49	46.28	0.73	19.58	16.77	21.61
	24 h	35.44	76.54	56.28	50.36	54.66	17.02
	48 h	61.72	85.04	79.05	68.09	73.48	10.52
	72 h	80.30	85.19	80.70	85.59	82.95	2.83
Cage Wash	12 h	0.39	0.52	0.06	0.24	0.30	0.20
	24 h	0.44	0.95	3.58	*0.30	°1.32	1.53
	48 h	0.52	1.03	3.80	0.76	1.53	1.53
	72 h	0.56	1.17	3.87	1.09	1.67	1.49
Expired Air-1	24 h	0.04	0.05	0.05	0.04	0.05	0.01
Expired Air-2	24 h	0.01	*0.01	*0.00	*0.00	°0.01	0.01
G.I. Tract + Contents	72 h	4.61	0.19	0.03	2.82	1.91	2.21
Carcass#	72 h	0.27	*0.12	*0.10	0.38	°0.22	0.13
Total		94.76	94.82	94.50	98.57	95.66	1.94

*=Results calculated from data less than 30 d.p.m. above background
°=Mean includes results calculated from data less than 30 d.p.m above background
= Includes all organs and tissues

Table 2.3-08 Cumulative Excretion of Total Radioactivity Following 14 Daily Oral Administrations of IR5885 Followed by a Single Oral Administration of [^{14}C]-IR5885 to Female Rats (Phase 3).

Target Dose Level: 100 mg.kg^{-1}.day^{-1}. Results expressed as % of the administered dose.

Sample	Timepoint	21F	22F	23F	24F	Mean	SD
Urine	6 h	23.14	6.86	27.05	8.87	16.48	10.11
	12 h	31.45	27.76	36.50	19.37	28.77	7.22
	24 h	36.10	29.48	38.50	24.91	32.25	6.21
	48 h	37.20	30.55	39.08	27.40	33.56	5.50
	72 h	37.25	30.58	39.21	27.58	33.65	5.48
Faeces	12 h	5.19	23.37	18.85	12.54	14.99	7.90
	24 h	31.07	50.47	44.40	55.27	45.30	10.48
	48 h	54.21	61.85	51.16	58.59	56.46	4.72
	72 h	54.34	62.46	52.03	61.96	57.70	5.30
Cage Wash	12 h	3.34	0.75	3.95	4.36	3.10	1.62
	24 h	3.92	1.47	4.50	5.11	3.75	1.60
	48 h	4.06	*1.51	4.74	5.35	°3.92	°1.69
	72 h	4.12	*1.55	5.07	5.49	°4.06	°1.77
Expired Air-1	24 h	0.05	0.04	0.16	0.03	0.07	0.06
Expired Air-2	24 h	*0.00	*0.01	0.12	*0.01	°0.04	°0.06
G.I. Tract + Contents	72 h	0.02	0.02	0.13	0.14	0.08	0.07
Carcass#	72 h	*0.22	*0.08	0.18	0.29	°0.19	°0.09
Total		96.00	94.74	96.91	95.50	95.79	0.91

*=Results calculated from data less than 30 d.p.m. above background
°=Mean includes results calculated from data less than 30 d.p.m above background 5
= Includes all organs and tissues

Blood Kinetics (Phases 4-6)

Phase 4

At 6 min after the administration of [^{14}C]-IR5885 at the lower dose level (100 mg.kg^{-1}), mean concentrations of total radioactivity in whole blood from male and female animals were 1.47 and 2.02 µg equiv.g^{-1}, respectively. Concentrations increased until a peak (T_{max}) was reached at 2 hours post dose in males and 1 hour post dose in females, with mean values (C_{max}) of 12.45 and 9.42 µg equiv.g^{-1}, respectively. Mean concentrations then declined in a biphasic manner with concentrations decreasing rapidly to 3.39 µg equiv.g^{-1} (males) and 1.17 µg equiv.g^{-1} (females) at 8 hours post dose. Thereafter, the rate of decline slowed, with low mean concentrations of radioactivity in the whole blood (<0.01 µg equiv.g^{-1} for both males and females) observed at 72 hours post dose.

As T_{max} was 2 hours post dose for males and 1 hour post dose for female rats, these times were selected as the respective sacrifice times for the animals in Phase 7. The elimination half-life ($T_{½}$) was 3.93 hours (males) and 5.46 hours (females) and AUC was 98.78 (males) and 49.20 (females) (Tables 2.3-09 and 2.3-10).

Table 2.3-09 Concentration of Total Radioactivity in Whole Blood Following a Single Oral Administration of [^{14}C]-IR5885 to Male Rats (Phase 4). Target Dose Level: 100 mg.kg^{-1}

Results expressed as µg equiv.g^{-1}

Sample	Timepoint	25M	26M	27M	28M	Mean	SD
Whole Blood	0.10 h	1.734	1.454	1.169	1.523	1.470	0.234
	0.20 h	3.176	2.702	3.598	3.618	3.273	0.432
	0.40 h	5.834	4.473	6.565	6.120	5.748	0.902
	1 h	10.151	9.031	9.260	14.791	10.808	2.699
	2 h	12.134	10.949	16.756	9.940	12.445	3.011
	4 h	12.071	9.433	7.072	8.227	9.201	2.143
	6 h	9.479	4.645	4.215	6.466	6.201	2.393
	8 h	4.480	2.738	2.914	3.406	3.385	0.783
	24 h	0.263	0.167	0.295	0.206	0.233	0.057
	48 h	*0.070	*0.066	*0.092	0.210	º0.109	º0.068
	72 h	*0.000	*0.000	*0.020	*0.000	º0.005	º0.010

*=Results calculated from data less than 30 d.p.m. above background
°=Mean includes results calculated from data less than 30 d.p.m above background

Table 2.3-10 Concentration of Total Radioactivity in Whole Blood Following a Single Oral Administration of [^{14}C]-IR5885 to Female Rats (Phase 4). Target Dose Level: 100 mg.kg^{-1}

Results expressed as µg equiv.g^{-1}

Sample	Timepoint	29F	30F	31F	32F	Mean	SD
Whole Blood	0.10 h	2.005	2.115	1.932	2.016	2.017	0.075
	0.20 h	4.220	3.854	5.093	3.158	4.081	0.806
	0.40 h	6.905	8.273	6.484	4.456	6.530	1.579
	1 h	9.246	9.694	9.380	9.346	9.417	0.194
	2 h	8.365	6.539	7.635	5.991	7.132	1.069
	4 h	5.126	2.753	2.964	3.299	3.535	1.084
	6 h	3.445	1.664	1.887	1.682	2.170	0.857
	8 h	1.700	0.875	1.236	0.869	1.170	0.393
	24 h	0.275	0.156	0.173	*0.157	º0.190	º0.057
	48 h	0.220	*0.051	*0.006	*0.022	º0.075	º0.098
	72 h	*0.015	*0.010	*0.000	*0.012	º0.009	º0.006

*=Results calculated from data less than 30 d.p.m. above background
°=Mean includes results calculated from data less than 30 d.p.m above background

Phase 5

Mean concentrations of radioactivity measured in whole blood at the first collection timepoint (6 min post dose at the higher dose level (1000 mg.kg^{-1})) were 2.84 and 2.69 µg equiv.g^{-1} for male and female animals, respectively. Concentrations increased rapidly until a peak (T_{max}) was reached at 2 hours post dose for both male and female animals, with mean values (C_{max}) of 30.24 and 19.86 µg equiv.g^{-1}, respectively. Mean concentrations then declined in a biphasic manner with concentrations decreasing rapidly to 1.34 µg equiv.g^{-1} (males) and 0.58 µg equiv.g^{-1} (females) at 24 hours post dose. Thereafter, the rate of decline slowed, with low mean concentrations of radioactivity in the whole blood (<0.01 µg equiv.g^{-1} for males and 0.50 µg equiv.g^{-1} for females) observed at 72 hours post dose.

As T_{max} was 2 hours post dose for both male and female rats, this time was selected as the sacrifice time for the animals in Phase 8. $T_{½}$ was 4.42 hours (males) and 4.50 hours (females) and AUC was 346.14 (males) and 175.12 (females). The AUC for both the male and female animals was *ca* 3.5-fold higher compared to the low level oral dosed animals (Tables 2.3-11 and 2.3-12).

Table 2.3-11 Concentration of Total Radioactivity in Whole Blood Following a Single Oral Administration of [^{14}C]-IR5885 to Male Rats (Phase 5). Target Dose Level: 1000 mg.kg^{-1}

Results expressed as µg equiv.g^{-1}

Sample	Timepoint	33M	34M	35M	36M	Mean	SD
Whole Blood	0.10 h	2.374	2.113	*2.724	4.163	°2.843	°0.915
	0.20 h	5.160	6.604	8.187	9.848	7.450	2.021
	0.40 h	10.323	13.509	12.272	13.494	12.399	1.501
	1 h	23.507	23.225	28.183	16.332	22.811	4.882
	2 h	46.370	25.684	31.004	17.904	30.240	12.023
	4 h	40.385	14.513	15.632	15.776	21.577	12.552
	6 h	37.895	12.764	17.417	15.680	20.939	11.466
	8 h	34.242	7.175	10.537	21.143	18.275	12.196
	24 h	*1.769	*0.232	*0.877	2.482	°1.340	°0.988
	48 h	*0.000	*0.000	*0.000	*0.000	°0.000	°0.000
	72 h	*0.000	*0.000	*0.000	*0.000	°0.000	°0.000

*=Results calculated from data less than 30 d.p.m. above background
°=Mean includes results calculated from data less than 30 d.p.m above background

Table 2.3-12 Concentration of Total Radioactivity in Whole Blood Following a Single Oral Administration of [^{14}C]-IR5885 to Female Rats (Phase 5). Target Dose Level: 1000 mg.kg^{-1}

Results expressed as µg equiv.g^{-1}

Sample	Timepoint	37F	38F	39F	40F	Mean	SD
Whole Blood	0.10 h	*1.247	3.981	3.205	*2.319	°2.688	°1.177
	0.20 h	*3.065	7.574	8.352	6.167	°6.290	°2.332
	0.40 h	12.837	12.349	12.294	12.057	12.384	0.327
	1 h	20.354	19.239	18.977	19.106	19.419	0.633
	2 h	18.634	23.402	21.017	16.366	19.855	3.033
	4 h	12.721	18.027	12.140	13.256	14.036	2.700
	6 h	10.462	9.797	6.620	11.530	9.602	2.112
	8 h	7.094	6.903	2.799	9.335	6.533	2.723
	24 h	*0.280	*1.238	*0.786	*0.029	°0.583	°0.538
	48 h	*0.000	*0.000	*0.000	*0.000	°0.000	°0.000
	72 h	*0.709	*0.459	*0.539	*0.306	°0.503	°0.168

*=Results calculated from data less than 30 d.p.m. above background
°=Mean includes results calculated from data less than 30 d.p.m above background

Phase 6

Mean concentrations of total radioactivity in whole blood following an oral administration of [^{14}C]-IR5885 (100 mg.kg^{-1}) after multiple oral administrations of non-radiolabelled IR5885 were 1.34 µg equiv.g^{-1} (males) and 1.85 µg equiv.g^{-1} (females) 6 min after the final dose. In both male and female animals the mean peak (C_{max}) concentration was reached at 1 hour post final dose (T_{max}). These were 9.14 and 8.20 µg equiv.g^{-1} respectively. Mean levels of radioactivity then decreased rapidly until 24 hours post final dose to a level of 0.24 and 0.19 µg equiv.g^{-1}, respectively, and thereafter continued to decline at a slower rate. The mean concentrations of total radioactivity at 72 hours post final dose were 0.05 µg equiv.g^{-1} and 0.04 µg equiv.g^{-1} respectively for male and females (Tables 2.3-13 and 2.3-14).

As T_{max} was 1 hours post dose for both male and female rats, this time was selected as the sacrifice time for the animals in Phase 9. $T_{½}$ was 5.60 hours (males) and 6.60 hours (females) and AUC was 56.76 (males) and 38.72 (females) (Table 2.3-15).

Table 2.3-13 Concentration of Total Radioactivity in Whole Blood Following 14 Daily Oral Administrations of IR5885 Followed by a Single Oral Administration of [^{14}C]-IR5885 to Male Rats (Phase 6). Target Dose Level: 100 mg.kg^{-1}.day^{-1}. Results expressed as µg equiv.g^{-1}

Sample	Timepoint	41M	42M	43M	44M	Mean	SD
Whole Blood	0.10 h	1.241	0.876	1.819	1.430	1.341	0.393
	0.20 h	2.576	1.550	3.413	2.684	2.556	0.766
	0.40 h	3.496	2.664	5.181	3.654	3.749	1.049
	1 h	5.759	8.601	11.540	10.676	9.144	2.572
	2 h	5.023	9.553	8.959	8.370	7.977	2.027
	4 h	2.727	5.821	5.135	3.630	4.329	1.406
	6 h	1.588	3.660	2.421	1.964	2.408	0.901
	8 h	1.373	1.643	1.605	1.505	1.532	0.121
	24 h	0.417	*0.121	0.220	0.198	º0.239	º0.126
	48 h	*0.093	*0.122	0.182	*0.087	º0.121	º0.043
	72 h	*0.056	*0.053	*0.039	*0.047	º0.049	º0.007

*=Results calculated from data less than 30 d.p.m. above background
°=Mean includes results calculated from data less than 30 d.p.m above background

Table 2.3-14 Concentration of Total Radioactivity in Whole Blood Following 14 Daily Oral Administrations of IR5885 Followed by a Single Oral Administration of [^{14}C]-IR5885 to Female Rats (Phase 6).

Target Dose Level: 100 mg.kg^{-1}.day^{-1}. Results expressed as µg equiv.g^{-1}

Sample	Timepoint	45F	46F	47F	48F	Mean	SD
Whole Blood	0.10 h	2.045	2.096	1.839	1.421	1.850	0.307
	0.20 h	4.609	3.501	2.750	2.668	3.382	0.900
	0.40 h	7.711	6.442	6.222	4.663	6.259	1.250
	1 h	7.496	7.611	8.795	8.891	8.198	0.747
	2 h	4.521	6.079	5.432	6.007	5.510	0.720
	4 h	1.812	2.490	2.292	2.465	2.265	0.314
	6 h	1.164	1.120	1.370	2.192	1.462	0.499
	8 h	0.537	0.948	0.882	1.052	0.855	0.223
	24 h	0.191	0.311	*0.122	*0.147	º0.193	º0.084
	48 h	*0.042	*0.080	*0.037	*0.055	º0.053	º0.019
	72 h	*0.027	*0.055	*0.025	*0.067	º0.043	º0.021

*=Results calculated from data less than 30 d.p.m. above background
°=Mean includes results calculated from data less than 30 d.p.m above background

Table 2.3-15 Pharmacokinetic Parameters for Whole Blood

Phase	Target Dose Level	Animals	C_{max}	T_{max}	$T_{½}$ (6-24 h)	r^2 (6-24 h)	AUC
4	100 mg.kg^{-1}	Male	12.45	2 h	3.93	0.995	98.78
		Female	9.42	1 h	5.46	0.979	49.20
5	1000 mg.kg^{-1}	Male	30.24	2 h	4.42	0.997	346.14
		Female	19.86	2 h	4.50	0.999	175.12
6	100 mg.kg^{-1}.day^{-1}	Male	9.14	1 h	5.60	0.993	56.76
		Female	8.20	1 h	6.60	0.976	38.72

C_{max} µg equiv.g^{-1}
$T_{½}$ elimination half life *(h)*
r^2 line of fit on curve

AUC(0-72 h) the area under the whole blood drug concentration *vs* time curve from time zero to 72 h calculated using the linear or log/linear trapezoidal method *(h x µg.g^{-1})*

Tissue Distribution (Phases 7-9)

Phase 7

Following administration of [^{14}C]-IR5885 at a target dose level of 100 mg.kg^{-1}, the distribution of total radioactivity was similar for male and female animals. The highest mean concentration of total radioactivity was found in the gastrointestinal tract with mean values of 930.95 µg equiv.g^{-1} (84.91% dose) and 806.45 µg equiv.g^{-1} (72.84% dose) for male and female animals, respectively.

Lower concentrations were found in the liver (mean 171.27 and 173.67 µg equiv.g^{-1}, respectively) which accounted for a mean of 7.64% and 7.00% of the dose, respectively, and kidneys (mean 35.01 and 56.93 µg equiv.g^{-1}, respectively) which accounted for a mean of 0.30% and 0.46% dose, respectively. With the exception of whole blood, which contained mean concentrations of 10.20 and 9.71 µg equiv.g^{-1}, all other tissues contained mean concentrations of less than 6.5 µg equiv.g^{-1} and accounted for 0.032% of the dose or less for both male and female animals. The carcass contained a mean of 3.19 µg equiv.g^{-1} (males) and 3.06 µg equiv.g^{-1} (females). The recovery of radioactivity in carcass was 2.48% (males) and 2.45% (females) of the administered dose (Tables 2.3-16 to 2.3-19).

Table 2.3- 16 Concentration of Total Radioactivity in Tissues Following a Single Oral Administration of [^{14}C]-IR5885 to Male Rats (Phase 7).Target Dose Level: 100 mg.kg^{-1}

Results expressed as µg equiv.g^{-1}

Sample	49M	50M	51M	52M	Mean	SD
Bone	1.262	1.169	2.186	*1.118	°1.434	°0.505
Brain	0.591	0.486	0.677	0.831	0.646	0.146
Fat-Brown	2.689	2.686	3.504	3.405	3.071	0.445
Fat- Subcutaneous	1.744	2.465	2.613	3.649	2.618	0.785
Heart	4.714	5.177	4.512	6.013	5.104	0.667
Kidneys	33.471	27.952	37.305	41.320	35.012	5.694
Liver	149.820	175.990	154.250	205.000	171.270	25.233
Lungs	4.963	6.109	5.509	6.810	5.848	0.794
Muscle	2.504	1.928	2.531	3.122	2.521	0.488
Spleen	2.261	2.276	3.419	3.849	2.951	0.807
G.I. Tract + Contents	741.060	978.020	1073.900	930.820	930.950	139.900
Carcass#	2.602	2.982	3.452	3.709	3.186	0.493
Whole Blood	9.044	9.329	9.217	13.191	10.195	2.000

*=Results calculated from data less than 30 d.p.m. above background
°=Mean includes results calculated from data less than 30 d.p.m above background
= Includes all the bone, muscle, fat and other tissues not completely sampled at necropsy

Table 2.3-17 Amounts of Total Radioactivity in Tissues Following a Single Oral Administration of [^{14}C]-IR5885 to Male Rats (Phase 7). Target Dose Level: 100 mg.kg^{-1}

Results expressed as % of the administered dose.

Sample	49M	50M	51M	52M	Mean	SD
Brain	0.004	0.003	0.005	0.005	0.004	0.001
Heart	0.021	0.027	0.016	0.025	0.022	0.005
Kidneys	0.263	0.271	0.326	0.338	0.300	0.038
Liver	7.560	7.234	6.879	8.875	7.637	0.871
Lungs	0.024	0.030	0.028	0.048	0.032	0.011
Spleen	0.005	0.005	0.007	0.010	0.007	0.002
G.I. Tract + Contents	84.217	85.426	87.753	82.235	84.908	2.308
Carcass#	1.975	2.409	2.598	2.919	2.475	0.394
Total	94.070	95.405	97.611	94.454	95.385	1.587

*=Results calculated from data less than 30 d.p.m. above background
°=Mean includes results calculated from data less than 30 d.p.m above background
= Includes all the bone, muscle, fat and other tissues not completely sampled at necropsy

Table 2.3-18 Concentration of Total Radioactivity in Tissues Following a Single Oral Administration of [^{14}C]-IR5885 to Female Rats (Phase 7). Target Dose Level: 100 mg.kg^{-1}

Results expressed as µg equiv.g^{-1}

Sample	53F	54F	55F	56F	Mean	SD
Bone	1.234	1.949	2.416	1.742	1.835	0.490
Brain	0.803	1.105	0.953	0.614	0.869	0.210
Fat-Brown	4.374	3.875	5.173	2.917	4.085	0.945
Fat- Subcutaneous	4.189	4.054	4.675	2.320	3.809	1.028
Heart	5.258	5.295	4.936	4.351	4.960	0.437
Kidneys	67.988	63.570	51.727	44.451	56.934	10.788
Liver	201.550	191.500	109.900	191.730	173.670	42.769
Lungs	6.590	7.048	6.800	5.641	6.520	0.615
Muscle	2.712	2.980	2.630	2.094	2.604	0.371
Spleen	3.979	3.949	3.312	2.488	3.432	0.701
G.I. Tract + Contents	831.120	858.330	800.470	735.880	806.450	52.651
Carcass#	4.106	2.518	2.924	2.702	3.063	0.715
Whole Blood	9.970	11.225	7.667	9.967	9.707	1.483

*=Results calculated from data less than 30 d.p.m. above background
°=Mean includes results calculated from data less than 30 d.p.m above background
= Includes all the bone, muscle, fat and other tissues not completely sampled at necropsy

Table 2.3-19 Amounts of Total Radioactivity in Tissues Following a Single Oral Administration of [^{14}C]-IR5885 to Female Rats (Phase 7). Target Dose Level: 100 mg.kg^{-1}

Results expressed as % of the administered dose

Sample	53F	54F	55F	56F	Mean	SD
Brain	0.005	0.008	0.007	0.003	0.006	0.002
Heart	0.020	0.021	0.019	0.018	0.020	0.001
Kidneys	0.554	0.568	0.395	0.325	0.460	0.120
Liver	7.813	7.888	4.158	8.143	7.000	1.900
Lungs	0.032	0.039	0.026	0.034	0.032	0.005
Spleen	0.006	0.009	0.006	0.005	0.007	0.002
G.I. Tract + Contents	62.693	49.012	98.935	80.725	72.841	21.709
Carcass#	3.197	2.225	2.286	2.087	2.449	0.506
Total	74.320	59.771	105.830	91.339	82.815	20.046

*=Results calculated from data less than 30 d.p.m. above background
°=Mean includes results calculated from data less than 30 d.p.m above background
= Includes all the bone, muscle, fat and other tissues not completely sampled at necropsy

Phase 8

As with Phase 7, the highest mean concentration of total radioactivity following administration of the test item at the higher dose level (1000 mg.kg^{-1}) was observed in the gastrointestinal tract, with mean values of 7547.70 µg equiv.g^{-1} (89.64% dose) and 9007.40 µg equiv.g^{-1} (83.76% dose) for male and female animals, respectively. Mean concentrations in whole blood, kidneys and liver were 74.12 (males) and 25.31 µg equiv.g^{-1} (females) for whole blood, 199.18 (males) and 128.44 µg equiv.g^{-1} (females) for kidneys, and 351.65 (males) and 269.22 µg equiv.g^{-1} (females) for liver. The mean recoveries of radioactivity from liver were 1.52% (males) and 1.18% (females) and for kidneys were 0.17% (males) and 0.11% (females) of the administered dose. Residues in the remaining tissues were lower, with mean values ranging between 5.60-36.44 µg equiv.g^{-1} (0.02% dose or less) for the male animals and 3.37-16.50 µg equiv.g^{-1} (0.01% dose or less) for the female animals. Carcass was an exception:, although mean concentration values of 28.34 and 6.16 µg equiv.g^{-1} for male and female animals, respectively, levels of radioactivity accounted for 2.12% and 0.46% of the administered dose, respectively. The distribution of total radioactivity was similar for male and female animals (Tables 2.3-20 to 2.3-23).

Table 2.3-20 Concentration of Total Radioactivity in Tissues Following a Single Oral Administration of [^{14}C]-IR5885 to Male Rats (Phase 8). Target Dose Level: 1000 mg.kg^{-1}

Results expressed as µg equiv.g^{-1}

Sample	57M	58M	59M	60M	Mean	SD
Bone	7.232	14.274	25.504	25.118	18.032	8.885
Brain	4.443	10.318	4.563	3.085	5.602	3.214
Fat-Brown	16.612	9.070	24.226	18.874	17.196	6.287
Fat- Subcutaneous	12.831	16.479	16.805	9.005	13.780	3.658
Heart	37.109	26.580	36.169	29.777	32.409	5.071
Kidneys	198.260	193.380	208.360	196.730	199.180	6.450
Liver	386.570	301.580	388.190	330.250	351.650	42.894
Lungs	43.365	28.660	39.377	34.350	36.438	6.363
Muscle	16.237	14.420	16.722	16.426	15.951	1.040
Spleen	17.939	16.235	19.908	19.983	18.516	1.791
G.I. Tract + Contents	7569.800	7878.000	7953.100	6789.900	7547.700	531.750
Carcass#	20.089	23.381	29.323	40.573	28.341	9.005
Whole Blood	110.800	41.881	79.290	64.501	74.117	28.890

*=Results calculated from data less than 30 d.p.m. above background
°=Mean includes results calculated from data less than 30 d.p.m above background
= Includes all the bone, muscle, fat and other tissues not completely sampled at necropsy

Table 2.3-21 Amounts of Total Radioactivity in Tissues Following a Single Oral Administration of [^{14}C]-IR5885 to Male Rats (Phase 8). Target Dose Level: 1000 mg.kg^{-1}

Results expressed as % of the administered dose

Sample	57M	58M	59M	60M	Mean	SD
Brain	0.003	0.004	0.003	0.002	0.003	0.001
Heart	0.013	0.012	0.013	0.010	0.012	0.001
Kidneys	0.175	0.159	0.182	0.159	0.168	0.012
Liver	1.909	0.816	1.730	1.619	1.519	0.483
Lungs	0.018	0.014	0.015	0.020	0.017	0.003
Spleen	0.003	0.004	0.005	0.006	0.004	0.001
G.I. Tract + Contents	87.851	95.004	85.556	90.149	89.640	4.038
Carcass#	1.509	1.731	2.229	3.027	2.124	0.673
Total	91.481	97.743	89.731	94.993	93.487	3.583

*=Results calculated from data less than 30 d.p.m. above background

°=Mean includes results calculated from data less than 30 d.p.m above background
= Includes all the bone, muscle, fat and other tissues not completely sampled at necropsy

Table 2.3-22 Concentration of Total Radioactivity in Tissues Following a Single Oral Administration of [^{14}C]-IR5885 to Female Rats (Phase 8). Target Dose Level: 1000 mg.kg^{-1}

Results expressed as µg equiv.g^{-1}

Sample	61F	62F	63F	64F	Mean	SD
Bone	6.421	5.863	7.121	8.973	7.095	1.354
Brain	3.061	3.152	3.967	3.305	3.371	0.410
Fat-Brown	10.754	8.354	13.298	6.215	9.655	3.055
Fat- Subcutaneous	6.882	7.716	10.641	7.670	8.227	1.654
Heart	13.072	11.550	17.088	10.667	13.094	2.842
Kidneys	111.290	111.840	180.570	110.060	128.440	34.761
Liver	254.910	255.870	317.540	248.550	269.220	32.379
Lungs	15.509	15.867	23.150	11.471	16.499	4.861
Muscle	7.529	8.251	10.319	5.042	7.785	2.178
Spleen	8.883	8.321	12.210	5.880	8.824	2.607
G.I. Tract + Contents	8608.100	8854.700	8652.700	9914.000	9007.400	613.870
Carcass#	8.306	*4.875	7.196	4.265	º6.160	º1.908
Whole Blood	24.844	27.631	30.453	18.306	25.308	5.200

*=Results calculated from data less than 30 d.p.m. above background
°=Mean includes results calculated from data less than 30 d.p.m above background
= Includes all the bone, muscle, fat and other tissues not completely sampled at necropsy

Table 2.3-23 Amounts of Total Radioactivity in Tissues Following a Single Oral Administration of [^{14}C]-IR5885 to Female Rats (Phase 8). Target Dose Level: 1000 mg.kg^{-1}

Results expressed as % of the administered dose

Sample	61F	62F	63F	64F	Mean	SD
Brain	0.002	0.002	0.003	0.003	0.002	0.001
Heart	0.005	0.006	0.006	0.005	0.006	0.001
Kidneys	0.100	0.092	0.148	0.099	0.110	0.026
Liver	1.108	1.232	1.420	0.958	1.180	0.196
Lungs	0.006	0.013	0.011	0.008	0.009	0.003
Spleen	0.001	0.002	0.002	0.002	0.002	0.001
G.I. Tract + Contents	90.139	74.482	91.690	78.738	83.762	8.463
Carcass#	0.620	*0.358	0.542	0.323	º0.461	º0.143
Total	91.981	76.187	93.824	80.135	85.532	8.695

*=Results calculated from data less than 30 d.p.m. above background
°=Mean includes results calculated from data less than 30 d.p.m above background
= Includes all the bone, muscle, fat and other tissues not completely sampled at necropsy

Phase 9

As with Phases 7 and 8, the highest mean concentration of total radioactivity following an oral administration of [^{14}C]-IR5885 (100 mg.kg^{-1}) after multiple oral administrations of non-radiolabelled IR5885 was observed in the gastrointestinal tract. The mean concentration levels in the gastrointestinal tract were 852.15 µg equiv.g^{-1} (males) and 806.99 µg equiv.g^{-1} (females) and accounted for 86.75% (males) and 74.61% (females) of dose. Liver and kidneys contained 222.77 µg equiv.g^{-1} (10.08% dose) and 55.31 µg equiv.g^{-1} (0.42% dose) for the male animals, respectively, and 124.48 µg equiv.g^{-1} (5.09% dose) and 31.53 µg equiv.g^{-1} (0.26% dose) for the female animals, respectively. With the exception of whole blood, which contained mean concentrations of 11.47 and 5.33 µg equiv.g^{-1}, all other tissues contained mean concentrations of less than 7.2 µg equiv.g^{-1} and accounted for 0.03% of the dose or less for both male and female animals. The carcass contained a mean of 3.09 µg equiv.g^{-1} (males) and 1.67 µg equiv.g^{-1} (females). The recovery of radioactivity in carcass was 2.41% (males) and 1.30% (females) of the administered dose (Tables 2.3-24 to 2.3-27).

Table 2.3-24 Concentration of Total Radioactivity in Tissues Following 14 Daily Oral Administrations of IR5885 Followed by a Single Oral Administration of [^{14}C]-IR5885 to Male Rats (Phase 9)

Target Dose Level: 100 mg.kg^{-1}.day^{-1}. Results expressed as µg equiv.g^{-1}

Sample	65M	66M	67M	68M	Mean	SD
Bone	1.207	1.679	1.405	1.425	1.429	0.194
Brain	0.389	0.424	*0.371	0.753	º0.484	º0.181
Fat-Brown	3.198	4.241	3.923	4.248	3.902	0.493
Fat- Subcutaneous	2.484	2.666	2.236	3.702	2.772	0.645
Heart	4.942	6.389	5.045	5.992	5.592	0.711
Kidneys	39.408	65.299	42.068	74.470	55.311	17.273
Liver	213.710	214.780	225.060	237.530	222.770	11.094
Lungs	6.391	8.173	5.961	8.310	7.209	1.207
Muscle	2.564	3.201	2.474	3.254	2.873	0.411
Spleen	3.359	4.164	3.020	4.537	3.770	0.701
G.I. Tract + Contents	884.180	824.140	960.180	740.090	852.150	93.163
Carcass[#]	2.766	2.752	3.133	3.723	3.093	0.455
Whole Blood	9.851	12.692	11.360	11.993	11.474	1.211

*=Results calculated from data less than 30 d.p.m. above background
°=Mean includes results calculated from data less than 30 d.p.m above background
[#] = Includes all the bone, muscle, fat and other tissues not completely sampled at necropsy

Table 2.3-25 Amounts of Total Radioactivity in Tissues Following 14 Daily Oral Administrations of IR5885 Followed by a Single Oral Administration of [^{14}C]-IR5885 to Male Rats (Phase 9)

Target Dose Level: 100 mg.kg^{-1}.day^{-1}. Results expressed as % of the administered dose

Sample	65M	66M	67M	68M	Mean	SD
Brain	0.002	0.002	*0.002	0.004	º0.002	º0.001
Heart	0.015	0.022	0.020	0.020	0.019	0.003
Kidneys	0.305	0.450	0.402	0.526	0.421	0.092
Liver	9.183	10.389	9.792	10.972	10.084	0.770
Lungs	0.025	0.034	0.021	0.034	0.029	0.006
Spleen	0.007	0.008	0.007	0.008	0.007	0.001
G.I. Tract + Contents	87.748	85.780	89.208	84.279	86.753	2.167
Carcass[#]	2.142	2.127	2.530	2.821	2.405	0.334
Total	99.426	98.812	101.980	98.663	99.720	1.543

*=Results calculated from data less than 30 d.p.m. above background
°=Mean includes results calculated from data less than 30 d.p.m above background
[#] = Includes all the bone, muscle, fat and other tissues not completely sampled at necropsy

Table 2.3-26 Concentration of Total Radioactivity in Tissues Following 14 Daily Oral Administrations of IR5885 Followed by a Single Oral Administration of [^{14}C]-IR5885 to Female Rats (Phase 9)

Target Dose Level: 100 mg.kg^{-1}.day^{-1}. Results expressed as µg equiv.g^{-1}

Sample	69F	70F	71F	72F	Mean	SD
Bone	0.795	*1.008	3.302	*0.160	°1.317	°1.372
Brain	0.436	0.365	0.392	0.444	0.409	0.037
Fat-Brown	2.388	2.765	1.879	2.165	2.299	0.374
Fat- Subcutaneous	1.896	2.961	1.478	1.512	1.962	0.692
Heart	2.989	3.576	2.069	2.920	2.888	0.620
Kidneys	32.759	44.886	19.843	28.630	31.529	10.406
Liver	123.460	137.670	96.001	140.780	124.480	20.427
Lungs	3.485	4.274	2.494	3.152	3.351	0.740
Muscle	2.339	1.939	1.133	1.448	1.715	0.532
Spleen	1.775	2.098	1.350	1.673	1.724	0.308
G.I. Tract + Contents	930.180	681.290	720.780	895.690	806.990	124.200
Carcass#	1.659	2.426	1.370	1.213	1.667	0.539
Whole Blood	6.361	6.721	3.447	4.772	5.325	1.511

*=Results calculated from data less than 30 d.p.m. above background
°=Mean includes results calculated from data less than 30 d.p.m above background
= Includes all the bone, muscle, fat and other tissues not completely sampled at necropsy

Table 2.3-27 Amounts of Total Radioactivity in Tissues Following 14 Daily Oral Administrations of IR5885 Followed by a Single Oral Administration of [^{14}C]-IR5885 to Female Rats (Phase 9)

Target Dose Level: 100 mg.kg^{-1}.day^{-1}. Results expressed as % of the administered dose

Sample	69F	70F	71F	72F	Mean	SD
Brain	0.003	0.003	0.003	0.003	0.003	0.000
Heart	0.012	0.016	0.008	0.014	0.013	0.003
Kidneys	0.289	0.337	0.183	0.246	0.264	0.066
Liver	4.953	5.410	4.189	5.805	5.090	0.694
Lungs	0.014	0.021	0.011	0.016	0.015	0.004
Spleen	0.004	0.004	0.004	0.004	0.004	0.000
G.I. Tract + Contents	71.660	70.693	73.453	82.638	74.611	5.472
Carcass#	1.309	1.885	1.067	0.930	1.298	0.422
Total	78.244	78.370	78.918	89.656	81.297	5.580

*=Results calculated from data less than 30 d.p.m. above background
°=Mean includes results calculated from data less than 30 d.p.m above background
= Includes all the bone, muscle, fat and other tissues not completely sampled at necropsy

Biliary Elimination (Phase 10)

At 48 hours after dose administration, the overall mean recovery of radioactivity was 96.02% and 97.73% in male and female animals, respectively. Urine contained 12.84% (males) and 30.79% (females) of the administered dose and bile contained 64.55% (males) and 48.68% (females) of the administered dose. Excretion in faeces accounted for 16.63% and 15.55% of the dose in male and female animals, respectively. The total recovery of radioactivity in bile and faeces represented 81.18% and 64.23% of dose in male and female animals, respectively. These values and those for urine were consistent with the recovery of radioactivity in faeces and urine observed during Phases 1 to 3, where the rats had not been fitted with indwelling bile duct cannulae. The excretion of total radioactivity in urine, bile and faeces was very rapid, with the majority of the radioactivity being excreted within *ca* 12 hours of dose administration and a plateau was reached by *ca* 24 hours post dose.

Cagewash samples accounted for a mean of 1.48% (male) and 2.19% (female). Very small amounts of radioactivity were detected in the gastrointestinal tract and carcass. In the male animals, the mean values were

0.16 % and 0.36% of the dose, respectively. In the female animals, mean values were 0.45% and 0.08%, respectively (Tables 2.3-28 and 2.3-29).

Table 2.3-28 Cumulative Excretion of Total Radioactivity Following a Single Oral Administration of [^{14}C]-IR5885 to Male Bile Duct Cannulated Rats (Phase 10)

Target Dose Level: 100 mg.kg^{-1}. Results expressed as % of the administered dose.

Sample	Timepoint	73M	74M	75M	76M	Mean	SD
Urine	6 h	3.65	5.82	16.06	6.58	8.03	5.50
	12 h	5.00	11.87	21.57	10.20	12.16	6.92
	24 h	5.28	12.22	22.95	10.47	12.73	7.42
	48 h	5.32	12.27	23.28	10.50	12.84	7.56
Faeces	12 h	12.84	10.02	16.39	8.74	12.00	3.39
	24 h	15.85	14.18	20.33	11.78	15.54	3.61
	48 h	16.61	15.10	22.40	12.42	16.63	4.22
Bile	6 h	61.81	59.05	30.54	64.57	53.99	15.79
	12 h	72.35	65.35	42.75	69.64	62.52	13.49
	24 h	74.12	66.75	45.31	70.89	64.27	12.99
	48 h	74.22	67.10	45.85	71.02	64.55	12.80
Cage Wash	12 h	0.76	0.54	3.41	0.32	1.26	1.45
	24 h	0.79	*0.58	3.71	*0.34	°1.35	°1.58
	48 h	0.86	*0.62	4.09	*0.35	°1.48	°1.75
G.I. Tract + Contents	48 h	0.06	0.14	0.40	0.05	0.16	0.16
Carcass#	48 h	0.32	0.34	0.48	0.30	0.36	0.08
Total		97.40	95.57	96.49	94.64	96.02	1.19

*=Results calculated from data less than 30 d.p.m. above background
°=Mean includes results calculated from data less than 30 d.p.m above background
= Includes all the bone, muscle, fat and other tissues not completely sampled at necropsy

Table 2.3-29 Cumulative Excretion of Total Radioactivity Following a Single Oral Administration of [^{14}C]-IR5885 to Female Bile Duct Cannulated Rats (Phase 10)

Target Dose Level: 100 mg.kg^{-1}. Results expressed as % of the administered dose.

Sample	Timepoint	77F	78F	79F	80F	Mean	SD
Urine	6 h	*0.00	23.61	*0.00	*0.00	°5.90	°11.80
	12 h	26.14	30.30	21.37	24.74	25.64	3.70
	24 h	27.27	31.61	30.99	28.54	29.61	2.04
	48 h	27.52	31.76	34.56	29.34	30.79	3.05
Faeces	12 h	9.01	18.01	2.11	0.26	7.35	8.04
	24 h	17.00	21.56	2.93	8.65	12.54	8.34
	48 h	17.58	21.90	10.33	12.38	15.55	5.22
Bile	6 h	42.99	34.70	20.45	36.65	33.70	9.51
	12 h	50.27	40.80	28.49	52.76	43.08	11.01
	24 h	52.01	41.25	43.67	55.13	48.01	6.62
	48 h	52.20	41.28	45.41	55.81	48.68	6.55
Cage Wash	12 h	0.96	3.89	1.08	0.85	1.70	1.46
	24 h	*0.99	3.98	1.79	0.97	°1.93	°1.41
	48 h	1.05	4.09	2.38	1.22	2.19	1.40
G.I. Tract + Contents	48 h	0.37	0.02	1.06	0.35	0.45	0.44
Carcass#	48 h	*0.02	*0.04	*0.17	*0.08	°0.08	°0.07
Total		98.74	99.09	93.91	99.20	97.73	2.56

*=Results calculated from data less than 30 d.p.m. above background
°=Mean includes results calculated from data less than 30 d.p.m above background
= Includes all the bone, muscle, fat and other tissues not completely sampled at necropsy

Conclusions

The excretion of radioactivity following a single oral administration of 100 mg [^{14}C]-IR5885.kg^{-1} was rapid with over 90% of the administered radioactivity recovered within 48 hours of dose administration. At 72 hours post dose, faeces accounted for the highest proportion of the dose with mean values of 86.82% and 50.71% of dose recovered in the male and female animals, respectively. However, bile was an important route of elimination for radioactivity with a mean of 64.55% (males) and 48.68% (females) of dose recovered in bile over 48 hours.

At the higher dose level (1000 mg.kg^{-1}) the excretion pattern following single oral administration was consistent with the single low dose level. Approximately 90% of the administered dose was recovered by 48 hours post dose with the majority of the administered radioactivity being recovered in faeces with mean recoveries of 76.22% (males) and 65.35% (females) of dose at 72 hours post dose. Urine contained mean values of 15.87% and 27.04% of the dose at 72 hours post dose in male and female animals, respectively.

The excretion of radioactivity was not markedly different after the administration of repeated low doses of non-radiolabelled IR-5885 followed by a single radiolabelled dose. Again the greater proportion of the administered dose was excreted in faeces with mean recoveries of 82.95% (males) and 57.70% (females) of dose at 72 hours post dose. Urine contained mean values of 8.86% and 33.65% of dose in male and female animals, respectively.

Elimination *via* urine was a more of important route of elimination for female animals compared with male animals for all dosing groups.

Following a single oral dose of [^{14}C]-IR5885 at the low dose level (100 mg.kg^{-1}) concentrations of radioactivity in whole blood increased rapidly to mean peak concentrations of 12.45 µg equiv.g^{-1} at 2 hours post dose in males and 9.42 µg equiv.g^{-1} at 1 hour post dose in females. By 48 hours post dose, concentrations were low (approaching the limit of quantification).

The maximum concentration reached in whole blood following a single oral administration at the high dose level (1000 mg.kg^{-1}) was at 2 hours post dose for both male and female animals, 30.24 µg equiv.g^{-1} and 19.86 respectively. In comparison to the low dose level this increase is far less than the 10 fold difference in dose levels.

Following a single low dose of [^{14}C]-IR5885 to male and female rats the tissue containing the highest concentration of radioactivity at C_{max} was the gastrointestinal tract. The only other tissues to contain a concentration greater than that associated with whole blood were liver and kidney. This pattern of distribution was also observed at the higher dose level and in animals which received repeated administrations of IR5885. At 72 hours post dose the carcass, that included all the organs and the tissues, showed clearance of radioactivity as it contained amounts always lower than 0.22%.

2.4 Single and repeated oral administration in rats – IR5885

Reference Number: KII- Section 3, Volume 1, Annex IIA, point 5.1.3/02

Report:	See annex conf. 37

Guidelines: US EPA Health Effects Test Guidelines, OPPTS 870.7485
Commission Directive 94/79/EEC, Annex I, Section 5
Council Directive 91/414/EEC, Part A, Annex II, Section 5

Deviation: none

GLP: Yes (certified laboratory)

Executive summary:

Profiling of metabolites of [^{14}C-U-phenyl] IR5885 in urine and faeces of male and female rats following single and repeated oral administration was carried out on samples generated during the Inveresk Research Study No.202787: The Disposition of [^{14}C] IR5885 in the rat following single and repeated oral administration.

The excretion of radioactivity following single administration at low (100 mg/kg) and high (1000 mg/kg) doses and repeated administration at low dose was mainly *via* faeces for both male and female animals. Radioactivity was almost completely excreted *via* urine within 24 hours and *via* faeces within 48-72 hours. The excretion patterns following the three administration doses were not markedly different except for the radioactivity eliminated in faeces which was higher in male than in female rats.

Following single administration at **low dose** the radioactivity was eliminated mainly in faeces (86.23% of administered dose [AD] for males and 50.48% AD for females) with an appreciable amount excreted *via* urine (9.21% AD for males and 40.59% AD for females).

Following single administration at **high dose** the radioactivity was eliminated mainly in faeces (76.22% AD for males and 64.52% AD for females) and in lower amount *via* urine (14.42% AD for males and 24.77% AD for females).

The recovery of radioactivity following **repeated oral administration** was very similar to that obtained following a single low administration. The greater proportion of the administered dose was excreted in faeces (82.94% and 56.46% AD for males and females, respectively) while a considerable amount was eliminated in urine (8.18% and 32.25% AD).

In **bile-duct-cannulated** male and female rats, the radioactivity was eliminated mainly *via* urine and bile (77.00% AD for male and 77.63% AD for female animals). Bile was an important route of elimination for radioactivity with a mean of 64.27% AD (males) and 48.02% AD (females) within 24 h from dose administration. Excretion in urine accounted for 12.73% AD (males) and 29.61% AD (females) and in faeces accounted for 15.54% AD (males) and 12.54% AD (females) always by 24 h post dose.

Following low, high and repeated oral administrations, the only tissues containing significative radioactivity, besides gastrointestinal tract, were **liver** and **kidneys** for both male and female rats.

The urine samples of the same sex up to 24 hours were pooled per time interval, relatively to each administration dose, and analysed for radioactivity content by LSC. Aliquots of pooled urine samples were analysed directly by TLC and HPLC for radioactivity distribution.

The faeces samples of the same sex up to 48-72 hours were pooled per time interval, relatively to each administration dose, and extracted twice with acetone and then once with acetone-H_2O (1-1). The extracts were analyzed for radioactivity content by LSC and the profile of the metabolites was obtained by TLC and HPLC. The dried faeces residues were oxidized to determine the unextractable radioactivity content by LSC.

The unextractable radioactivity was always lower than 2% AD.

Chromatographic analyses established that IR5885 (R1) was extensively metabolized and six compounds were pointed out: R2, R3, R4, R5, R6, and R7.

Study results showed that the metabolic profile was almost the same following single oral (low and high) administration and repeated oral administration although the amounts of some compounds were different especially between low and high doses.

The metabolic profile was the same in male and female rats treated at the same dose, although the amounts of some compounds were slightly different in the two sexes.

Three metabolites were found both in urine and faeces, while three compounds were observed only in urine.

IR5885 (R1) was largely degraded following single low administration (it amounted to 5.30% AD in males and 6.17% AD in females) and repeated administration at low dose (7.80% AD in males and 5.47% AD in females) while it was less degraded in rats administered with single high dose (40.41% AD in males and 9.50% AD in females).

Compound R2 was the major metabolite following all administration doses: it amounted to 75.88% AD in males and 76.67% AD in females at low dose, 42.04% and 72.55% AD at high dose, 68.46% and 74.12% AD at repeated administration.

None of the other metabolites reached 6% AD in the excreta.

Among these, compound R3 was the main reaching 5.14% AD and 2.45% AD in the excreta of male and female rats administered with single low dose, 3.18% AD and 3.20% AD in male and female rats administered with single high dose and 5.90% AD and 3.28% AD in males and females administered with repeated dose.

TLC analysis of biliary excretion phase (from rats administered with single low dose) established that IR5885 was largely degraded (8.08% AD and 6.65% AD in male and female rats, respectively) producing mainly IR5885 acid (R2, 70.73% AD in male and 76.93% AD in female rats).

Other metabolites never reached 6% AD in the excreta, with compound R3 as main product reaching 5.67% AD in males and 2.46% AD in females.

Liver and kidneys from each animal of the same sex administered with the same dose were pooled and extracted twice with acetone and then once with acetone-H_2O (1-1). The extracts were analysed for radioactivity content by LSC and the profile of the metabolites was obtained by TLC and HPLC. The dried residues were oxidized to determine the unextractable radioactivity content by LSC.

Chromatographic analyses established that compounds found in extracts of liver and kidneys were the same found in urine.

In liver was found almost only IR5885 acid (R2) both in male and female rats following all doses.

In kidneys the principal compounds found was IR5885 acid (R2) both in male and female rats following all administration doses while other compounds (already found in urine) were present only as traces.

In conclusion, study results showed that:

- the metabolic profile was the same in male and female rats, although the amounts of some compounds were slightly different in the two sexes;
- all the compounds identified in faeces were observed in urine but three compounds were found only in urine (all lower than 4%);
- unchanged IR5885 (R1) was only found in faeces both for male and female rats;
- R2 was the main degradation products found both in faeces and urine; it was identified as IR5885 acid;
- none of the other metabolites, reached 6% AD in the excreta; among these R3 (or R4) was identified RS-β-alanine, N-[(1-methylethoxy)carbonyl]-L-valyl-3-(2-hydroxy-4- chlorophenyl), R4 (or R3) was identified as RS-β-alanine, N-[(1-methylethoxy)carbonyl]-L-valyl-3-(3-hydroxy-4- chlorophenyl), and R5 was identified as 3-amino-3-(4-chlorophenyl) propionic acid;
- the sum of metabolites found in bile and urine from bile-duct-cannulated rats was ≥ than 77% both in male and female and it was exclusively represented by degradation compounds.

<u>Materials and Methods</u>

Labelled Test Material

[^{14}C-U-phenyl]-IR5885	Lot # 199
Specific Activity:	4.435 MBq/µg
	119.858 µCi/µg
	266084 dpm/µg
Radiochemical purity:	>97% (TLC and HPLC)
Diastereoisomeric ratio	S,R/S,S (by HPLC):1.07

Unlabelled Test Material

IR5885	
Batch No.	FCF/T/180-00 (ex ZI 068)
Chemical purity (HPLC)	> 98 %

Source of the biological samples

The biological samples (urine, faeces, bile and tissues) used in this study were generated during the in-life portion of the study Inveresk Project No. 202787: The Disposition of [^{14}C]-IR5885 in the Rat Following Single and Repeated Oral Administration.

In particular, 3 groups of 8 rats each (4 males and 4 females Sprague Dawley rats), were administered orally with a single dose of 100 mg/kg and the following biological samples were collected:

- urine and faeces (Phase 1)
- tissues (Phase 7)
- bile and faeces (Phase 10)

Other 2 groups of 8 rats each (4 males and 4 females), were administered orally with a single dose of 1000 mg/kg and the following biological samples were collected:

- urine and faeces (Phase 2)
- tissues (Phase 8)

Finally 2 groups of 8 rats each (4 males and 4 females), were administered orally with a dose of 100 mg/kg of [^{14}C] IR5885 after multiple oral administrations of non-radiolabelled IR5885 at 100 mg/kg for 14 days. The following biological samples were collected:

- urine and faeces (Phase 3)
- tissues (Phase 9)

<u>Study design and Methods</u>

Identification of samples

Each sample from Inveresk Research was unequivocally identified by a label reporting:

Inveresk Project No.
Urine or Faeces, animal No. and sex
Collection interval

Inveresk Project No. 202787 was conducted in 10 phases. The phases concerning excretion kinetics, tissue distribution and biliary elimination are outlined in the Table 2.4-01:

Table 2.4-01

	Excretion Kinetics			**Tissue Distribution**			**Biliary elimination**
Phase	**1**	**2**	**3**	**7**	**8**	**9**	**10**
Dose regime	Single oral	Single oral	Multiple oral	Single oral	Single oral	Multiple oral	Single oral
Dose level of IR5885 (mg/kg)	100	1000	100	100	1000	100	100
Nominal radioactive dose (µCi/rat)	20	20	20	20	20	20	20
Rat No./sex	1-4M (♂) 5-8F (♀)	9-12M (♂) 13-16F (♀)	81, 18-20M (♂) 21-24F (♀)	49-52M (♂) 53-56F (♀)	57-60M (♂) 61-64F (♀)	65-68M (♂) 69-72F (♀)	73-76M (♂) 77-80F (♀)
Urine collection (hours)	0-6 6-12 12-24 24-48 48-72	0-6 6-12 12-24 24-48 48-72	0-6 6-12 12-24 24-48 48-72	not collected	not collected	not collected	0-6 6-12 12-24 24-48
Faeces collection	0-12	0-12	0-12	not	not	not	0-12

	Excretion Kinetics			**Tissue Distribution**			**Biliary elimination**
(hours)	12-24 24-48 48-72	12-24 24-48 48-72	12-24 24-48 48-72	collected	collected	collected	12-24 24-48
Tissue collection (hours)	not collecte d	not collected	not collected	2	2	2	not collected
Bile collection (hours)	not collecte d	not collected	not collected	not collected	not collected	not collected	0-6 6-12 12-24 24-48

Transport and storage of samples

The urine, faeces, tissues and bile were sent from Inveresk Research to Isagro Ricerca on August 29, 2002 packed in a suitable amount of dry ice so that they arrived frozen.

The samples were successively stored at –20°C until the analyses were performed.

Analysis of samples

Analysis of urine from Phases 1-3

The urine from each animal of the same sex and at the same time interval (0-6, 6-12 and 12-24 hours) from Phases 1, 2 and 3 were thawed and pooled by combining 30% of the original weight of the individual samples. The pooled samples were identified with a label reporting the Phase (**L** for Phase 1, **H** for Phase 2, **R** for Phase 3), the sex (**M** for males and **F** for females), the substrate (**U** for Urine), and the collection time (e.g. **6** for 0-6 hours):

Table 2.4-02

LMU6, LMU12, LMU24	Low dose (Phase 1), Male rats, Urine, 0-6, 6-12, 12-24 h
LFU6, LFU12, LFU24	Low dose (Phase 1), Female rats, Urine, 0-6, 6-12, 12-24 h
HMU6, HMU12, HMU24	High dose (Phase 2), Male rats, Urine, 0-6, 6-12, 12-24 h
HFU6, HFU12, HFU24	High dose (Phase 2), Female rats, Urine, 0-6, 6-12, 12-24 h
RMU6, RMU12, RMU24	Repeated dose (Phase 3), Male rats, Urine, 0-6, 6-12, 12-24 h
RFU6, RFU12, RFU24	Repeated dose (Phase 3), Female rats, Urine, 0-6, 6-12, 12-24 h

0.05-mL duplicate aliquots of each pooled urine sample were analyzed for radioactivity content by LSC. The profile of the radiolabelled urinary compounds was obtained by TLC and HPLC.

Analysis of faeces from Phases 1-3

The faeces from each animal of the same sex and at the same time interval (0-12, 12-24, 24-48, and, for Phases 2 and 3 male rats only, 48-72 hours) were thawed and pooled by combining 30% of the original weight of the individual samples. The pooled samples were identified with a label reporting the Phase (**L** for Phase 1, **H** for Phase 2, **R** for Phase 3), the sex (**M** for males and **F** for females), the substrate (**F** for Faeces) and the collection time (e.g. **12** for 0-12 hours):

Table 2.4-03

LMF12, LMF24, LMF48	Low dose (Phase 1), Male rats, Faeces, 0-12, 12-24, 24-48 h
LFF12, LFF24, LFF48	Low dose (Phase 1), Female rats, Faeces, 0-12, 12-24, 24-48 h
HMF12, HMF24, HMF48, HMF72	High dose (Phase 2), Male rats, Faeces, 0-12, 12-24, 24-48, 48-72 h
HFF12, HFF24, HFF48	High dose (Phase 2), Female rats, Faeces, 0-12, 12-24, 24-48 h
RMF12, RMF24, RMF48, RMF72	Repeated dose (Phase 3), Male rats, Faeces, 0-12, 12-24, 24-48, 48-72 h
RFF12, RFF24, RFF48	Repeated dose (Phase 3), Female rats, Faeces, 0-12, 12-24, 24-48 h

Pooled faecal samples were extracted twice by shaking at 300 strokes/min for 45 minutes with acetone and once with acetone-H_2O 1-1 (ratio faeces/solvent 1/5 w/v).

After each extraction, the residue was separated by centrifugation (15 minutes at 7500 rcf), the supernatants **[Extract I]**, **[Extract II]** and **[Extract III]** were separately collected in volumetric flasks and made up to the mark (100 mL) with acetone.

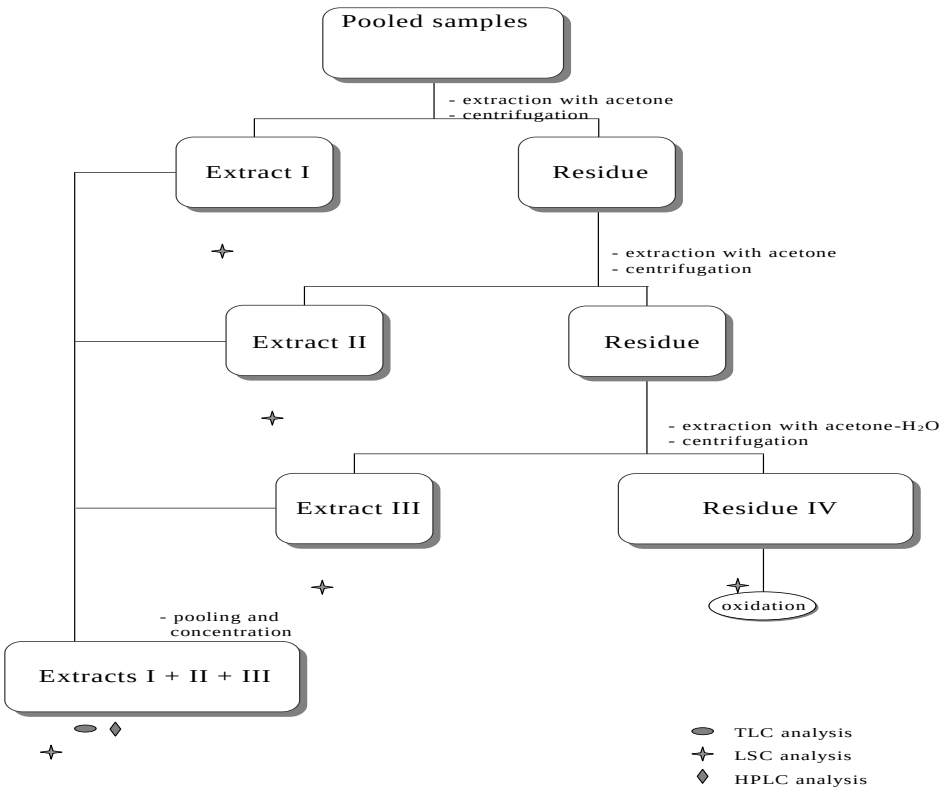
Radioactivity content was determined by LSC.

The profile of the radiolabelled faecal compounds was obtained by HPLC and TLC after concentration of a mixture of Extracts I+II+III for each pooled sample.

The **[Residues IV]** were allowed to dry to constant weight at room temperature and four aliquots (approximately 0.10 g) were then oxidized by means of a biological oxidizer. The radioactivity was determined by LSC.

The complete procedure of extraction/analysis is shown in Scheme 1.

SCHEME 1. Procedure of extraction and analysis of faeces and tissue samples.



Analysis of liver and kidneys from Phases 7-9

Liver and kidneys from each animal of the same Phase and sex were pooled by combining 30% of the original weight of the homogenized sample.

The pooled samples were identified with a label reporting the Phase (**L** for Phase 7, **H** for Phase 8, **R** for Phase 9), the sex (**M** for males and **F** for females), and the substrate (**L** for Liver and **K** for Kidneys):

Table 2.4-04

LML, LFL	Low dose (Phase 7), Male and Female rats, Liver
HML, HFL	High dose (Phase 8), Male and Female rats, Liver
RML, RFL	Repeated dose (Phase 9), Male and Female rats, Liver
LMK, LFK	Low dose (Phase 7), Male and Female rats, Kidneys
HMK, HFK	High dose (Phase 8), Male and Female rats, Kidneys
RMK, RFK	Repeated dose (Phase 9), Male and Female rats, Kidneys

Pooled tissue samples were extracted twice by shaking at 300 strokes/min for 45 minutes with acetone and once with acetone-H_2O 1-1 (ratio tissue/solvent 1/5 w/v).

After each extraction, the residue was separated by centrifugation (15 minutes at 7500 rcf), the supernatants **[Extract I]**, **[Extract II]** and **[Extract III]** were separately collected in volumetric flasks and made up to the mark (100 mL) with acetone. Radioactivity content was determined by LSC.

The profile of the radiolabelled compounds in tissues was obtained by HPLC and TLC after concentration of a mixture of Extracts I+II+III for each pooled sample.

The **[Residues IV]** were allowed to dry to constant weight at room temperature and four aliquots (approximately 0.10 g) were then oxidized by means of a biological oxidizer. The radioactivity was determined by LSC.

The complete procedure of extraction/analysis is shown in Scheme 1.

Analysis of bile, urine, and faeces from Phase 10

Bile samples from each animal of the same sex and at the same time interval (0-6, 6-12 and 12-24 hours) from Phase 10 (Biliary elimination) were thawed and pooled by combining 30% of the original weight of the individual samples. The pooled samples were identified with a label reporting **B** (Phase 10), the sex (**M** for males and **F** for females), the substrate (**B** for Bile), and the collection time (e.g. **6** for 0-6 hours):

Table 2.4-05

BMB6, BMB12, BMB24	Biliary elimination (Phase 10), Male rats, Bile, 0-6, 6-12, 12-24 h
BFB6, BFB12, BFB24	Biliary elimination (Phase 10), Female rats, Bile, 0-6, 6-12, 12-24 h

Radioactivity content was determined by LSC.

The profile of the radiolabelled biliary compounds was obtained by TLC and HPLC.

The urine samples from each animal of the same sex and at the same time interval (0-6, 6-12 and 12-24 hours) from Phase 10 (Biliary elimination) were thawed and pooled by combining 30% of the original weight of the individual samples. The pooled samples were identified with a label reporting **B** (Phase 10), the sex (**M** for males and **F** for females), the substrate (**U** for Urine), and the collection time (e.g. **6** for 0-6 hours):

Table 2.4-06

BMU6, BMU12, BMU24	Biliary elimination (Phase 10), Male rats, Urine, 0-6, 6-12, 12-24 h
BFU6, BFU12, BFU24	Biliary elimination (Phase 10), Female rats, Urine, 0-6, 6-12, 12-24 h

Radioactivity content was determined by LSC.

The profile of the radiolabelled urinary compounds was obtained by TLC and HPLC.

The faeces from each animal of the same sex and at the same time interval (0-12 and 12-24 hours) were thawed and pooled by combining 30% of the original weight of the individual samples. The pooled samples were identified with a label reporting **B** (Phase 10), the sex (**M** for males and **F** for females), the substrate (**F** for Faeces), and the collection time (e.g. **12** for 0-12 hours):

Table 2.4-07

BMF12, BMF24	Biliary elimination (Phase 10), Male rats, Faeces, 0-12, 12-24 h
BFF12, BFF24	Biliary elimination (Phase 10), Female rats, Faeces, 0-12, 12-24 h

Results and Discusssion

Inveresk excretion results (phases 1-3)

The study No. 202787 established that excretion after single oral administrations of 100 mg/kg and 1000 mg/kg and repeated oral administration at low dose was rapid with about 90% of the administered radioactivity excreted within 48 h from dose administration. In particular, the majority of the administered radioactivity was recovered in faeces. The remaining part was completely excreted in urine within 24 h. Differences were marked between sexes at the same dose as female animals eliminated more radioactivity in urine than male animals (Tables 2.4-08 to 2.4-10).

Table 2.4-08 Single low dose. Radioactivity found in urine within 24 hours, in faeces within 48 hours and analysed radioactivity.

Results are expressed as percentage of ^{14}C-IR5885 equivalents referred to administered dose.The data are derived from Inveresk Research Project No.202787).

Animal	**Urine**			**Faeces**		
	0-6 h	**6-12 h**	**12-24 h**	**0-12 h**	**12-24 h**	**24-48 h**
1M	9.36	1.68	0.87	37.29	38.39	5.38
2M	6.24	0.99	1.33	0.27	66.58	22.13
3M	5.57	2.84	1.08	0.21	68.63	16.98
4M	2.57	3.39	0.92	0.19	83.59	5.27
Mean value	**5.93**	**2.22**	**1.05**	**9.49**	**64.30**	**12.44**
	(LMU6)	(LMU12)	(LMU24)	(LMF12)	(LMF24)	(LMF48)
Total analyzed	9.21			86.23		
5F	33.50	7.68	2.49	0.00	45.82	0.91
6F	26.08	7.62	2.04	11.56	33.41	6.03
7F	33.82	8.14	2.88	18.26	28.85	3.24
8F	26.89	8.19	3.00	15.38	33.68	4.77
Mean value	**30.07**	**7.91**	**2.61**	**11.30**	**35.44**	**3.74**
	(LFU6)	(LFU12)	(LFU24)	(LFF12)	(LFF24)	(LFF48)
Total analyzed	40.59			50.48		

Table 2.4-09 Single high dose. Radioactivity found in urine within 24 hours, in faeces within 48-72 hours and analysed radioactivity.

Results are expressed as percentage of ^{14}C-IR5885 equivalents referred to administered dose. The data are derived from Inveresk Research Project No.202787.

Animal	**Urine**			**Faeces**			
	0-6 h	**6-12 h**	**12-24 h**	**0-12 h**	**12-24 h**	**24-48 h**	**48-72 h**
9M	6.95	5.44	2.91	34.27	24.06	6.42	8.55
10M	7.98	7.39	1.16	30.04	37.64	8.27	0.81
11M	7.02	3.88	1.98	28.86	38.88	10.63	3.67
12M	3.82	5.12	4.00	13.16	30.83	21.47	7.33
Mean value	**6.44**	**5.46**	**2.52**	**26.58**	**32.85**	**11.70**	**5.09**
	(HMU6)	(HMU12)	(HMU24)	(HMF12)	(HMF24)	(HMF48)	(HMF72)

Animal	Urine			Faeces			
	0-6 h	**6-12 h**	**12-24 h**	**0-12 h**	**12-24 h**	**24-48 h**	**48-72 h**
Total analyzed	14.42			76.22			
13F	6.92	13.61	4.79	1.57	55.78	7.79	
14F	9.43	10.56	4.89	0.25	53.45	10.27	
15F	13.32	8.64	4.89	0.31	50.68	13.09	
16F	10.21	7.93	3.88	0.55	40.08	24.25	
Mean value	**9.97**	**10.19**	**4.61**	**0.67**	**50.00**	**13.85**	
	(HFU6)	(HFU12)	(HFU24)	(HFF12)	(HFF24)	(HFF48)	
Total analyzed	24.77			64.52			

Table 2.4-10 Repeated dose. Radioactivity found in urine within 24 hours, in faeces within 48-72 hours and analysed radioactivity.

Results are expressed as percentage of ^{14}C-IR5885 equivalents referred to administered dose. The data are derived from Inveresk Research Project No.202787.

Animal	Urine			Faeces			
	0-6 h	**6-12 h**	**12-24 h**	**0-12 h**	**12-24 h**	**24-48 h**	**48-72 h**
81M	3.76	2.57	1.28	19.58	30.78	17.74	17.49
18M	4.56	2.66	0.83	0.49	34.95	26.27	18.58
19M	5.85	1.41	0.66	46.28	30.26	8.51	0.15
20M	5.42	2.47	1.25	0.73	55.56	22.77	1.65
Mean value	**4.90**	**2.28**	**1.01**	**16.77**	**37.89**	**18.82**	**9.47**
	(RMU6)	(RMU12)	(RMU24)	(RMF12)	(RMF24)	(RMF48)	(RMF72)
Total analyzed	8.18			82.94			
21F	23.14	8.31	4.65	5.19	25.89	23.14	
22F	6.86	20.90	1.72	23.37	27.10	11.38	
23F	27.06	9.44	2.01	18.85	25.55	6.76	
24F	8.87	10.50	5.54	12.54	42.74	3.32	
Mean value	**16.48**	**12.29**	**3.48**	**14.99**	**30.32**	**11.15**	
	(RFU6)	(RFU12)	(RFU24)	(RFF12)	(RFF24)	(RFF48)	
Total analyzed	32.25			56.46			

Analysis of ^{14}C-compounds in urine (phases 1-3)

TLC and HPLC analyses of the pooled urine samples from single **low dose** showed 6 degradation compounds of IR5885 (Table 5.1.3-40): R2, R3, R4, R5, R6, and R7. No traces of IR5885 were found.

The main degradation compounds were R2 (amounting to 2.68% and 36.25% AD in male and female rats, respectively) and R5 (3.60% and 2.07% AD).

The other minor compounds were all lower than 1% of the dose except compound R6 in male rats (1.33% AD).

TLC and HPLC analyses of the pooled urine samples from single **high dose** showed 5 degradation compounds of IR5885 (Table 5.1.3-41): R2, R3, R4, R5, and R6. No traces of IR5885 were found.

The main degradation compounds were R2 (amounting to 10.38% and 21.40% AD in male and female rats, respectively) and R5 (2.11% and 1.64% AD).The other minor compounds were all lower than 1% of the dose.

TLC and HPLC analyses of the pooled urine samples from **repeated administration of low dose** showed 6 degradation compounds of IR5885 (Table 5.1.3-42): R2, R3, R4, R5, R6, and R7.

No traces of IR5885 were found.

The main degradation compounds were R2 (amounting to 2.20% and 27.91% AD in male and female rats, respectively) and R5 (3.15% and 2.23% AD).

The other minor compounds were all lower than 1% of the dose except compound R6 in male rats (1.32% AD).

Table 2.4-11 Single low dose. Proportion of compounds in urine within 24 hours.

Data are expressed as percentage of ^{14}C-IR5885 equivalents referred to administered radioactivity.

Compound	Urine sample							
	Male				Female			
	LMU6	LMU12	LMU24	Total	LFU6	LFU12	LFU24	Total
R1 (IR5885)	nd	nd	nd	nd	nd	nd	nd	nd
R2	2.12	0.38	0.19	2.68	26.77	7.14	2.35	36.25
R3	0.56	0.20	0.06	0.82	0.36	0.13	0.05	0.54
R4	0.52	0.13	0.03	0.68	0.59	0.12	0.04	0.75
R5	1.73	1.22	0.65	3.60	1.52	0.43	0.12	2.07
R6	0.91	0.29	0.12	1.33	0.70	0.09	0.05	0.85
R7	0.10	nd	nd	0.10	0.13	nd	nd	0.13

Table 2.4-12 Single high dose. Proportion of compounds in urine within 24 hours.

Data are expressed as percentage of ^{14}C-IR5885 equivalents referred to administered radioactivity.

Compound	Urine sample							
	Male				Female			
	HMU6	HMU12	HMU24	Total	HFU6	HFU12	HFU24	Total
R1 (IR5885)	nd	nd	nd	nd	nd	nd	nd	nd
R2	4.52	4.24	1.61	10.38	8.09	9.05	4.26	21.40
R3	0.28	0.20	0.11	0.59	0.25	0.19	0.08	0.52
R4	0.20	0.14	0.10	0.44	0.22	0.14	0.06	0.41
R5	0.90	0.63	0.58	2.11	0.88	0.60	0.16	1.64
R6	0.54	0.25	0.11	0.90	0.54	0.21	0.05	0.79
R7	nd	nd	nd	nd	nd	nd	nd	nd

Table 2.4-13 Repeated dose. Proportion of compounds in urine within 24 hours.

Data are expressed as percentage of ^{14}C-IR5885 equivalents referred to administered radioactivity.

Compound	Urine sample							
	Male				Female			
	RMU6	RMU12	RMU24	Total	RFU6	RFU12	RFU24	Total
R1 (IR5885)	nd	nd	nd	nd	nd	nd	nd	nd
R2	1.49	0.50	0.21	2.20	13.76	10.94	3.21	27.91
R3	0.42	0.20	0.11	0.73	0.36	0.13	0.07	0.56
R4	0.48	0.12	0.09	0.69	0.47	0.27	0.05	0.79
R5	1.47	1.20	0.49	3.15	1.36	0.77	0.10	2.23
R6	0.95	0.26	0.11	1.32	0.52	0.19	0.06	0.76
R7	0.09	nd	nd	0.09	nd	nd	nd	nd

Analyses of ^{14}C-compounds in faeces (phases 1-3)

Extractable radioactivity in faeces

The radioactivity in faeces was almost completely extracted with acetone and acetone-H_2O; the percentages of radioactivity, extractable and unextractable, referred to found radioactivity and to administered dose are shown in Tables 2.4-14 to 2.4-19.

Table 2.4-14 Single low dose. Radioactivity distribution in faeces (see Scheme 1).

Data are expressed as percentage referred to found radioactivity.

Phase	Male			Female			Mean value
	LMF12	LMF24	LMF48	LFF12	LFF24	LFF48	±SD
EXTRACTABLE	99.26	98.57	98.04	99.34	98.78	99.41	98.90
(Extracts I + II + III)							0.54
UNEXTRACTABLE	0.74	1.43	1.96	0.66	1.22	0.59	1.10
(Faeces Residue IV)							0.54
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00

Table 2.4-15 Single low dose. Radioactivity distribution in faeces (see Scheme 1).

Data are expressed as percentage referred to administered dose.

Phase	Male			Female		
	LMF12	LMF24	LMF48	LFF12	LFF24	LFF48
EXTRACTABLE	9.42	63.38	12.20	11.23	35.01	3.72
(Extracts I + II + III)						
UNEXTRACTABLE	0.07	0.92	0.24	0.07	0.43	0.02
(Faeces Residue IV)						
Total	9.49	64.30	12.44	11.30	35.44	3.74

Table 2.4-16 Single high dose. Radioactivity distribution in faeces (see Scheme 1).

Data are expressed as percentage referred to found radioactivity.

Phase	Male				Female			Mean value
	HMF12	HMF24	HMF48	HMF72	HFF12	HFF24	HFF48	±SD
EXTRACTABLE	98.86	98.67	98.15	98.16	99.32	98.93	98.35	98.63
(Extracts I + II + III)								0.44
UNEXTRACTABLE	1.14	1.33	1.85	1.84	0.68	1.07	1.65	1.37
(Faeces Residue IV)								0.44
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

Table 2.4-17 Single high dose. Radioactivity distribution in faeces (see Scheme 1).

Data are expressed as percentage referred to administered dose.

Phase	Male				Female		
	HMF12	HMF24	HMF48	HMF72	HFF12	HFF24	HFF48
EXTRACTABLE	26.28	32.41	11.48	5.00	0.67	49.47	13.62
(Extracts I + II + III)							
UNEXTRACTABLE	0.30	0.44	0.22	0.09	0.00	0.53	0.23
(Faeces Residue IV)							
Total	26.58	32.85	11.70	5.09	0.67	50.00	13.85

Table 2.4-18 Repeated dose. Radioactivity distribution in Faeces (see Scheme 1).

Data are expressed as percentage referred to found radioactivity.

Phase	Male				Female			Mean value
	RMF12	RMF24	RMF48	RMF72	RFF12	RFF24	RFF48	±SD
EXTRACTABLE	99.14	98.97	98.26	98.54	98.51	98.91	98.19	98.65
(Extracts I + II + III)								0.37
UNEXTRACTABLE	0.86	1.03	1.74	1.46	1.49	1.09	1.81	1.35
(Faeces Residue IV)								0.37
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

Table 2.4-19 Repeated dose. Radioactivity distribution in Faeces (see Scheme 1).

Data are expressed as percentage referred to administered dose.

Phase	Male				Female		
	RMF12	RMF24	RMF48	RMF72	RFF12	RFF24	RFF48
EXTRACTABLE (Extracts I + II + III)	16.63	37.50	18.49	9.33	14.77	29.99	10.95
UNEXTRACTABLE (Faeces Residue IV)	0.14	0.39	0.33	0.14	0.22	0.33	0.20
Total	16.77	37.89	18.82	9.47	14.99	30.32	11.15

Faeces extracts

TLC and HPLC analyses of the pooled faeces extracts from single **low dose** showed unchanged IR5885 (R1) and 3 degradation compounds (Table 2.4-20): R2, R3, and R4.

IR5885 (compound R1) amounted to 5.30% and 6.17% AD in male and female rats, respectively.

Compound R2 was the main degradation compound amounting to 73.20% and 40.42% AD.

The other minor compounds were R3 (4.32% and 1.91% AD) and R4 (2.18% and 1.46% AD).

TLC and HPLC analyses of the pooled faeces extracts from single **high dose** showed unchanged IR5885 (R1) and 3 degradation compounds (Table 2.4-21): R2, R3, and R4.

IR5885 (compound R1) amounted to 40.41% and 9.50% AD in male and female rats, respectively. Compound R2 was the main degradation compound amounting to 31.66% and 51.15% AD. The other minor compounds were R3 (2.59% and 2.68% AD) and R4 (0.51% and 0.44% AD).

TLC and HPLC analyses of the pooled faeces extracts from **repeated administration at low dose** showed unchanged IR5885 (R1) and 3 degradation compounds (Table 2.4-22): R2, R3, and R4.

IR5885 (compound R1) amounted to 7.80% and 5.47% AD in male and female rats, respectively.

Compound R2 was the main degradation compound amounting to 66.26% and 46.21% AD.

The other minor compounds were R3 (5.17% and 2.72% AD) and R4 (2.72% and 1.32% AD).

Table 2.4-20 Single low dose. Proportion of compounds in faeces within 48 hours.

Data are expressed as percentage of ^{14}C-IR5885 equivalents referred to administered radioactivity.

Compound	Faeces sample							
	Male				Female			
	LMF12	LMF24	LMF48	Total	LFF12	LFF24	LFF48	Total
R1 (IR5885)	1.16	3.95	0.19	5.30	3.35	2.82	nd	6.17
R2	7.25	55.17	10.78	73.20	6.96	30.14	3.32	40.42
R3	0.71	2.73	0.88	4.32	0.48	1.17	0.27	1.91
R4	0.30	1.53	0.35	2.18	0.45	0.88	0.13	1.46
R5	nd	nd	nd	nd	nd	nd	nd	nd
R6	nd	nd	nd	nd	nd	nd	nd	nd
R7	nd	nd	nd	nd	nd	nd	nd	nd

Table 2.4-21 Single high dose. Proportion of compounds in faeces within 72 (male) and 48 (female) hours.

Data are expressed as percentage of ^{14}C-IR5885 equivalents referred to administered radioactivity.

Compound	Faeces sample								
	Male					Female			
	HMF12	HMF24	HMF48	HMF72	Total	HFF12	HFF24	HFF48	Total
R1 (IR5885)	22.02	18.12	0.26	nd	40.41	0.47	8.74	0.29	9.50
R2	3.82	13.29	10.02	4.54	31.66	0.18	38.87	12.09	51.15
R3	0.44	0.99	0.82	0.33	2.59	0.02	1.87	0.80	2.68
R4	nd	nd	0.38	0.13	0.51	nd	nd	0.44	0.44
R5	nd	nd	nd	nd	nd	nd	nd	nd	nd
R6	nd	nd	nd	nd	nd	nd	nd	nd	nd
R7	nd	nd	nd	nd	nd	nd	nd	nd	nd

Table 2.4-22 Repeated dose. Proportion of compounds in faeces within 72 (male) and 48 (female) hours.

Data are expressed as percentage of ^{14}C-IR5885 equivalents referred to administered radioactivity.

Compound	Faeces sample								
	Male					Female			
	RMF12	RMF24	RMF48	RMF72	Total	RFF12	RFF24	RFF48	Total
R1 (IR5885)	4.04	3.57	0.19	nd	7.80	1.82	3.65	nd	5.47
R2	10.80	30.84	16.29	8.33	66.26	11.72	24.60	9.89	46.21
R3	1.12	1.88	1.46	0.71	5.17	0.78	1.15	0.79	2.72
R4	0.67	1.22	0.55	0.29	2.72	0.45	0.59	0.27	1.32
R5	nd	nd	nd	nd	nd	nd	nd	nd	nd
R6	nd	nd	nd	nd	nd	nd	nd	nd	nd
R7	nd	nd	nd	nd	nd	nd	nd	nd	nd

Comparison of compounds from excreta

Comparison between urinary and faecal compounds from male and female rats was performed by co-chromatographic analyses of representative samples. Urinary metabolites from male rats corresponded to urinary metabolites from female rats.

Faecal metabolites from male rats corresponded to faecal metabolites from female rats. All faecal metabolites were present in urine (R5, R6, and R7, instead, were only in urine).

Metabolites from rats administered with low dose corresponded to compounds from rats administered with high dose.

Metabolites from rats administered with single low dose corresponded to compounds from rats administered with repeated low dose.

Inveresk tissue distribution results (phases 7-9)

Following low, high and repeated oral administrations, the highest mean concentration of radioactivity was found in gastrointestinal tract for both male and female rats. Lower concentrations were found also in liver and kidneys, the only organs with significative radioactivity.

Analysis of ^{14}C-compounds in liver and kidneys

Extractable radioactivity in liver and kidneys

The radioactivity in liver and kidneys was almost completely extracted with acetone and acetone-H_2O; the percentages of radioactivity, extractable and unextractable, referred to found radioactivity and to administered dose are shown in Tables 2.4-23 to 2.4-25.

Table 2.4-23 Single low dose. Radioactivity distribution in liver and kidneys (see Scheme 1).

Data are expressed as percentage referred to found radioactivity.

Phase	**Liver**		**Mean value**	**Kidneys**		**Mean value**
	LML	LFL	±SD	LMK	LFK	±SD
EXTRACTABLE	99.09	98.39	98.74	98.01	98.07	98.04
(Extracts I + II + III)			0.49			0.04
UNEXTRACTABLE	0.91	1.61	1.26	1.99	1.93	1.96
(Faeces Residue IV)			0.49			0.04
Total	100.00	100.00	100.00	100.00	100.00	100.00

Table 2.4-24 Single low dose. Radioactivity distribution in liver and kidneys (see Scheme 1).

Data are expressed as percentage referred to administered dose.

Phase	**Liver**		**Kidneys**	
	LML	LFL	LMK	LFK
EXTRACTABLE	7.57	6.89	0.29	0.45
(Extracts I + II + III)				
UNEXTRACTABLE	0.07	0.11	0.01	0.01
(Faeces Residue IV)				
Total	7.64	7.00	0.30	0.46

Table 2.4-25 Single high dose. Radioactivity distribution in liver and kidneys (see Scheme 1).

Data are expressed as percentage referred to found radioactivity.

Phase	**Liver**		**Mean value**	**Kidneys**		**Mean value**
	HML	HFL	±SD	HMK	HFK	±SD
EXTRACTABLE	98.15	98.43	98.29	98.87	98.09	98.48
(Extracts I + II + III)			0.20			0.55
UNEXTRACTABLE	1.85	1.57	1.71	1.13	1.91	1.52
(Faeces Residue IV)			0.20			0.55
Total	100.00	100.00	100.00	100.00	100.00	100.00

Table 2.4-26 Single high dose. Radioactivity distribution in liver and kidneys (see Scheme 1).

Data are expressed as percentage referred to administered dose.

Phase	**Liver**		**Kidneys**	
	HML	HFL	HMK	HFK
EXTRACTABLE	1.49	1.16	0.17	0.11
(Extracts I + II + III)				
UNEXTRACTABLE	0.03	0.02	0.00	0.00
(Faeces Residue IV)				
Total	1.52	1.18	0.17	0.11

Table 2.4-27 Repeated dose. Radioactivity distribution in liver and kidneys (see Scheme 1).

Data are expressed as percentage referred to found radioactivity.

Phase	**Liver**		**Mean value**	**Kidneys**		**Mean value**
	RML	RFL	±SD	RMK	RFK	±SD
EXTRACTABLE	98.16	98.93	98.55	98.95	98.15	98.55
(Extracts I + II + III)			0.54			0.57
UNEXTRACTABLE	1.84	1.07	1.46	1.05	1.85	1.45
(Faeces Residue IV)			0.54			0.57

Total	100.00	100.00	100.00	100.00	100.00	100.00

Table 2.4-28 Repeated dose. Radioactivity distribution in liver and kidneys (see Scheme 1).

Data are expressed as percentage referred to administered dose.

Phase	**Liver**		**Kidneys**	
	RML	RFL	RMK	RFK
EXTRACTABLE (Extracts I + II + III)	9.89	5.04	0.42	0.26
UNEXTRACTABLE (Faeces Residue IV)	0.19	0.05	0.00	0.00
Total	10.08	5.09	0.42	0.26

Liver and kidneys extracts

TLC and HPLC analyses of extracts from **liver** of rats administered with **low dose** showed 2 degradation compounds of IR5885: R2 and R3 (Table 2.4-29). No traces of IR5885 were found.

The main degradation compound was R2 (amounting to 7.28% and 6.58% AD in male and female rats, respectively).

The other minor compound R3 was lower than 1% of the dose (0.29% and 0.31% AD).

TLC analysis of extracts from **kidneys** of rats administered with **low dose** showed 4 degradation compounds of IR5885: R2, R3, R4, and R5 (Table 2.4-29).

No traces of IR5885 were found.

All the degradation compounds were lower than 1% and R2 was the main (amounting to 0.16% and 0.35% AD in male and female rats, respectively). Compound R4 was found only in male rats.

TLC and HPLC analyses of extracts from **liver** of rats administered with **high dose** showed 2 degradation compounds of IR5885: R2 and R3 (Table 2.4-30).

No traces of IR5885 were found.

The main degradation compound was R2 (amounting to 1.42% and 1.16% AD in male and female rats, respectively).

The other minor compound R3 was present only in male rats and was lower than 1% of the dose (0.07% AD).

TLC analysis of extracts from **kidneys** of rats administered with **high dose** showed 4 degradation compounds of IR5885: R2, R3, R4, and R5 (Table 2.4-30).

No traces of IR5885 were found.

All the degradation compounds were lower than 1% and R2 was the main (amounting to 0.10% both in male and female rats). Compound R4 was found only in male rats.

TLC and HPLC analyses of extracts from **liver** of rats administered with **repeated dose** showed 2 degradation compounds of IR5885: R2 and R3 (Table 2.4-31).

No traces of IR5885 were found.

The main degradation compound was R2 (amounting to 9.62% and 5.04% AD in male and female rats, respectively).

The other minor compound R3 was present only in male rats and was lower than 1% of the dose (0.27% AD).

TLC analysis of extracts from **kidneys** of rats administered with **repeated dose** showed 3 degradation compounds of IR5885: R2, R3, and R5 (Table 2.4-31).

No traces of IR5885 were found.

All the degradation compounds were lower than 1% and R2 was the main (amounting to 0.33% and 0.15% AD in male and female rats, respectively).

Table 2.4-29 Single low dose. Proportion of compounds in liver and kidneys of rats.

Data expressed as percentage of ^{14}C-IR5885 equivalents referred to administered radioactivity and as µg of ^{14}C-IR5885 equivalents g^{-1}.

%AD

Compound	Tissue sample			
	Liver		Kidneys	
	Male	Female	Male	Female
R1 (IR5885)	nd	nd	nd	nd
R2	7.28	6.58	0.16	0.35
R3	0.29	0.31	0.02	0.03
R4	nd	nd	0.01	nd
R5	nd	nd	0.10	0.07
R6	nd	nd	nd	nd
R7	nd	nd	nd	nd

µg/g

Compound	Tissue sample			
	Liver		Kidneys	
	Male	Female	Male	Female
R1 (IR5885)	nd	nd	nd	nd
R2	163.161	163.236	19.368	43.630
R3	6.551	7.638	2.038	3.858
R4	nd	nd	1.204	nd
R5	nd	nd	11.705	8.347
R6	nd	nd	nd	nd
R7	nd	nd	nd	nd

Table 2.4-30 Single high dose. Proportion of compounds in liver and kidneys of rats.

Data expressed as percentage of ^{14}C-IR5885 equivalents referred to administered radioactivity and as µg of ^{14}C-IR5885 equivalents g^{-1}.

%AD

Compound	Tissue sample			
	Liver		Kidneys	
	Male	Female	Male	Female
R1 (IR5885)	nd	nd	nd	nd
R2	1.42	1.16	0.10	0.10
R3	0.07	nd	0.02	0.00
R4	nd	nd	0.01	nd
R5	nd	nd	0.04	0.01
R6	nd	nd	nd	nd
R7	nd	nd	nd	nd

µg/g

Compound	Tissue sample			
	Liver		Kidneys	
	Male	Female	Male	Female
R1 (IR58885	nd	nd	nd	nd

R2	330.062	264.993	118.158	114.547
R3	15.083	nd	18.177	5.279
R4	nd	nd	10.437	nd
R5	nd	nd	50.158	6.161
R6	nd	nd	nd	nd
R7	nd	nd	nd	nd

Table 2.4-31 Repeated dose. Proportion of compounds in liver and kidneys of male rats.

Data expressed as percentage of ^{14}C-IR5885 equivalents referred to administered radioactivity and as µg of ^{14}C-IR5885 equivalents g^{-1}.

%AD:

Compound	**Tissue sample**			
	Liver		**Kidneys**	
	Male	**Female**	**Male**	**Female**
R1 (IR5885)	nd	nd	nd	nd
R2	9.62	5.04	0.33	0.15
R3	0.27	nd	0.03	0.02
R4	nd	nd	nd	nd
R5	nd	nd	0.06	0.09
R6	nd	nd	nd	nd
R7	nd	nd	nd	nd

µg/g:

Compound	**Tissue sample**			
	Liver		**Kidneys**	
	Male	**Female**	**Male**	**Female**
R1 (IR5885)	nd	nd	nd	nd
R2	212.658	123.148	43.023	17.506
R3	6.013	nd	3.711	2.237
R4	nd	nd	nd	nd
R5	nd	nd	7.996	11.202
R6	nd	nd	nd	nd
R7	nd	nd	nd	nd

Table 2.4-32 Proportion of compounds in bile (Phase 10).

Data are expressed as percentage of ^{14}C-IR5885 equivalents referred to administered radioactivity.

Compound	**Bile sample**							
	Male				**Female**			
	BMB6	BMB12	BMB24	Total	BFB6	BFB12	BFB24	Total
R1 (IR5885)	nd	nd	nd	nd	nd	nd	nd	nd
R2	50.37	7.56	1.66	59.58	32.56	9.03	4.76	46.35
R3	2.42	0.58	0.09	3.09	1.14	0.35	0.18	1.67
R4	1.20	0.39	nd	1.59	nd	nd	nd	nd
R5	nd	nd	nd	nd	nd	nd	nd	nd
R6	nd	nd	nd	nd	nd	nd	nd	nd
R7	nd	nd	nd	nd	nd	nd	nd	nd

Table 2.4-33 Proportion of compounds in urine (Phase 10).

Data are expressed as percentage of ^{14}C-IR5885 equivalents referred to administered radioactivity.

Compound	Urine sample							
	Male				Female			
	BMU6	BMU12	BMU24	Total	BFU6	BFU12	BFU24	Total
R1 (IR5885)	nd	nd	nd	nd	nd	nd	nd	nd
R2	2.60	1.24	0.12	3.96	4.91	16.51	3.47	24.90
R3	1.73	0.79	0.07	2.58	0.13	0.56	0.10	0.79
R4	0.71	0.21	0.05	0.97	0.26	0.60	0.13	1.00
R5	1.79	1.51	0.27	3.56	0.43	1.44	0.20	2.07
R6	1.08	0.39	0.07	1.54	0.17	0.61	0.06	0.84
R7	0.13	nd	nd	0.13	nd	nd	nd	nd

Table 2.4-34 Proportion of compounds in faeces (Phase 10).

Data are expressed as percentage of ^{14}C-IR5885 equivalents referred to administered radioactivity.

Compound	Faeces sample					
	Male			Female		
	BMF12	BMF24	Total	BFF12	BFF24	Total
R1 (IR5885)	7.36	0.71	8.08	5.50	1.15	6.65
R2	4.43	2.77	7.19	1.74	3.94	5.68
R3	nd	nd	nd	nd	nd	nd
R4	nd	nd	nd	nd	nd	nd
R5	nd	nd	nd	nd	nd	nd
R6	nd	nd	nd	nd	nd	nd
R7	nd	nd	nd	nd	nd	nd

Inveresk biliary elimination results (phase 10)

Inveresk Project No. 202787 established that bile was an important route of elimination for radioactivity with a mean of 64.27% AD (males) and 48.02%AD (females) detected over 24 h. The urine of bile-duct-cannulated animals contained mean values of 12.73% AD and 29.61% AD (male and female respectively) at 24 h post dose, consistent with similarly dosed non-cannulated animals. Faeces samples contained up to a mean of 15.54% AD (males) and 12.54% AD (females) at 24 h post dose (Table 5.1.3-64)

Table 2.4-35 Analysed radioactivity in bile, in urine and in faeces within 24 hours. (Phase 10)

Results are expressed as percentage of ^{14}C-IR5885 equivalents referred to administered dose.

The data are derived from Inveresk Research Project No.202787.

Animal	Bile			Urine			Faeces		Total analysed
	0-6 h	6-12 h	12-24 h	0-6 h	6-12 h	12-24 h	0-12 h	12-24 h	
73M	61.81	10.54	1.77	3.65	1.35	0.28	12.84	3.02	
74M	59.05	6.31	1.40	5.82	6.06	0.34	10.02	4.17	
75M	30.55	12.21	2.56	16.07	5.50	1.38	16.39	3.94	
76M	64.57	5.07	1.26	6.58	3.62	0.27	8.75	3.04	
Mean value	**53.99**	**8.53**	**1.75**	**8.03**	**4.13**	**0.57**	**12.00**	**3.54**	
	(BMB6)	(BMB12)	(BMB24)	(BMU6)	(BMU12)	(BMU24)	(BMF12)	(BMF24)	
Total analyzed	64.27			12.73			15.54		92.54
77F	42.99	7.29	1.74	0.00	26.14	1.13	9.01	7.99	
78F	34.70	6.10	0.45	23.61	6.69	1.32	18.01	3.55	
79F	20.45	8.04	15.18	0.00	21.37	9.63	2.12	0.82	
80F	36.65	16.11	2.38	0.00	24.74	3.80	0.26	8.39	
Mean value	**33.70**	**9.38**	**4.94**	**5.90**	**19.73**	**3.97**	**7.35**	**5.19**	
	(BFB6)	(BFB12)	(BFB24)	(BFU6)	(BFU12)	(BFU24)	(BFF12)	(BFF24)	
Total analyzed	48.02			29.61			12.54		90.16

Extractable radioactivity in faeces (Phase 10)

The radioactivity in faeces was almost completely extracted with acetone and acetone-H_2O; the percentages of radioactivity, extractable and unextractable, referred to found radioactivity and to administered dose are shown in Tables 2.4-36 and 2.4-37.

Table 2.4-36 Radioactivity distribution in faeces. (Phase 10) (see Scheme 1).

Data are expressed as percentage referred to found radioactivity.

Phase	Male		Female		Mean value
	BMF12	BMF24	BFF12	BFF24	±SD
EXTRACTABLE	98.24	98.29	98.52	98.10	98.29
(Extracts I + II + III)					0.17
UNEXTRACTABLE	1.76	1.71	1.48	1.90	1.71
(Faeces Residue IV)					0.17
Total	100.00	100.00	100.00	100.00	100.00

Table 2.4-37 Radioactivity distribution in faeces. (Phase 10) (see Scheme 1).

Data are expressed as percentage referred to administered dose.

Phase	Male		Female	
	BMF12	BMF24	BFF12	BFF24
EXTRACTABLE	11.79	3.48	7.24	5.09
(Extracts I + II + III)				
UNEXTRACTABLE	0.21	0.06	0.11	0.10
(Faeces Residue IV)				

Total	12.00	3.54	7.35	5.19

Analysis of ^{14}C-compounds in bile, urine, and faeces

TLC analysis of pooled bile samples (Table 2.4-32) showed that the main compound eliminated via bile was compound R2, accounting for 59.58% and 46.35% AD in male and female rats, respectively.

Other compounds were R3 (3.09% and 1.67% AD) and, only in male rats, R4 (1.59% AD).

No traces of IR5885 were found.

TLC analysis of pooled urine samples (Table 2.4-32) showed that the main compound was compound R2, accounting for 3.96% and 24.90% AD in males and females, respectively.

Other compounds were R3, R4, R5, R6, and, only in male rats, R7.

No traces of IR5885 were found. The faeces extracts, analysed by TLC, showed the presence of R1 (unchanged IR5885, accounting for 8.08% and 6.65% AD in male and female rats respectively) and of R2 (7.19% and 5.68) (Table 2.4-35).

Comparison of compounds in liver, kidneys, bile, urine, and excreta

Comparison between compounds found in bile, liver, kidneys and excreta was performed by co-chromatographic analyses of representative samples.

The only metabolite in liver was also present in kidneys (compound R2) which also contained compound R3, R4, R5, and R6.

The only common compound in bile and faeces of cannulated rats was compound R2.

All bile metabolites were present in faeces of no cannulated rats which also contained compound R1 (unchanged IR5885), completely absent in bile sample.

Identification of IR5885 and its degradation compounds

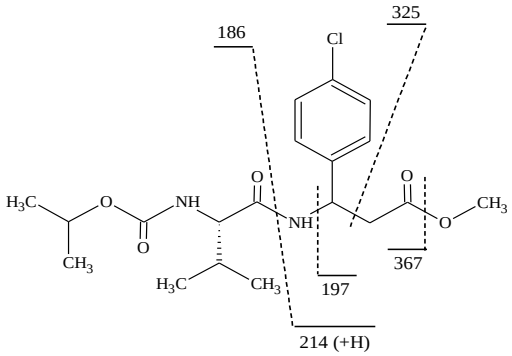
Identification of the ^{14}C-compounds was performed by co-chromatographic analyses (both TLC and HPLC) of representative samples and reference standards and by LC-MS analysis.

Compound R1 (unchanged IR5885)

The TLC radiochromatogram of sample LMF12 with authentic ^{14}C-IR5885 reference standard and the HPLC radiochromatogram of sample HMF24 with authentic ^{14}C-IR5885 reference standard show that the compound R1 had the same chromatographic properties of IR5885.

The MS spectra of compound R1 show the same molecular fragmentation of IR5885 reference standard:

m/z	*attribution*
399	$[M+H]^+$



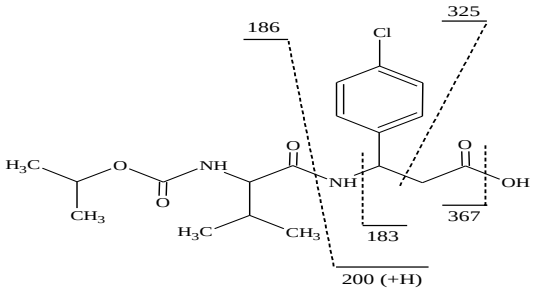
On the basis of co-HPLC, co-TLC, and LC-MS analyses, the compound R1 was identified as unchanged IR5885: RS-β-Alanine, N-[(1-methylethoxy)carbonyl]-L-valyl-3-(4-chlorophenyl) methyl ester.

Diastereoisomeric ratio (*S*,*R*/*S*,*S*) of unchanged IR5885 (R1) did not change remarkably in all the samples.

Compound R2

The TLC radiochromatograms of samples LMF12 and LMU6 with authentic ^{14}C-IR5885 acid reference standard and the HPLC radiochromatograms of samples HMF24 and RMU6 with authentic ^{14}C-IR5885 acid reference standard show that the compound R2 had the same chromatographic properties of IR5885 acid. The MS spectra of compound R2 show the same molecular fragmentation of IR5885 acid reference standard:

m/z	*attribution*
385	$[M+H]^+$



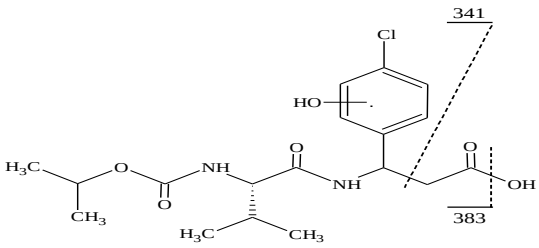
On the basis of co-HPLC, co-TLC, and LC-MS analyses, the compound R2 was identified as: RS-β-alanine, N-[(1-methylethoxy)carbonyl]-L-valyl-3-(4-chlorophenyl).

Diastereoisomeric ratio (*S*,*R*/*S*,*S*) of IR5885 acid (R2) did not change remarkably in all the samples.

Compounds R3/R4

TLC analysis showed that R3 and R4 had similar properties (similar Rf); HPLC analysis showed for both the compounds (R3 and R4) a couple of peaks meaning that the two chiral centers were again present; MS spectra of compounds R3 and R4 showed the same molecular fragmentation.

m/z	*attribution*
401	$[M+H]^+$

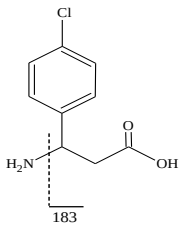


On the basis of TLC, HPLC and LC-MS analyses, the compounds R3 and R4 were likely identified as: RS-β-alanine, N-[(1-methylethoxy)carbonyl]-L-valyl-3-(2-hydroxy-4-chlorophenyl) and RS-β-alanine, N-[(1-methylethoxy)carbonyl]-L-valyl-3-(3-hydroxy-4-chlorophenyl). The difference is only in the ring position of hydroxyl group.

Compound R5

The MS spectra of compound R5 showed the same molecular fragmentation of 3-amino-3-(4-chlorophenyl) propionic acid (IR6459) reference standard.

m/z	*attribution*
200	$[M+H]^+$



Then the compound R5 was identified as 3-amino-3-(4-chlorophenyl) propionic acid.

Conclusions

Study results showed that IR5885 was extensively metabolized following oral administration to rats.

The qualitative metabolic profile was almost the same following single oral (low and high) administration and repeated oral administration although the amounts of some compounds were different especially between low and high doses.

The qualitative metabolic profile was the same in male and female rats treated at the same dose, although the amounts of some compounds were slightly different in the two sexes; identical metabolites were found both in urine and faeces.

The radioactivity was mainly excreted *via* faeces.

IR5885 acid was the main degradation product both in faeces and urine at all administration doses except in urine of male rats at low dose (single or repeated).

Other five metabolites were found never reaching 6% AD in the excreta.

Among these R3 and R4 were identified as RS-β-alanine, N-[(1-methylethoxy)carbonyl]-L-valyl-3-(2-hydroxy-4- chlorophenyl) and RS-β-alanine, N-[(1-methylethoxy)carbonyl]-L-valyl-3-(3-hydroxy-4-chlorophenyl) and R5 was identified as 3-amino-3-(4-chlorophenyl) propionic acid.

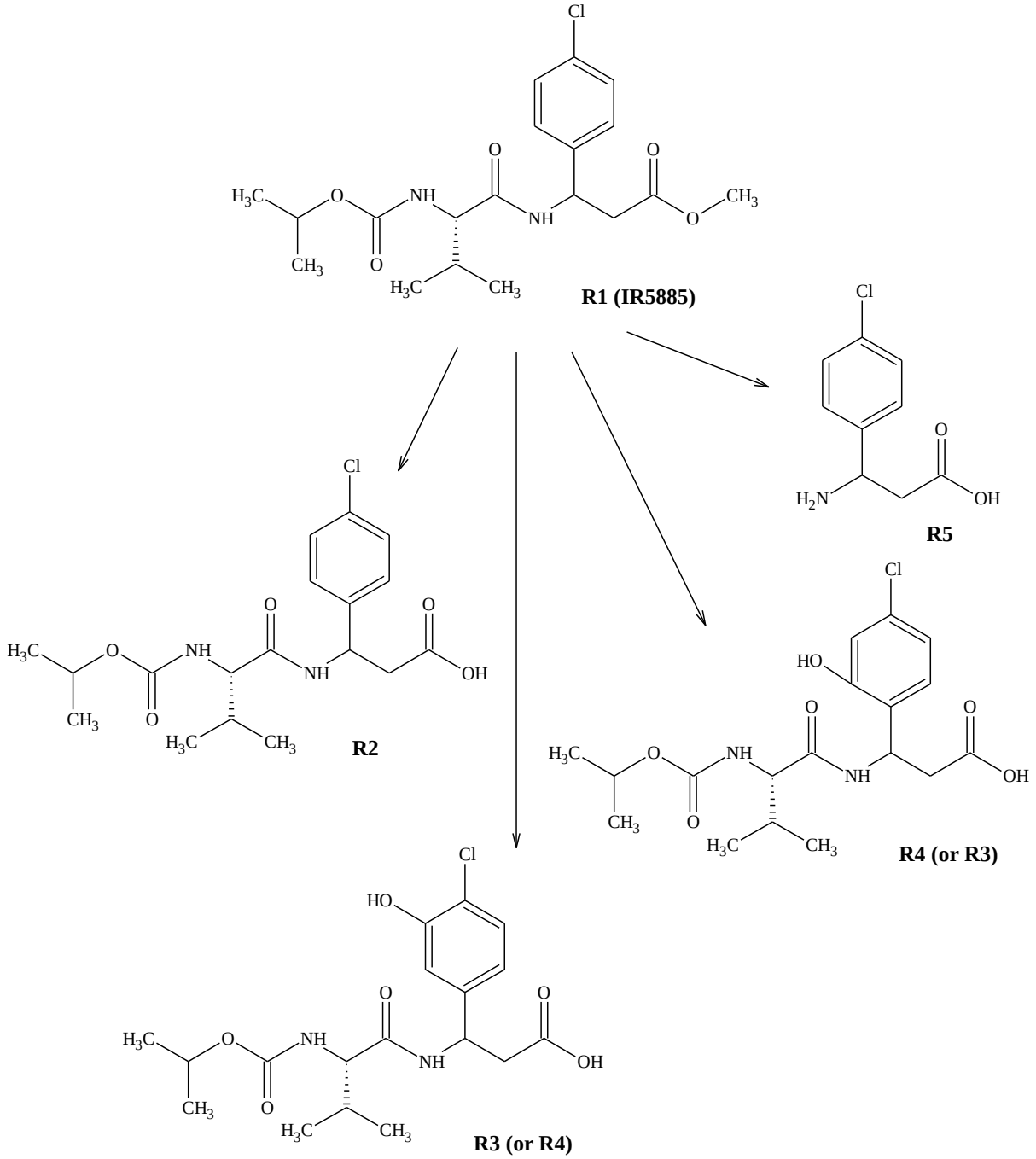
The elimination of radioactivity following a single oral administration at low dose to bile-duct-cannulated male and female rats was mainly via urine and bile.

The main metabolite found in bile was IR5885 acid while no traces of IR5885 were observed. The radioactivity excreted in faeces consisted essentially of IR5885 and IR5885 acid in the same proportion.

Liver and kidneys of animals administered with single (low and high) and repeated (low) doses were the only tissues containing significative radioactivity. The analyses of liver extracts showed only the presence of IR5885 acid, while in kidneys extracts IR5885 acid was the main compound but other metabolites already found in urine were observed at very low amounts.

The proposed degradation pathway for IR5885 in Rat is shown in Scheme 2.

Scheme 2: Proposed degradation pathway of IR5885 in rat



3 HEALTH HAZARDS

Acute toxicity

3.1 Acute toxicity - oral route

3.1.1 Animal data

3.1.1.1 Acute Oral toxicity to rat – IR5885

Reference Number: KII- Section 3, Volume 1, Annex IIA, point 5.2.1/01

Report:	See annex conf. 62

Guidelines: OECD 401, EEC B.1

Deviation: none

GLP: Yes (certified laboratory)

Executive summary:

In an acute oral toxicity study, groups of fasted young adult Sprague Dawley Crl: CD (SD) BR rats (5/sex) received a single oral administration (by gavage) of the test item IR5885 (98.9% w/w purity) in aqueous solution of Methocel (0.5% w/v) at the dose of 5000 mg/kg bw. All rats received a constant administration volume of 10 mL/kg. Food was re-offered to the rats about 3 hours after administration. Following dosing, the rats were observed for 14 days.

During the period of observation there were no mortalities. Transient piloerection was observed in all animals the day after the treatment. No abnormalities were found thereafter. Normal body weight gain was recorded in the animals during the study. No macroscopic findings were evident at autopsy. The acute oral LD_{50} value for Sprague Dawley Crl: CD (SD) BR rats exposed to IR5885 as a single dose was determined to be higher than 5000 mg/kg bw. On the basis of this study, IR5885 does not warrant classification as being harmful or toxic.

MATERIALS AND METHODS
MATERIALS

Test Item	IR5885
Description	White powder
Lot/Batch #	FCF/T/180-00 (ex ZI 068)
Purity	98.9%
CAS #	283159-90-0
Vehicle and/or positive control:	aqueous solution of 0.5% (w/v) Methocel
Test animals –	
Species:	Rat
Strain:	Crl: CD (SD) BR
Age:	< 3 months
Weight at dosing:	275-304 males; 170-188 females
Source:	Charles River Italia S.p.A., Via Indipendenza, 11 – 23885 CALCO (Lecco) – Italy
Acclimation period:	≥ 5 days
Diet:	GLP 4RF21, ad libitum
Water:	Tap water from the municipal water main system, ad libitum

Housing: 5 animals/sex in grill cages 40.5x38.5x18h cm with stainless steel feeder

Environmental conditions -
Temperature: 22 °C ± 2
Humidity: 55% ± 10
Air changes: About 15-20 / hour filtered on HEPA 99.97%
Photoperiod: 12-hour cycle (7.00 a.m. – 7 p.m.)

STUDY DESIGN AND METHODS

In life dates: March 2 – April 4, 2001

Animal assignment and treatment

Animals were assigned to the test group as listed in Table 5.2.1-01. Following an overnight fast (16 hours), rats were given a single dose of IR5885 (98.9% pure) by gavage. The test item was administered in 0.5% Methocel water solution (w/v) at the volume of 10 mL/kg bw. Food was re-offered to the rats about 3 hours after administration. Animals were observed for mortality and clinical signs at 30 minutes, 2, 4, 6 hours after dosing and twice a day up to the termination of the observation period. Body weights were recorded just before dosing (day 1) and on day 3, 8 and 14. On day 14, surviving animals were sacrificed by excision of the femoral arteries, after i.p. over dosage anesthesia with 5% sodium pentobarbital. All animals were necropsied and examined for gross pathology changes.

Dose (mg/kg bw)	Males	Female	Combined
5000	0/5	0/5	0/10

Statistics

The data did not warrant statistical analysis

RESULTS AND DISCUSSION

Mortality

No mortalities occurred at 5000 mg/kg bw, the only dose level tested.

The oral LD_{50} for males was > 5000 mg/kg bw
for females was > 5000 mg/kg bw
combined was > 5000 mg/kg bw

Clinical observations

Transient piloerection was observed in all animals the day after the treatment. No abnormalities were found thereafter.

Body weight

Normal weight gain was recorded in the animals during the study

Necropsy

At autopsy carried out at the end of the observation period, no appreciable macroscopic findings were evident in any treated rat.

Deficiencies - None

CONCLUSIONS:

The acute oral LD_{50} of IR5885 was found to be higher than 5000 mg/kg bw. IR5885 does not warrant classification as being toxic or harmful on the basis of its acute oral toxicity.

3.1.2 Human data

No human data available on acute oral toxicity

3.1.3 Other data

No other data available on acute oral toxicity

3.2 Acute toxicity - dermal route

3.2.1 Animal data

3.2.1.1 Acute percutaneous toxicity to rat – IR5885

Reference Number:	KII- Section 3, Volume 1, Annex IIA, point 5.2.2/01
Report:	See annex conf. 63
Guidelines:	OECD 402, EEC B.3
Deviation:	None
GLP:	Yes (certified laboratory)

Executive summary:

In an acute percutaneous toxicity study, groups of young adult Sprague Dawley Crl: CD (SD) BR rats (5/sex) received a single epidermal administration of the test item IR5885 (98.9% w/w purity). The test item was applied uniformly to 10% body surface of each animal onto a porous gauze which was moistened with 0.9% (w/v) NaCl solution, at the dose of 2000 mg/kg bw for about 24 hours. After the administration, the rats were observed for the following 15 days.

During the period of observation there were no mortalities. No general or local clinical abnormalities were seen in any animal. Body weights of both males and females were found to be unaffected by the test item administration. No macroscopic findings were evident at autopsy. The acute dermal LD_{50} value for Sprague Dawley Crl: CD (SD) BR rats exposed to IR5885 as a single dose was determined to be higher than 2000 mg/kg bw. On the basis of this study, IR5885 does not warrant classification as being harmful or toxic.

MATERIALS AND METHODS
MATERIALS

Test Item	IR5885
Description	White powder
Lot/Batch #	FCF/T/180-00 (ex ZI 068)
Purity	98.9%
CAS #	283159-90-0
Vehicle and/or positive control:	0.9% (w/v) NaCl solution
Test animals –	
Species:	Rat
Strain:	Crl: CD (SD) BR
Age:	< 3 months
Weight at dosing:	198-210 males; 174-188 females

Source:	Charles River Italia S.p.A., Via Indipendenza, 11 – 23885 CALCO (Lecco) – Italy
Acclimation period:	≥ 5 days
Diet:	GLP 4RF21, *ad libitum*
Water:	Tap water from the municipal water main system, *ad libitum*
Housing:	Individually during the 24-hour treatment period and then 5 animals/sex in grill cages 40.5x38.5x18h cm with stainless steel feeder
Environmental conditions -	
Temperature:	22 °C ± 2
Humidity:	55% ± 10
Air changes:	About 20 / hour filtered on HEPA 99.97%
Photoperiod:	12-hour cycle (7.00 a.m. – 7 p.m.)

STUDY DESIGN AND METHODS

In life dates: March 2 – April 12, 2001

Animal assignment and treatment

Animals were assigned to the test group as listed in Table 5.2.2-01. Approximately 24 hours before the test, fur was clipped from the dorsal and ventral area of the trunk of each animal. The clipped area accounted for about 10% of the total body surface of each animal. Care was taken to avoid abrading the skin. The test item was applied uniformly onto a porous gauze moistened with 0.9% NaCl solution. The test site was covered with the porous gauze dressing fixed to the skin with hypoallergenic nom-irritating tape. The test site was then further covered in a suitable manner in order to ensure the animals could not ingest the test item. At the end of exposure period the occlusion was removed and the residual test item was wiped off with water. Animals were observed for mortality and clinical signs at 30 minutes, 2, 4, 6 hours after dosing and twice a day up to the termination of the observation period. Body weights were recorded just before dosing (day 1), on day 8 and 15. On day 15, surviving animals were sacrificed by excision of the femoral arteries, after i.p. overdosage anaesthesia with 5% sodium pentobarbital. All animals were necropsied and examined for gross pathology changes.

Dose (mg/kg bw)	Males	Female	Combined
2000	0/5	0/5	0/10

Statistics

The data did not warrant statistical analysis

RESULTS AND DISCUSSION

Mortality

No mortalities occurred at 2000 mg/kg bw, the only dose level tested.

The dermal LD_{50}	for males was	> 2000 mg/kg bw
	for females was	> 2000 mg/kg bw
	combined was	> 2000 mg/kg bw

Clinical observation

No general or local abnormalities were seen in any animal.

Body weight

Body weights of both males and females were found to be unaffected by the test item administration.

Necropsy

At autopsy carried out at the end of observation period no appreciable macroscopic findings were evident in any treated rat.

Deficiencies - None

CONCLUSIONS

The acute dermal LD_{50} of IR5885 was found to be higher than 2000 mg/kg bw. IR5885 does not warrant classification as being toxic or harmful on the basis of its acute dermal toxicity.

3.3 Acute toxicity - inhalation route

3.3.1 Animal data

3.3.1.1 Acute inhalation toxicity to rat – IR5885

Reference Number:	KII- Section 3, Volume 1, Annex IIA, point 5.2.3/01
Report:	See annex conf. 11
Guidelines:	OECD 403, EEC B.2
Deviation:	None
GLP:	Yes (certified laboratory)

Executive summary:

In an acute inhalation toxicity study groups of young adult Wistar rats, Han-Ibm (5/sex), were exposed for 4 hours by nose-only flow-past inhalation route to IR5885 (98.6% purity) at a concentration of 3.118 ± 0.045 mg/L air, the highest technically achievable concentration. Animals were observed during exposure and for the following 14 days.

There were no mortalities. During the last two hours of exposure all animals showed moderate salivation. No clinical signs were observed in all animals after exposure. Slight stagnation of body weight or slight body weight loss (in 1 male and 2 females) was observed between day 1 and day 4. No macroscopic findings were evident at necropsy. The acute inhalation LC_{50} value for Wistar Han-Ibm rats exposed to IR5885 resulted to be higher than the highest technically achievable aerosol concentration of 3.118 mg/L air (gravimetrically determined mean aerosol concentration).

MATERIALS AND METHODS

MATERIALS

Test Item	IR5885
Description	White powder
Lot/Batch #	FCF/T/180-00 (ex ZI 069)
Purity	98.6%
CAS #	283159-90-0
Stability of test compound	stable in the dark at room temperature and also for at least 14 days at 54°C
Vehicle and/or positive control:	0.9% (w/v) NaCl solution

Test animals –

Species:	Rat
Strain:	Wistar Han-Ibm outbread
Age:	6-8 weeks males, 10-12 weeks females
Weight at dosing:	240.5-254.6 males, 208.0-215.6 females
Source:	M & B A/S, P.O. box 39 - DK-8680 Ry, Denmark
Acclimation period:	6 days

Diet:	Kliba 3433 (Provini Kliba AG – Switzerland**)** *ad libitum,* except during exposure period
Water:	Tap water from FJllinsdorf, *ad libitum,* except during exposure period
Housing:	Individually during the 4-hour exposure period and then 5 animals/sex in cages with wire mesh top and standard softwood bedding
Environmental conditions -	
Temperature:	22 °C ± 3. During 4-hour exposure 21 ± 0.28 °C
Humidity:	30% to 70%. During 4-hour exposure 12.0% ± 12.92%
Air changes:	About 10 / hour
Photoperiod:	Alternating 12-hour light and dark cycles. Music played during light period

STUDY DESIGN AND METHODS

In life dates: March 22 – April 11, 2001

Animal assignment and treatment

Animals were assigned to the test group as listed in Table 5.2.3-01. Animals were observed for mortality approximately once per hour during exposure, once after exposure on the day of treatment and twice daily thereafter. Observations for signs of toxicity were conducted once per hour during exposure, once after exposure and once daily thereafter. Individual body weights were recorded just before exposure (on day 1), on day 4, 8 and 15. On day 15, surviving animals were sacrificed by exsanguination, after i.p. injection of NARCOREN at a dose of at least 2.0 mL/kg body weight (equivalent to at least 320 mg sodium pentobarbitone/kg bw).

Dose (mg/L air)	Males	Female	Combined
3.118	0/5	0/5	0/10

Generation of the test atmosphere / chamber description

Animals were confined separately in restraint tubes positioned radially around the exposure chamber. This ensures a uniform test item distribution and precludes re-breathing the exhaled air. The test item was generated using a CR 3020 powder generator feeding a micronizing Jet-Mill. The test atmosphere was sampled from an empty port of the exposure chamber. Gravimetric concentrations were performed four times during exposure using a glass fibre filter. Oxygen concentration was measured and recorded continuously during exposure using calibrated Oxopac RD device. Particle size distribution and Mass Median Aerodynamic Diameter (MMAD) were measured twice during exposure using a 7-stage cascade Mercer Impactor.

1st measure: MMAD 2.42 - Geometrical Standard Deviation 2.95
2nd measure: MMAD 2.45 - Geometrical Standard Deviation 2.89
% particles < 10 microns: 80
% particles < 1 micron: 20

Statistics

The data did not warrant statistical analysis

RESULTS AND DISCUSSION

Mortality

Details are provided in Table 5.2.3-01. No mortalities occurred at 3.118 mg/L, the only dose level tested.

The 4-hour inhalation LC_{50}	for males was	> 3.118 mg/L
	for females was	> 3.118 mg/L
	combined sexes was	> 3.118 mg/L

Clinical observation

During the last two hours of exposure all animals showed moderate salivation. After exposure no clinical signs were ever observed in all animals.

Body weight

Between days 1 and 4, slight stagnation of the body weight gain or even slightly body weight loss were noted in all animals. Thereafter the body weight gain in both males and females was considered to be normal.

Necropsy

At autopsy carried out at the end of the observation period no abnormal macroscopic findings were evident in any treated rat.

Deficiencies - None

CONCLUSIONS

The acute inhalation LC_{50} of IR5885 was found to be higher than 3.118 mg/L, the highest technically achievable concentration. IR5885 does not warrant classification as being toxic or harmful on the basis of its acute inhalation toxicity.

3.3.2 Human data

No human data available on acute inhalation toxicity

3.3.3 Other data

No other studies available on acute inhalation toxicity

3.4 Skin corrosion/irritation

3.4.1 Animal data

3.4.1.1 Skin irritation study on rabbits – IR5885

Reference Number:	KII- Section 3, Volume 1, Annex IIA, point 5.2.4/01
Report:	See annex conf. 33
Guidelines:	OECD 404, EEC B.4
Deviation:	None
GLP:	Yes (certified laboratory)

Executive summary:

In a primary dermal irritation study, 3 young adult New Zealand White rabbits (males) were exposed by dermal route to 0.5 g of IR5885 (98.6% purity) per animal. The test item was moistened with about 1 mL of water and then applied to a small area (approximately 6 cm^2) for 3 minutes, 1 hour (first rabbit) and 4 hours (all rabbits). Animals were observed for 72 hours. Irritation was scored using the Draize scheme.

Neither mortality nor adverse general clinical modifications were seen during the study. No changes were seen at any evaluation of the application sites. On the basis of this study, IR5885 does not warrant classification as being irritating to the skin.

MATERIALS AND METHODS

MATERIALS

Test Item	IR5885
Description	White crystalline solid
Lot/Batch #	FCF/T/180-00 (ex ZI 069)
Purity	98.6%
CAS #	283159-90-0
Stability of test compound	stable in the dark at room temperature and also for at least 14 days at 54°
Vehicle and/or positive control:	used as supplied by the Sponsor
Test animals –	
Species:	Rabbit
Strain:	New Zealand White
Age:	about 5 months
Weight at dosing:	2.7-3.2 kg
Source:	Charles River Italia S.p.A., Via Indipendenza, 11 – 23885 CALCO (Lecco) – Italy
Acclimation period:	> 5 days
Diet:	2 RB 15 GLP Certificate produced by Charles River, *ad libitum*
Water:	Filtered water from the municipal water main system, *ad libitum*
Housing:	Individually in T05C cages (metal cages with stainless steel feeders)
Environmental conditions -	
Temperature:	19 °C ± 2
Humidity:	55% ± 10
Air changes:	About 15 - 20 / hour filtered on HEPA 99.97%
Photoperiod:	12-hour cycle (7.00 a.m. – 7 p.m.)

STUDY DESIGN AND METHODS

In life dates: October 27, 2000 - March 23, 2001

Animal assignment and treatment

Approximately 24 hours before the test, fur was clipped from the trunk of the animals. 0.5 g of the test item were moistened with about 1mL of water and then applied to a small area (about 6cm^2) of skin. Firstly one rabbit was used and three treatment sites of the skin were prepared. On the first site the test item was applied for an exposure time of 3 minutes. Since no gross skin reactions were noted, the test item was applied for an exposure period of 1 hour on the second site. The application area was covered with a gauze patch. Since no gross reactions were observed after the 1-hour exposure period, the test item was applied for an exposure period of 4 hours on the third skin site and the test was completed using two additional animals. The application area was covered with a gauze patch. At the end of each exposure period residual test item was wiped off. Adjacent areas of untreated skin of each animal served as control. Treatment sites were examined

for signs of erythema and edema immediately following the 3 minutes and 1-hour exposure period. These sites were also evaluated after 72 hours. Following the 4-hour exposure period, animals were examined for erythema and edema at 60 minutes and 24, 48 and 72 hours after patch removal.

RESULTS AND DISCUSSION
Findings
No changes were seen at any of the skin evaluation of the test item application sites**.**

Table 3.4.1.1-01 Individual skin irritation scores according to Draize scheme (n.a. = not applicable)

	OEDEMA					ERYTHEMA				
Animal No.	85M			86M	88M	85M			86M	88M
Exposure period	3 min	1 h	4 h	4 h	4 h	3 min	1 h	4 h	4 h	4 h
Evaluation at:										
• Immediately	0	0	n.a.	n.a.	n.a.	0	0	n.a.	n.a.	n.a.
• 1 hour	n.a.	n.a.	0	0	0	n.a.	n.a.	0	0	0
• 24 hour	n.a.	n.a.	0	0	0	n.a.	n.a.	0	0	0
• 48 hours	n.a.	n.a.	0	0	0	n.a.	n.a.	0	0	0
• 72 hours	0	0	0	0	0	0	0	0	0	0

CONCLUSIONS

IR5885 was non-irritant to the rabbit skin. On the basis of this study IR5885 does not warrant classification as being irritating to the skin.

3.4.2 Human data

No human data available on skin corrosion/irritation.

3.4.3 Other data

No other data available on skin corrosion/irritation.

3.5 Serious eye damage/eye irritation

3.5.1 Animal data

3.5.1.1 Eye irritation study on rabbits – IR5885

Reference Number: KII- Section 3, Volume 1, Annex IIA, point 5.2.5/01

Report: See annex conf. 34

Guidelines: OECD 405, EEC B.5

Deviation: None

GLP: Yes (certified laboratory)

Executive summary:

In a primary eye irritation study, 0.1 g of IR5885 (98.6% purity) was instilled into the conjunctival sac of the right eye of 3 young adult New Zealand White male rabbits. The other untreated eye was used as control. Animals were observed for 72 hours. After 24-hour reading, the cornea was examined after the instillation of one drop of 1% sodium fluorescent and successive washing with sterile saline solution. For evaluation of results, the Guide issued by the U.S. Consumer Product Safety Commission (CPSC) was used as reference source.

Slight conjunctival redness (grade 1) was noted in all rabbits at the reading carried out 1 hour after application and subsided within 24 hours of treatment. No other concomitant or subsequent ocular changes were noted. Negative results were obtained at the fluorescent staining performed 24 hours after the test item application. On the basis of this study, IR5885 does not warrant classification as being eye irritant.

MATERIALS AND METHODS

MATERIALS

Test Item	IR5885
Description	White crystalline solid
Lot/Batch #	FCF/T/180-00 (ex ZI 069)
Purity	98.6%
CAS #	283159-90-0
Stability of test compound	stable in the dark at room temperature and also for at least 14 days at 54°C.
Vehicle and/or positive control:	used as supplied by the Sponsor
Test animals –	
Species:	Rabbit
Strain:	New Zealand White
Age:	about 5 months
Weight at dosing:	2.7-3.2 kg
Source:	Charles River Italia S.p.A., Via Indipendenza, 11 – 23885 CALCO (Lecco) – Italy
Acclimation period:	> 5 days
Diet:	2 RB 15 GLP Certificate produced by Charles River, *ad libitum*
Water:	Filtered water from the municipal water main system, *ad libitum*
Housing:	Individually in T05C cages (metal cages with stainless steel feeders)
Environmental conditions -	
Temperature:	9 °C ± 2
Humidity:	55% ± 10
Air changes:	About 15 - 20 / hour filtered on HEPA 99.97%
Photoperiod:	12-hour cycle (7.00 a.m. – 7 p.m.)

STUDY DESIGN AND METHODS

In life dates: October 27, 2000 to March 30, 2001

Animal assignment and treatment

The test item was placed into the conjunctival sac of the right eye of each animal, after gently pulling the lower lid away from the eyeball. The lids were then gently held together for about 3-4 seconds in order to prevent loss of material. The other eye, remaining untreated, served as control. 24 hours later, after the 24-hour reading, the cornea was examined after instillation of one drop of 1% sodium fluorescent and successive washing with sterile saline solution.

RESULTS AND DISCUSSION

Findings

Slight conjunctival redness (grade 1) was noted in all rabbits at the reading carried out 1 hour after application and subsided within 24 hours of treatment. No other concomitant or subsequent ocular changes were noted. Negative results were obtained at the fluorescent staining performed 24 hours after the test item application.

Table 3.5.1.1-01 Individual eye irritation scores according to CPSC

	Conjunctive-redness			**Conjunctive-chemosis**			**Cornea**			**Iris**		
Time / rabbit No.	40M	41M	42M	40M	41M	42M	40M	41M	42M	40M	41M	42M
60 minutes	1	1	1	0	0	0	0	0	0	0	0	0
1 hour	0	0	0	0	0	0	0	0	0	0	0	0
24 hours	0	0	0	0	0	0	0	0	0	0	0	0
72 hours	0	0	0	0	0	0	0	0	0	0	0	0

CONCLUSIONS

Slight transient conjunctival redness, confined to the 1-hour reading, was the only finding. On the basis of this study IR5885 does not warrant classification as an eye irritant.

3.5.2 Human data

No human data available on eye irritation.

3.5.3 Other data

No other data available on skin corrosion/irritation.

3.6 Respiratory sensitisation

There is no evidence that valifenalate is a respiratory sensitiser. In relation to any assessment of respiratory sensitisation from repeat dose studies, there are no relevant data.

3.6.1 Human data

No human data available on respiratory sensitisation.

3.6.2 Other data

No other data available on respiratory sensitisation.

3.7 Skin sensitisation

3.7.1 Animal data

3.7.1.1 Dermal sensitisation study on guinea pigs – IR5885

Reference Number: KII- Section 3, Volume 1, Annex IIA, point 5.2.6/01

Report:	See annex conf. 47

Guidelines:	OECD 406, EEC B.6
Deviation:	None
GLP:	Yes (certified laboratory)

Executive summary:

In a dermal sensitisation study, IR5885 (98.6% purity) in Freund's complete adjuvant (FCA) was tested using Albino Dunkin Hartley guinea-pigs (10 males). The treatment regime involved induction by intradermal injection on day 0, induction by topical application on day 6 and challenge by topical application on day 20.

No death occurred and no signs of general toxicity were observed in any animal. The body weight gain of the animals throughout the test was normal There was no dermal response to either induction or challenge applications. On the basis of this study, IR5885 does not warrant classification as being a dermal sensitizer.

MATERIALS AND METHODS

MATERIALS

Test Item	IR5885
Description	White crystalline powder
Lot/Batch #	FCF/T/180-00 (ex ZI 069)
Purity	98.6%
CAS #	283159-90-0
Stability of test compound	stable in the dark at room temperature and also for at least 14 days at 54°C
Vehicle and/or positive control:	Corn seed oil, Freund's complete adjuvant (FCA)
Test animals –	
Species:	Albino Guinea-pig
Strain:	Dunkin Hartley
Age:	about 5 weeks
Weight at dosing:	351 – 425 g
Source:	Charles River Italia S.p.A., Via Indipendenza, 11 – 23885 CALCO (Lecco) – Italy
Acclimation period:	5 days
Diet:	8 GP 22, produced by Charles River, *ad libitum*
Water:	Filtered water from the municipal water main system, *ad libitum*
Housing:	2/3 animals/cage in wire 40.5x38.5x18h cages with stainless steel feeders
Environmental conditions -	
Temperature:	22 °C ± 2
Humidity:	55% ± 15
Air changes:	About 15 - 20 / hour filtered on HEPA 99.97%
Photoperiod:	12-hour cycle (7.00 a.m. – 7 p.m.)

STUDY DESIGN AND METHODS

In life dates:	February 16 - April 6, 2001

Animal assignment and treatment

The treatment regime involved induction of sensitisation by intradermal injection on day 0, induction of sensitisation by topical application on day 6 and challenge by topical application on day 20. The test concentration for dermal and intradermal induction and challenge were selected following a preliminary irritancy testing. On day 5, after clipping the area destined to treatment, the sites were pre-treated with 10% sodium lauryl sulfate in vaseline oil to elicit some dermal response. Corn seed oil was used alone for intradermal induction and mixed to IR5885 to produce a 1% w/v mixture for intradermal induction. Freund's Complete Adjuvant (FCA) Emulsion was mixed 50% v/v in distilled water for intradermal induction and mixed with IR5885 to produce a 1% w/v mixture for intradermal induction. The test item was administered to

10 male Dunkin Hartley guinea-pigs at 5% for the intradermal induction, the highest technically administrable concentration via intradermal route, and at 10% for the topical induction, the highest concentration technically administrable by topical application.

RESULTS AND DISCUSSION

Findings

No deaths occurred and no signs of general toxicity were observed in any animal. No animals showed positive reactions to either the induction or challenge application. No skin reactivity was observed in the negative control group.

CONCLUSIONS

IR5885 did not exhibit dermal sensitisation potential in a Maximization test. On the basis of this study IR5885 does not warrant classification as being a skin sensitizer.

3.7.2 Human data

No human data available on skin sensitisation.

3.7.3 Other data

No other data available on skin sensitisation.

3.8 Germ cell mutagenicity

3.8.1 In vitro data

3.8.1.1 Bacterial assay for gene mutation – IR5885

Reference Number: KII- Section 3, Volume 5, Annex IIA, point 5.4.1/01

Report:	See annex conf. 53

Guidelines: OECD 471 and L 1362000 4D Commission Directive 2000/32/EC

Deviation: None

GLP: Yes (certified laboratory)

Executive summary:

In a reverse gene mutation assay in bacteria, strains TA98, TA100, TA102, TA1535, TA1537 of *Salmonella typhimurium* were exposed to IR5885 (98.9% purity) at concentrations of 33, 100, 333, 1000, 2500 and 5000 µg/plate in the presence and absence of metabolic activation, using dimethylsulfoxide (DMSO) as solvent This study was performed according to the plate incorporation method and the pre-incubation method. Each concentration, including the controls, was tested in triplicate.

IR5885 was tested up to the limit concentration of 5000 µg/plate. No toxic effects, evident as a reduction in the number of revertants, occurred in the test groups with and without metabolic activation. The plates incubated with the test item showed normal background growth up to 5000 µg/plate with and without S9-mix in all strains used. No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with IR5885 at any dose level, neither in presence nor in the absence of metabolic activation. There was also no tendency to higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance. Appropriate reference mutagens were used as positive controls and showed a distinct increase of induced revertant colonies. In conclusion, it can be stated that under the experimental conditions reported, IR5885 did not induce gene mutations by pair changes or frameshifts in the genome of the strains used. Therefore, IR5885 is considered non mutagenic in this *Salmonella typhimurium* mutation assay.

MATERIALS AND METHODS

MATERIALS:

Test Item: IR5885
Description: White powder
Lot/Batch #: FCF/T/180-00 (ex ZI 068)
Purity: 98.9%
CAS #: 283159-90-0
Stability of test item: Stable in the dark at room temperature and also for at least 14 days at 54°C.
Solvent used: dimethylsulfoxide (DMSO)

Control Materials –
Negative: concurrent untreated and solvent controls were tested.
Solvent/final conc.: DMSO/0.1 mL/plate
Positive controls: without S9mix:

Sodium azide (NaN_3)	10 µg/plate	TA1535 TA100
4-nitro-o-phenylene-diamine (4-NOPD)	10 µg/plate	TA98
	50 µg/plate	TA1537
methyl methane sulfonate (MMS)	5 µL/plate	TA102
with S9 mix:		
2-aminoanthracene (2-AA)	2.5 µg/ plate	TA98, TA100 TA1535 TA1537
	10 µg/plate	TA102

Activation: S9 obtained from male Wistar Hanlbm rats (Phenobarbital/β-Naphtoflavone induced rat liver). Each rat was induced by application of 80 mg/kg bw Phenobarbital i.p. and β napthoflavone p.o. for three consecutive days.

The rat liver S9 (Lot No. R261001; protein content 31.5 mg/mL) was prepared by RCC Cytotest Cell Research

S9-mix composition:

Component:	**Concentration**
Sodium-ortho-phosphate buffer (pH 7.4)	100 mM
Glucose-6-phosphate	5 mM
NADP	5 mM
KCl	33 mM
$MgCl_2$	8 mM
S9	15 % (v/v)

Test Organisms: *S. typhimurium* strains TA98, TA100, TA102, TA1535, TA1537 – test organisms were properly maintained and regularly checked for the properties regarding membrane permeability, ampicillin- and tetracycline-resistance as well as spontaneous mutation rates

Test concentrations:

(a) Preliminary cytotoxicity assay: One preliminary assay was performed:

Plate incorporation assay: 3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate were evaluated with and without S9 activation in *S. typhimurium* strain TA98 and TA100. Three plates were used, per dose, per condition

(b) Mutation assay:

Plate incorporation assay: 33, 100, 333, 1000, 2500 and 5000 µg/plate were evaluated in triplicate in the presence and absence of S9 activation in *S. typhimurium* strain TA98, TA100, TA102, TA1535 and TA1537.

Pre-incubation assay: as above for the plate incorporation assay.

STUDY DESIGN AND METHODS

The experimental work was conducted during the period November 15 to 30, 2001 by the RCC Cytotest Cell Research Gmbh, Rossdorf, Germany.

Preliminary cytotoxicity/plate incorporation mutation assay

The pre-experiment is reported as part of the main plate incorporation mutation assay.

The following materials were mixed in a test tube and poured onto the selective agar plates and then incubated at 37 °C for 48 hours in the dark:

- 0.1 mL aliquot of the appropriate test strain
- 0.1 mL of appropriate test material dose, positive controls or solvent
- 0.5 mL of the S9 mix (for tests with metabolic activation) or S9 mix substitution buffer (for tests without metabolic activation).
- 2.0 mL of Overlay agar

Pre-incubation assay

The independently repeated mutation assay was conducted using the pre-incubation modification to the standard plate incorporation test. The pre-incubation assay was carried out as described above with the following exceptions: everything but the overlay agar was added and mixed into the test tubes and incubated at 37 °C for 60 minutes. After pre-incubation period, 2.0 mL overlay agar (45 °C) was added to each tube.

Statistics

A statistical analysis of the data was not performed.

Evaluation criteria

A test item is considered as a mutagen if a biologically relevant increase in the number of revertants exceeding the threshold of twice (TA98, TA100, TA102) or three times (TA1535 and TA1537) the colony count of the corresponding solvent control is observed.
A dose-dependent increase is considered biologically relevant if the threshold is exceeded at more than one concentration.
An increase exceeding the threshold at only one concentration is judged as biologically relevant if reproduced in a second independent experiment.
A dose-dependent increase in the number of revertant colonies below the threshold is regarded as indication of mutagenic potential if reproduced in a second independent experiment. However, whenever the colony counts remain within the historical range of negative and solvent controls such an increase is not considered biologically relevant.

RESULTS AND DISCUSSION

Preliminary cytotoxicity

Eight doses of the test material ranging from 3 to 5000 µg/plate ± S9 were evaluated in the plate incorporation cytotoxicity test. No precipitation was observed up to the limit dose, 5000 µg/plate. No cytotoxicity was observed up to the highest dose tested (HDT) with and without S9. Hence, 5000 µg/plate was chosen as the HDT for mutagenicity testing using both the plate incorporation and pre-incubation methods.

Mutation assays

No toxic effects, evident as a reduction in the number of revertants, occurred in any test group with and without metabolic activation. The plates incubated with the test item showed normal background growth up to 5000 µg/plate.
No substantial increase in revertant colony numbers of any of the five tester strains was observed following the treatment with IR5885 at any concentration level, neither in presence nor in absence of metabolic activation (S9 mix). There was also no tendency to higher mutations rate with increasing concentrations in the range below the generally acknowledge border of biological relevance. In contrast, positive controls responded appropriately with a distinct increase in induced revertant colonies.

CONCLUSIONS

In conclusion, it can be stated that under the experimental conditions reported, IR5885 was not mutagenic since it did not induce gene mutations by base-pair changes or frameshifts in the genome of the *Salmonella typhimurium* strains used. Additionally, the sensitivity of both the plate incorporation and pre-incubation procedures to detect mutagenesis was adequately demonstrated by the results obtained with the positive controls.

3.8.1.2 Test for clastogenity in mammalian cells – IR5885

Reference Number: KII- Section 3, Volume 5, Annex IIA, point 5.4.2/01

Report: See annex conf. 41

Guidelines: OECD 473 and EEC B.10

Deviation: None

GLP: Yes (certified laboratory)

Executive summary:

In a mammalian cell cytogenetic assay, Chinese Hamster Ovary (CHO/D1) cells cultured *in vitro* were exposed to IR5885 (98.9 % w/w purity) in two independent experiments, with and without metabolic activation. Dimetylsulfoxide (DMSO) was used as solvent. The following experimental design was performed:

Table 3.8.1.2-01 Study design and respective concentrations applied (µg/mL)

	Without S9-mix			With S9-mix	
	Exp. I (50, 100, 200P)	Exp. II (37.5, 75, 100)	Exp. II (100)	Exp. I (100, 200, 400^{P})	Exp. II (50, 100, 200^{P})
Exposure period (h)	4	24	46	4	4
Recovery (h)	20	/	/	20	42
Preparation interval (h)	24	24	46	24	46

^{P}= precipitation occurred

Two parallel cultures were set up for each experimental group. Per culture, 100 metaphase plates were scored for structural chromosome aberrations. The highest applied concentration in the range-finding pre-test toxicity (1600 µg/mL) was chosen with regard to the solubility properties of the test item in DMSO. The concentrations to be used in the subsequent cytogenetic experiments were selected considering the toxicity data previously obtained and the occurrence of precipitation (Table 5.4.2-01).

In the cytogenetic experiments neither a reduced mitotic indices nor reduced cell numbers could be observed up to the highest concentration of test item evaluated, except in the experiment I, in absence of S9 at 200 µg/mL.
In both independent experiments, neither a statistically significant nor a biological relevant increase in cells carrying structural chromosome aberrations was observed after treatment with IR5885. No increase in polyploidy metaphases was found after treatment with the test item if compared to the frequencies of the controls. Positive controls induced the appropriate response.

In conclusion, it can be stated that under the experimental conditions reported, IR5885 did not induce structural chromosome aberrations as determined by the chromosome aberration test in CHO cells *in vitro*. Therefore, IR5885 is considered to be non-clastogenic in this chromosome aberration test in the absence and in the presence of S9-mix.

MATERIALS AND METHODS

MATERIALS:

Test Item:	IR5885
Description:	White powder
Lot/Batch #:	FCF/T/180-00 (ex ZI 068)
Purity:	98.9%
CAS #:	283159-90-0
Stability of test item:	Stable in the dark at room temperature and also for at least 14 days at 54°C.
Solvent used:	dimethylsulfoxide (DMSO)

Control Materials –		
Negative:	concurrent untreated and solvent controls were tested.	
Solvent/final conc.:	DMSO/0.5% (v/v)	
Positive controls:	without S9:	
	Ethylmethane sulfonate (EMS)	150 µg/mL (1.2 mM) Exp. II
		250 µg/mL (2.0 mM) Exp. I
	with S9:	
	Cyclophosphamide (CPA)	1 µg/mL (3.5 µM) Exp. I and II

Activation	S9 obtained from male Wistar Hanlbm rats (Phenobarbital/β Naphtoflavone induced rat liver). Each rat was induced by application of 80 mg/kg bw Phenobarbital i.p. and β-Napthoflavone p.o. for three consecutive days.

The rat liver S9 (Lot No. 270701; protein content 30 mg/mL) was prepared by RCC Cytotest Cell Research

S9 mix composition:	**Component:**	**Concentration**
	Sodium-ortho-phosphate buffer (pH 7.4)	100 mM
	Glucose-6-phosphate	5 mM
	NADP	5 mM
	KCl	33 mM
	$MgCl_2$	8 mM
	S9	0.75 mg/mL

Test Organisms:	CHO cells (supplied by laboratory for Mutagenicity Testing, LMP, Technical University Darmstadt) were stored in liquid nitrogen in the cell bank of RCC Cytotest Cell Research GmbH allowing a repeated use of the same batch in several experiments.
Culture medium	Minimal Essential Medium (MEM), supplemented with 10% foetal calf serum (FCS). Cells were incubated at 37 °C and 4.5% CO_2 and sub-cultured twice weekly.

Test item concentrations used

	Experiment I	Experiment II
	µg/mL	
Without S9-mix	50 – 100 – 200	37.5 – 75 – 100
With S9 mix	100 – 200 - 400	50 – 100 - 200

STUDY DESIGN AND METHODS

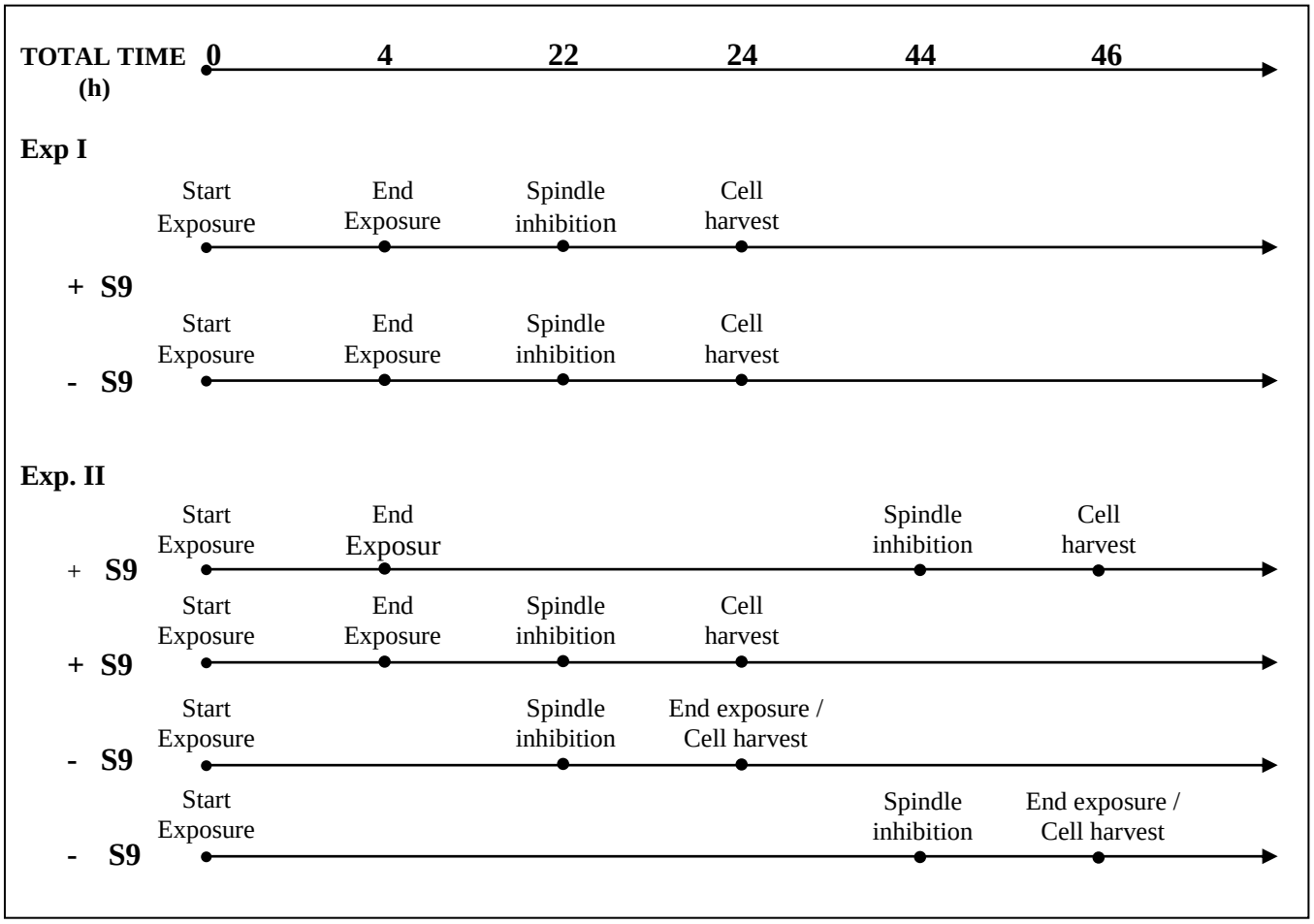
The experimental work was conducted during the period September 5, 2001 to January 25, 2002 by RCC Cytotest Cell Research GmbH, Rossdorf, Germany

Preliminary cytotoxicity/plate incorporation mutation assay

Cultures were exposed to a range of doses from 12.5 µg/mL to 1600 µg/mL for 4 hours ± S9-mix and for 24 hours without only S9-mix. The cytotoxicity was determined as reduction of % cells as compared to the control.

Cytogenetic assays:

Cell treatment: Cells were exposed to test item and positive/negative controls according to the following experimental design. Spindle inhibition was induced by introducing 0.2 µg/mL of colcemid solution into the cultures.



Slide preparation	Slides were prepared by dropping the harvested cultures on clean slides after ipotonic and fixative treatment. After preparation the slides were stained with Giemsa.
Metaphase analysis	Slides were coded prior to analysis. 100 well spread metaphase plates per culture were scored (200 per dose) for structural aberrations and numerical aberrations (polyploidy, where polyploid means a near tetraploid kariotype). Only metaphase with characteristic chromosome numbers of 21 ± 2 were included in the analysis.
Evaluation criteria	A test item is considered clastogenic if i) the number of induced structural chromosome aberrations in all evaluated dose groups is not in the range of performing laboratory's historical data. ii) either a concentration-related or a significant increase of the number of structural chromosome aberrations is observed. A test item can be classified as aneugenic if i) the number of induced numerical chromosome aberrations is not in the range of performing laboratory's historical data.
Definitions:	g=gap ig=iso-gap b=break f=fragment ib=iso-break if=iso-fragment d=deletion id=iso-deletion ma=multiple aberrations ex=chromatid type exchange cx=chromosome type exchange cd=chromosomal disintegration

Statistics

Data were evaluated for statistical significant at analysis at $p<0.01$ using Fisher's Exact test with adjustment for multiple comparison. However, both biological and statistical significance were considered together.

RESULTS AND DISCUSSION

In a range finding pre-test on toxicity, no clear toxic effects were observed after 4 hours of treatment up to the highest test item concentration in the absence and presence of S9-mix. 24 after continuous treatment with 100 µg/mL and above in the absence of S9-mix, strong toxicity effects were observed.
In the first experiment, precipitation of the test item in culture medium was observed 4 hours after start with 200 µg/mL and above with and without S9-mix. No influence of the test item on the pH value or osmolarity was observed.
In the cytogenetic experiments, neither reduced mitotic indices nor reduced cell numbers could be observed up to the highest evaluated concentrations of the test item except in experiment I, without S9 (Table 5.4.2-02 and Table 5.4.2-03), where the mitotic indices and cell numbers were reduced after treatment with 200 µg/mL (39% and 50% of control, respectively) at the 24 hours preparation interval. At 24 and 46 hours continuous treatment in the absence of S9-mix clear toxicity was observed at 100 µg/mL and above.

Table 3.8.1.2-02: Mitotic index (MI) and number of polyploid cells – Experiment I

Treatment group	WITHOUT S9-MIX exposure period 4 h					WITH S9-MIX exposure period 4 h				
	µg/mL	Mitotic index [A]		Polyploid cells[&]		µg/mL	Mitotic index [A]		Polyploid cells[&]	
		Mean [$] (%)	Relative MI (%)	Incidence mean [$]	Mean (%)		Mean [$] (%)	Relative MI (%) #	Incidence Mean [$]	Mean (%)
Negative control	/	11.1	100	29	2.9	/	6.9	100	37	3.7
Solvent control	/	9.9	100	22	2.2	/	5.0	100	31	3.1
Positive control @	250	7.6	68.3	26	2.6	1.0	5.8	84.1	39	3.9
IR5885	50	6.8	68.7	38	3.8	100	7.6	151.0	39	3.9
IR5885	100	5.0	50.5	36	3.6	200	5.5	110.0	21	2.1
IR5885	200	4.9	49.5	37	3.7	400	6.2	124.0	26	2.6

Table 3.8.1.2-03: Mitotic index (MI) and number of polyploid cells – Experiment II

Treatment group	WITHOUT S9-MIX exposure period 24 h					WITH S9-MIX exposure period 4 h				
	µg/mL	Mitotic index [A]		Polyploid cells[&]		µg/mL	Mitotic index [A]		Polyploid cells[&]	
		Mean [$] (%)	Relative MI (%)	Incidence mean [$]	Mean (%)			Mean [$] (%)	Relative MI (%)	Incidence mean [$]
Negative control		5.2	100	28	2.8		7.8	100	29	2.9
Solvent control		5.2	100	17	1.7		9.5	100	18	1.8
Positive control @	150	3.5	66.3	25	2.5	1.0	6.6	84	18	1.8
IR5885	37.5	3.2	61.5	18	1.8	50	5.9	62.4	24	2.4
IR5885	75	6.7	127.9	32	3.2	100	6.6	69.8	28	2.8
IR5885	100	4.7	89.4	31	3.1	200	5.6	58.7	31	3.1

Treatment group	WITHOUT S9-MIX exposure period 46 h				
	µg/mL	Mitotic index [A]		Polyploid cells[&]	
		Mean [$] (%)	Relative MI (%)		Mean [$] (%)
Negative control		5.1	100	9	1.8
Solvent control		8.2	100	7	1.4
Positive control @	150	3.8	74.3	11	2.2
IR5885	100	6.6	80.4	11	2.2

$ = these data are the means the values obtained in the two cultures tested for each concentration group.
& = the number of polyploid cells was determined of each test group in a sample of 500 cells per culture, except after 46 h continuous treatment 250 cells per culture were scored.

A = the mitotic index was determined in a sample of 1000 cells per culture of each test group in %.
@ =Positive Control was: without S9 mix Ethylmethane sulfonate (EMS) and with S9 mix Cyclophospamide (CPA).
# = for the positive control groups, the relative values of the mitotic index are related to the Negative controls, for the test item treatment groups the values are related to the Solvent controls.

In both cytogenetic experiments, in the presence and absence of S9-mix, no biologically relevant or statistically significant increase in the number of cells carrying structural chromosome aberrations was observed. The aberration rates of cells after treatment were near the range of control values and within the range of historical data. However, in absence of S9-mix after 24 continuous treatment a dose related increase in the number of cells carrying exchanges was observed at 37.5, 75 and 100 µg/mL (0, 0.5 and 1%, respectively). These values were in the range of historical control data and, therefore, this observation was regarded as biologically irrelevant.
In both experiments, no biologically relevant increase in the rate of polyploid metaphases was found after treatment with the test item as compared to the controls.
In both experiments, positive controls showed distinct increases in cells with structural aberrations.

CONCLUSIONS

In conclusion, it can be stated that in the study described under the experimental conditions reported, the test item IR5885 did not induce structural and numerical chromosome aberrations in CHO cells.

3.8.1.3 Test for gene mutation in mammalian cells – IR5885

Reference Number: KII- Section 3, Volume 5, Annex IIA, point 5.4.3/01

Report:	See annex conf. 54

Guidelines: OECD No. 476, and EEC B.17

Deviation: None

GLP: Yes (certified laboratory)

Executive summary:

In a mammalian cell cytogenetic assay, mouse lymphoma cell line was exposed to IR5885 (98.9 % w/w purity) up to 800 µg/mL in two independent experiments, using dimetylsulfoxide (DMSO) as solvent.
The first main experiment was performed with and without liver microsomal activation and for a period of 4 hours. The second main experiment was solely performed without metabolic activation with a treatment period of 24 hours. The highest applied concentration in the pre-test on toxicity (1600 µg/mL) was chosen with regard to the solubility properties of the test item in DMSO.

No substantial and reproducible dose-dependent increase in mutant colony numbers was observed in both main experiments. No relevant shift of the ratio of small versus large colonies was observed up to the maximal concentration of the test item.

Appropriate reference mutagens were used as positive controls and showed a distinct increase in induced mutant colonies, indicating sensivity and validity of the test system.
In conclusion it can be stated that under the experimental conditions reported the test item did not induce mutations in the thymidine kinase locus using the lymphoma cell line L5178Y with and without metabolic activation. Therefore, IR5885 is considered to be non mutagenic in this mouse lymphoma assay.

MATERIALS AND METHODS

MATERIALS:

Test Item:	IR5885
Description:	White powder
Lot/Batch #:	FCF/T/180-00 (ex ZI 068)
Purity:	98.9%
CAS #:	283159-90-0
Stability of test item:	Stable in the dark at room temperature and also for at least 14 days at 54 °C.
Solvent used:	dimethylsulfoxide (DMSO)

Control Materials –

Negative:	concurrent untreated and solvent controls were tested.	
Solvent/final conc.:	DMSO/0.5% (v/v)	
Positive controls:	without S9:	
	Methyl methane sulfonate (MMS)	13 µg/mL (0.12 mM)
	with S9:	
	3-methylcholanthrene (3-MC)	3 µg/mL

Activation S9 obtained from male Wistar Hanlbm rats (Phenobarbital/β Naphtoflavone induced rat liver). Each rat was induced by application of 80 mg/kg bw Phenobarbital i.p. and β-Napthoflavone p.o. for three consecutive days.

The rat liver S9 (Lot No. 270701; protein content 30 mg/mL) was prepared by RCC Cytotest Cell Research

S9-mix composition:

Component:	**Concentration**
Sodium-ortho-phosphate buffer (pH 7.4)	100 mM
Glucose-6-phosphate	5 mM
NADP	4 mM
KCl	33 mM
$MgCl_2$	8 mM
S9	0.75 mg/mL

Test Organisms: Prior to mutagenicity testing, the amount of spontaneous mutants is reduced by growing the L5178Y cell line in RPMI-HAT medium supplemented with hypoxantine, aminopterin and thymidine. This incubation is followed by a recovery period of 2 days in RPMI medium containing hypoxantine and thymidine. After this incubation cells are returned to normal RPMI 1640 complete medium.

Culture medium Roswell Park Memorial Institute (RPMI) 1640 medium**,** supplemented with 15% horse serum (HS), 100 U/100 µg/mL Penicillin/streptomycin, 220 µg/mL Sodium-Pyruvate and 1.25 U/mL amphotericin used as antifungal. Cells were incubated at 37 °C and 4.5% CO_2 and sub-cultured at least three times a week.

Test item concentrations applied:

	Experiment I (4 hours)	Experiment II (24 hours)
	µg/mL	
Non-Activated conditions	12.5* – 25.0 – 50.0 – 100.0 – 200.0P – 400.0P	25.0* – 50.0 – 100.0 – 200.0 – 400.0P – 800.0P
Activated conditions	12.5* – 25.0 – 50.0 – 100.0 – 200.0P – 400.0P	n.a.

* = culture was not continued since a minimum of four concentrations is required by the guidelines
P= precipitation occurred

METHODS:

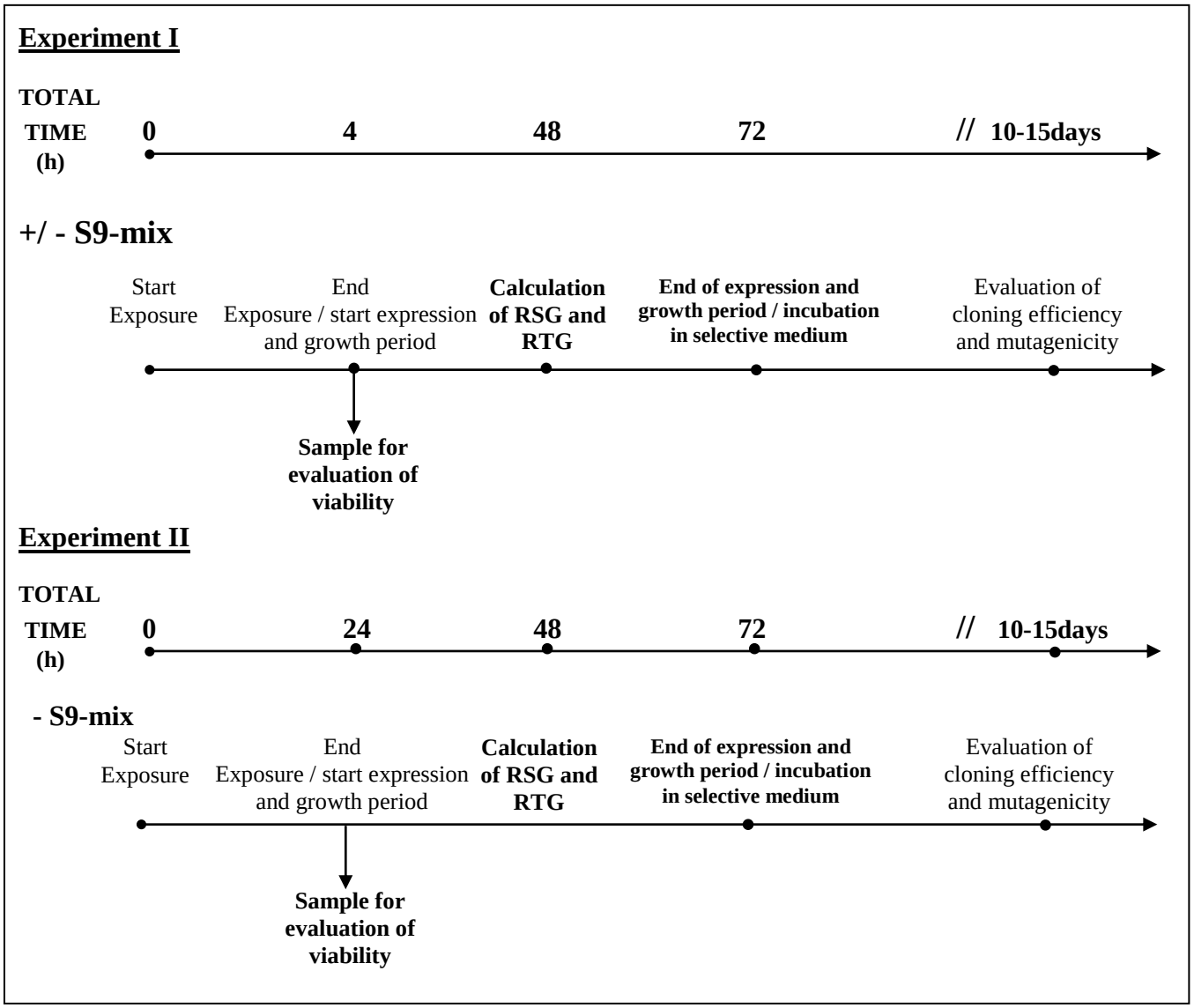
The experimental work was conducted between the period September 11, to November 5, 2001 by RCC Cytotest Cell Research GmbH, Rossdorf, Germany.

Preliminary cytotoxicity/plate incorporation mutation assay

Cultures were exposed to a range of doses from 12.5 µg/mL to 1600 µg/mL for 4 hours ± S9-mix and for 24 hours without S9-mix only. The cytotoxicity was determined as reduction of % cells as compared to the controls.

Cytogenetic assays:

Cell treatment: Cells were exposed to test compound, positive/negative controls according to the following experimental design. Selective medium was constituted by RPMI 1640 medium supplemented with trifluorothymidine (TFT) 5 µg/mL. Two parallel cultures were evaluated per test concentration and controls.



RSG: Relative Suspension Growth; RTG = Relative Total Growth

Evaluation criteria

The numbers of colonies are counted manually. In accordance with their size they are classified into two groups. The colony size distribution is determined in the controls and at all test item concentrations. A test item is considered positive if i) it induces either a reproducible concentration-related increase in the mutant frequency or a reproducible positive response for at least one of the test points. A significant response is described as follows: i) if the test item reproducibly induces with at least one of the concentrations a mutation frequency that is two times higher than the mean spontaneous mutation frequency in the experiment ii) if there is a reproducible concentration-related increase in the mutation frequency. Results of test groups are rejected if the RTG, RSG/or cloning efficiency 1 (evaluated immediately after treatment) is less than 10% of the solvent control or the cloning efficiency 2 (evaluated at the end of the expression period) is less than 20% of the respective control value.

Statistics

No statistical evaluation is required.

RESULTS AND DISCUSSION

In a range finding pre-test on toxicity, strong toxicity occurred at precipitating concentrations. The apparent reduced toxicity at the maximum concentration is considered due to erroneous determinations of the cell density caused by the heavy precipitation observed. Therefore, the concentration range of the main experiments was strongly limited by the low solubility of the test item. There was no shift in osmolarity and pH values of the medium even at the maximum concentration tested.
Precipitation occurred at 200 µg/mL and above in the firsts experiment with and without S9 and at 400 µg/mL and above in the second experiment.
In the first experiment relevant toxic effects reducing both RTG and survival were observed at the highest concentration of 400 µg/mL (both cultures) without S9. Only minor toxic effects occurred with S9. In the second experiment strong toxicity was also observed at the highest concentration tested in both cultures treated with 800 µg/mL. In one of these cultures, some data did not quite meet the acceptance criteria due to exceedingly severe toxic effects (RTG of only 4.2% versus a threshold of 10%). However, data are considered acceptable since the corresponding survival (relative cloning efficiency 1) was well above the limit of 10%.
No substantial and reproducible increase in mutant colony numbers was observed in both main experiments.
In this study, the range of the negative and vehicle controls was from 59 to 138 mutant colonies per 106 cells, the range of the groups treated with the test item was from 55 up to 184 mutant colonies per 106 cells.
MMS and 3-MC were used as positive controls and showed a distinct increase in induced total mutant colonies and an increase of small versus large colonies.

Table 3.8.1.3-01 Summary of results

Experiment I			Culture I				Culture II			
	Conc. (µg/mL)	S9 mix	Relative cloning efficiency 1	RTG	Mutant colonies/10^6 cells	Induction factor	Relative cloning efficiency 1	RTG	Mutant colonies/10^6 cells	Induction factor
Control		-	100.0	100.0	116		100.0	100.0	128	
DMSO		-	100.0	100.0	102	1.0	100.0	100.0	112	1.0
MMS	13	-	94.5	67.8	325	2.8	108.3	89.7	457	3.6
IR5885	12.5	-	86.4	Culture was not continued			108.4	Culture was not continued		
IR5885	25.0	-	97.1	118.4	139	1.4	124.7	65.7	137	1.2
IR5885	50.0	-	85.2	179.6	99	1.0	104.9	79.0	149	1.36
IR5885	100.0	-	94.3	158.0	75	0.7	103.2	73.5	110	1.0
IR5885	200.0	-	48.5	94.1	149	1.5	91.2	121.7	95	0.8
IR5885	400.0	-	20.1	38.6	80	0.8	54.3	44.7	137	1.2
Control		+	100.0	100.0	138		100.0	100.0	87	
DMSO		+	100.0	100.0	120	1.0	100.0	100.0	115	1.0
3-MC	3.0	+	95.5	68.6	294	2.4	97.0	73.4	257	2.2
IR5885	12.5	+	103.2	Culture was not continued			88.8	Culture was not continued		
IR5885	25.0	+	98.5	83.7	115	1.0	97.0	117.2	74	0.6
IR5885	50.0	+	111.8	139.8	115	1.0	123.2	93.0	119	1.0
IR5885	100.0	+	95.5	106.8	110	0.9	90.1	123.6	107	0.9
IR5885	200.0	+	75.6	108.8	138	1.1	85.0	69.4	93	0.8
IR5885	400.0	+	55.3	65.2	184	1.5	87.5	113.8	92	0.8
Experiment II			**Culture I**				**Culture II**			
Control		-	100.0	100.0	62		100.0	100.0	93	
DMSO		-	100.0	100.0	59	1.0	100.0	100.0	78	1.0
MMC	13.0	-	12.8	35.2	268	4.3	27.6	50.4	444	4.8
IR5885	25.0	-	277.2	Culture was not continued			83.2	Culture was not continued		
IR5885	50.0	-	152.7	75.5	58	1.0	90.2	147.6	75	1.0
IR5885	100.0	-	189.0	79.8	55	0.9	112.0	147.6	94	1.2
IR5885	200.0	-	310.8	66.4	68	1.2	67.3	154.8	104	1.3
IR5885	400.0	-	118.1	45.7	86	1.5	77.0	120.3	78	1.0
IR5885	800.0	-	24.0	4.2	94	1.6	42.6	56.2	73	0.9

CONCLUSIONS

In conclusion, it can be stated that in the study described under the experimental conditions reported, the test item IR5885 did not induce mutations in the thymidine kinase locus assay using the lymphoma cell line L5178Y with and without S9. Therefore, IR5885 is considered to be non mutagenic in this mouse lymphoma assay.

3.8.2 Animal data *In vivo* genotoxicity testing (somatic cells)

3.8.2.1 Metaphase analysis in rodent bone marrow, or micronucleus test in rodents – IR5885

Reference Number:	KII- Section 3, Volume 5, Annex IIA, point 5.4.4/01
Report:	See annex conf. 20
Guidelines:	OECD No. 474, ECC 2000/32 4C – B12, OPPTS 870.5395.
Deviation:	The experimental part with the female mice was repeated. In the scoring of the repeated experiment the reserve animals were mistakenly also evaluated. Therefore the number of animals tested per dose group was not 10 (5 males and 5 females), but 11 (5 males and 6 females). This deviation was not considered to have affected the validity of the study.
GLP:	Yes (certified laboratory)

Executive summary:

In a bone marrow micronucleus assay using CD-1 mice, IR5885 was administered by oral gavage to groups of male and female animals at doses of 500, 1000 and 2000 mg/kg bw for the 24 hours preparation interval and 2000 mg/kg bw for the 48 hours preparation interval. The highest dose (2000 mg/kg bw) was estimated by a pre-experiment to be suitable, causing no death but only slight clinical signs. The test item was formulated in corn oil, which was also used as vehicle control. Eleven animals (5 males, 6 females) per test group were evaluated for the occurrence of micronuclei after a single administration of the test item. At least 2000 polychromatic erythrocytes (PCEs) per animal were scored. For each sample, cytotoxic effect due to the treatment with the test item was evaluated by calculating the ratio between polychromatic and total erythrocytes and reported as PCEs/2000 erythrocytes.

After treatment with the test item the number of PCEs was not substantially decreased as compared to the mean value of PCEs of the vehicle control thus indicating that IR5885, did not exert any cytotoxic effects in the mouse bone marrow. In comparison to the corresponding vehicle controls there was no biologically relevant or statistically significant enhancement in the frequency of the detected micronuclei at any preparation interval after administration of IR5885 and at any dose level used.

40 mg/kg bw cyclophosphamide administered orally was used as positive control and showed a substantial increase of induced micronucleus frequency.

In conclusion, it can be stated that under the experimental conditions reported, IR5885 is considered to be non-mutagenic in this *in vivo* micronucleus assay.

MATERIALS AND METHODS

MATERIALS:

Test Item:	IR5885
Description:	White powder
Lot/Batch #:	T 025/02
Purity:	99.56%
CAS #:	283159-90-0
Stability of test item:	Stable in the dark at room temperature and also for at least 14 days at 54 °C.
Solvent used:	Corn oil (Sigma-Aldrich)

Control Materials –	
Negative:	None
Solvent/final conc.:	Corn oil (volume 10 mL/kg)
Positive controls:	Cyclophosphamide (CPA) – Sigma-Aldrich – 40 mg/kg bw by gavage (10 mL/kg bw)
Test animals –	
Species:	Mouse
Strain:	CD-1
Age:	8-10 weeks
Acclimatisation	At least 5 days
Weight at dosing	29.0 g ± 2.8 males; 27.1 g ± 2.0 females.
Number of animal	11 (5 males and 6 females) / dose / sacrifice time
Husbandry	Individually, in Makrolon Type I cages, with wire mesh top. Mice received pellet standard diet (ALTROMIN 1234) and tap water, *ad libitum*

Test item concentration:

Pre-Experiment for Toxicity:	2000 mg/kg bw IR5885 in corn oil administered by means of oral gavage to 4 animals (2 males, 2 females).
Micronucleus assay:	0, 500, 1000 and 2000 mg/kg bw/day for the 24 hours preparation interval and 2000 mg/kg bw for 48 the 48 hours preparation interval

METHODS

The experimental work was conducted between the period January 25, to March 07, 2003 by RCC Cytotest Cell Research GmbH, Rossdorf, Germany

Treatment and sampling time

Sampling took place at 24 and 48 hours after the single dose was administered. The positive control was sampled only at 24 hours.

Tissues and cells examined

Bone marrow; at least 2000 polychromatic erythrocytes (PCEs) examined per animal; the ratio between polychromatic and total erythrocytes was determined in each sample and expressed as polychromatic erythrocytes per 2000 total erythrocytes. The analysis was performed with coded slides.

Details of slide preparation

At 24 hours (also 48 hours for 2000 mg/kg bw) after the administration of test item or vehicle control, animals were sacrificed by cervical dislocation. The femora were removed, the epiphyses were cut off and the marrow was flushed out with faecal calf serum, using a syringe. Cells suspension was centrifuged and supernatant was discarded. A small drop of the resuspended cell pellet was spread on a slide. The smear was air-dried and then stained with May-Grünwald/Giemsa. Cover slips were mounted with EUKITT.

Evaluation criteria

A test item is classified as mutagenic if it induces either a dose-related increase or a clear increase in the number of micronucleated polychromatic erythrocytes in a single dose group. A test item that fails to produce a biological relevant increase in the number of micronucleated polycromatic erythrocytes is considered non-mutagenic in this system.

Statistical methods

Statistical methods (non-parametric Mann-Whitney test were used as an aid in evaluating the results. However, the primary point of consideration was the biological relevance of the results.

RESULTS AND DISCUSSION

Pre-experiment toxicity

In a pre-experiment 4 animals (2 males, 2 females) received orally single dose of 2000 mg/kg bw IR5885 formulated in 10 mL/kg corn oil. The animals treated with the up-mentioned dosage expressed toxic reactions as shown in the table.

Table 3.8.2.1-01 Pre-experiment toxicity

Toxic reactions	Hours post-treatment (male / female)					
	1	2-4	6	24	30	48
Ruffled fur	0/0	0/0	2/0	2/0	2/0	0/0

Micronucleus assay

Toxicity and mortalities

No death occurred. Clinical signs were observed as described in the Table below.

Table 3.8.2.1-02 Toxicity

Toxic reactions	Dose (mg/kg bw) No. of animals	Hours post-treatment (h) Male / female				
		1	2-4	6	24	48
Reductions of spontaneous activity	500 6/6	0/2	0/1	0/0	0/0	n.a.
	1000 6/6	0/4	1/3	2/2	0/0	n.a.
	2000 12/12	2/5	4/6	5/5	0/0	n.a.
Eyelid closure	1000 6/6	0/2	0/1	0/1	0/0	n.a.
	2000 12/12	0/3	0/4	0/2	0/0	n.a.
Ruffled fur	1000 6/6	0/4	2/3	1/2	0/0	n.a.
	2000 12/12	2/5	8/6	3/4	0/0	n.a.

n.a. = not applied

PCE ratio

There was no evidence of target cell cytotoxicity at any dose level or sacrifice time. The PCE/total erythrocytes ratio was not increased after treatment with the test item as compared to the mean value of this value for the vehicle control (Table 5.4.4-03)

Micronucleated polychromatic erythrocytes (MPCEs)

In comparison to the corresponding vehicle controls there was no statistically significant or biologically relevant enhancement in the frequency of the detected micronuclei at any preparation interval and dose level after administration of the test item. The mean values of micronuclei observed after treatment with IR5885 were below or near to the value of the control group.
40 mg/kg cyclophosphamide administered orally was used as positive control and it showed a statistically significant increase of induced micronucleus frequency.

Table 3.8.2.1-03 Summary of results

Test group	Animals scored	Dose (mg/kg bw)	Sampling time (h)	PCEs with micronuclei (%)	Range	PCE / 2000 erythrocytes
Vehicle	11	0	24	0.036	0-2	1103
IR5885	11	500	24	0.068	0-5	1104
IR5885	11	1000	24	0.045	0-3	1048
IR5885	11	2000	24	0.073	0-3	1073
Positive control	11	40	24	0.995*	7-34	1042
IR5885	11	2000	48	0.032	0-2	1009

* $p<0.0001$ by Mann-Whitney test

CONCLUSIONS

In conclusion, it can be stated that during the study described and under the experimental conditions reported, IR5885 is considered to be non-mutagenic in this micronucleus assay.

3.8.3 Human data

No human data available on Germ cell mutagenicity

3.8.4 Other data

No other data available on Germ cell mutagenicity

3.9 Carcinogenicity

3.9.1 Animal data

3.9.1.1 Combined long-term and carcinogenicity study – IR5885

Reference Number:	KII- Section 3, Volume 5-6-7, Annex IIA, point 5.5.1/01
Report:	See annex conf. 51
Guidelines:	OECD 453 – US OPPTS 870.4300
Deviation:	None
GLP:	Yes (Certified laboratory)

Executive summary:

The carcinogenic and toxic potential of the fungicide IR5885, given by dietary administration to Han Wistar rats over a period of 104 and 52 weeks, respectively, was assessed in this study. Three groups of 50 male and 50 female rats received the test item at concentrations designed to achieve dosages of 15, 150 or 1000 mg/kg bw/day for 104 weeks. Together with a similarly constituted group that received the vehicle (untreated diet), these animals comprised the Carcinogenicity phase of the study. A further 20 male and 20 female rats assigned to each group were sacrificed after completion of 52 weeks of treatment and comprised the Toxicity phase of the study.
During the study, clinical condition, detailed physical and arena observations, sensory reactivity, grip strength, motor activity, bodyweight, food consumption, water consumption, ophthalmic examination, haematology, blood chemistry, urinalysis, organ weight, macropathology and histopathology investigations were undertaken. Mortality was unaffected by treatment.
Slight increased incidence of yellow staining in the perigenital area was observed in females receiving 1000 mg/kg bw/day. Brown urine staining on the cage paper was observed in males and females at this dosage, up to Week 23
The group distribution, multiplicity and mean onset time of palpable swellings were unaffected by treatment.
There was no evidence of neurotoxicity from arena observations or assessment of sensory reactivity and grip strength. Animals receiving 1000 and (to a lesser extent) 150 mg/kg bw/day showed slightly increased motor activity scores during Week 50.
Overall body weight gain was slightly low in males receiving 150 or 1000 mg/kg bw/day assigned to the Carcinogenicity phase and food consumption was slightly low during the first two weeks of treatment in males receiving the two highest doses.
There were no treatment-related ophthalmic findings.
Hematological investigations indicated persistently low hemoglobin concentration in males receiving 1000 mg/kg bw/day. In Week 13 and 26 this finding was associated with reduced erythrocytes counts and mean cell hemoglobin concentration. Hematocrit, hemoglobin concentration and erythrocyte counts were slightly low during Weeks 13, 26 and 52, respectively, also in females receiving 1000 mg/kg bw/day.
Examination of tail vein smears taken from Carcinogenicity phase animals did not reveal any treatment-related change.
There were no toxicological changes in the blood plasma.
Urinalysis investigations revealed some alterations in pH, ketones (in males) and urine volume (in females) treated at the two highest concentrations, which resulted to be slightly higher than respective control values. Specific gravity and protein concentration, in general, resulted to be slightly lower in females at 150 and 1000 mg/kg bw/day during Week 25 whereas a slight increase in males treated at 1000 mg/kg bw/day during weeks 25 and 51 was observed. The appearance and composition of urine during weeks 77 and 103 were unaffected by treatment.

Slight high absolute and bodyweight-relative liver weight was registered in both Carcinogenicity and Toxicity animals treated at 1000 mg/kg bw/day. Bodyweight-relative higher kidney weights were also observed in Toxicity phase males treated at 1000 mg/kg bw/day.
Macroscopic examinations after 52 and 104 weeks of treatment did not reveal any findings related to treatment.
There were no neoplastic changes due to treatment with IR5885.
Histopathology examinations performed in animals killed after 52 weeks of treatment indicated an increased incidence of pelvic/papillary epithelial hyperplasia in the kidney of females at 1000 mg/kg bw/day and follicular cell hypertrophy in the thyroids of males receiving 1000 mg/kg bw/day. This latter change was not present in Carcinogenicyty phase animals.
It is concluded that oral administration of IR5885, a fungicide, via the diet to Han Wistar rats for 104 weeks at concentrations designed to achieve dosages of up to 1000 mg/kg bw/day was generally well tolerated, with no evidence of significant toxicity or any increase in tumour incidence. The thyroid and kidney were identified as target organs. Based on the above-reported findings, the NOAEL in this study was considered to be 150 mg/kg bw/day in males and 1000 mg/kg bw/day in females.

MATERIALS AND METHODS

MATERIALS:

Test Item:	IR5885
Description:	White powder
Lot/Batch #:	T 025/02 (Total amount of test item was deliver in two times)
Purity:	99.56% (weeks 1-103) – 99.63% (weeks 104-106)
CAS #:	283159-90-0
Stability of test item:	Stable in the dark at room temperature
Vehicle:	None
Test animals –	
Species:	Rat
Strain:	Han Wistar
Age:	26-30 days at arrival; 37-41 at beginning of dosing
Weight:	109-150 g males; 90-122 g females
Source:	Harlan UK Ltd, Bicester, Oxfordshire, England.
Acclimation period:	11 days
Diet:	Rat and mouse No.1 Maintenance Diet, *ad libitum*
Water:	Tap water from the municipal water main system, *ad libitum*
Housing:	Five per sex/cage. Cages were made of stainless steel body with a stainless steel mesh lid and floor.
Environmental conditions -	
Temperature:	21 °C ± 2
Humidity:	55% ± 15
Air changes:	Periodically (frequency not specified)
Photoperiod:	12-hour light : 12-hour dark cycle (7 a.m. – 7 p.m.)

STUDY DESIGN AND METHODS:

In life dates:	February 28, 2002 to March 18, 2004

Animal assignment and treatment

As shown in table 3.9.1.1-01, IR5885 was administered over a period of 52 consecutive weeks to animals assigned to the Toxicity phase and 104 consecutive weeks to animals assigned to the Carcinogenicity phase.

On completion of the respective phases the animals were sacrificed. The animal numbering system of the Toxicity phase was such that it was not easy to identify a treatment group from the animal number.

Table 3.9.1.1-01 Study design

Test group	Toxicity phase (52 weeks)		Carcinogenicity phase (104 weeks)		Dosage (mg/kg bw/day)
	Males	Females	Males	Females	
1	20	20	50	50	0
2	20	20	50	50	15
3	20	20	50	50	150
4	20	20	50	50	1000

Test item formulate preparation and analysis

IR5885 was incorporated into the diet to provide the required concentration by dilution of an appropriate premix. All formulated diets were prepared freshly each week, with diets for males and females being prepared separately. The concentrations for each group were adjusted at the same frequency as food and bodyweight recordings were made in order to achieve the required dosage in terms of mg/kg bw/day.
Two primary premixes were prepared in order to cover the preparation of the high and low concentration diets. For each premix the required amount of test item was gradually mixed with half the required final weight of diet, which was finely sieved and finally mixed in a coffee grinder. The resultant mix was diluted with coarse diet to achieve the final weight and blended in a Turbula mixer for 100 revolutions. All formulations were stored at room temperature. The unused residue at the end of each week was discarded for all groups.
The suitability of the mixing procedure and the homogeneity and stability of the test material in the diet matrix following room temperature storage for 22 days and frozen storage (-20 °C) for 21 days, were demonstrated at concentrations of 50 and 20000 ppm (Report No. IGA 012/013038). Further homogeneity and stability assessments were performed on the current study at a dietary concentration of 30000 ppm to cover the range of concentrations expected to be used in this study. The homogeneity was assessed as analysed concentration at the top, middle and bottom of the freshly prepared diet formulation. The stability was assessed on samples taken from the homogeneity mix and was reported as the concentration of samples taken at intervals during ambient temperature storage up to 22 days. Each homogeneity and stability sample taken on day 8 and 22 was analysed in duplicate. The precision of the duplicate analyses of each sample and of the homogeneity samples was expected to be within 5%. The accuracy of each formulation was expected to be within –15% and +10% of nominal concentrations.

Statistics

All statistical analyses were carried out separately for males and females and considering the individual animal as the basic experimental unit. For categorical data, including pathological findings, the proportion of animals was analysed using Fisher Exact test for each treated group versus control.
For continuous data, Bartlett's test was first applied to test the homogeneity of variance among the groups. Using tests dependent on the outcome of Bartlett's test, treated groups were then compared to the control group, incorporating adjustment for multiple comparisons where necessary. For bodyweight gains and organ weights, whenever Bartlett's test was found to be statistically significant, a Behrens Fisher test was used to perform pairwise comparisons, otherwise a Dunnett's test was used.
Significant differences between control and treated groups were expressed at the 5% ($p<0.05$), 1% ($p<0.01$) or 0.1% ($p<0.01$) level. The following statistical ciphers were used throughout the report:
a – $p<0.05$; b – $p<0.01$ – using categorical or parametric tests
A – $p<0.05$; B – $p<0.01$ – using non-parametric tests
Inter-group differences in mortality and tumour incidence were performed using the approach of Peto *et al*. (British J. Cancer 29 (1974) pp. 101-105).

METHODS:

Mortality and clinical observations

Animals were inspected visually at least twice a day for evidence of ill-health or reaction to treatment. Cages were inspected daily for evidence of any abnormality, such as loose faeces. Before treatment commenced and weekly thereafter a detailed physical examination, which included palpation, was performed on all animals to monitor general health.
Debilitated animals were observed carefully and, where necessary, isolated to prevent cannibalism. Where possible, blood smears were taken *ante mortem* from the Carcinogenicity phase animals.

Detailed physical examination

For Toxicity phase animals, arena observations were performed each week in addition to the physical examination, at approximately the same time of a day by an observer unaware of the experimental group to which the animal belong. Particular attention was paid to possible signs of neurotoxicity such as convulsion, tremor and abnormalities of gait or behaviour.

Sensory reactivity and grip strength

During Week 50 of treatment sensory reactivity and grip strength assessment was performed on the 10 males and 10 females with the lowest animal numbers in each group of the Toxicity phase by an observer unaware of the experimental group to which the animal belonged. The following measurements, reflexes and responses were recorded: approach response – grip strength - auditory startle reflex – tail pinch response – touch response.

Motor activity

During Week 50 of treatment the motor activity of the 10 males and 10 females with the lowest animal numbers in each group of the toxicity phase was measured using an automated equipment. Activity measurements were recorded for ten six-minute periods (a total of one hour).

Bodyweight

The weight of each rat was recorded on the day that treatment commenced (Week 0), at weekly intervals for the first 16 weeks, and once every four weeks thereafter (in coincidence with the end of a food consumption measurement period) and before necropsy.

Food consumption

The weight of food supplied to each cage, that remaining and an estimate of any spilled was recorded weekly for the first 16 weeks and every four week throughout the remaining treatment period. From these records the mean weekly consumption per animal (g/rat/week) was calculated for each cage.

Water consumption

During Week 24 of treatment, water consumption was recorded by weight (over a 3-day period) for each cage of animals, using water bottles fitted with sipper tubes.

Ophthalmic examination

Before treatment commenced, the eyes of all animals allocated to the study (including spare animals) were examined by means of a binocular indirect ophthalmoscope. Rejected animals were replaced with animals with no adverse ocular abnormality. During Week 51 of treatment the eyes of all animals of groups 1 and 4 assigned to the Toxicity phase were similarly examined.

Hematology, peripheral blood

During Weeks 13, 26 and 52 of treatment, blood samples were obtained from all surviving Toxicity phase animals after overnight starvation. Similarly, during Weeks 78 and 104 of treatment, blood samples were obtained from the 10 males and 10 females from each group with the highest animal numbers assigned to the Carcinogenicity phase. Animals were held under light general anaesthesia induced by isofluorane and blood samples were withdrawn from the retro-orbital sinus. Blood samples were collected into EDTA as anticoagulant and examined for the following characteristics: haematocrit (Hct), haemoglobin concentration (Hb), erythrocyte count (RBC), total and differential leucocyte count (WBC), platelet count (Plt), mean cell haemoglobin concentration (MCHC), mean cell haemoglobin (MCH), mean cell volume (MCV). Additional blood sample was taken into citrate anticoagulant and examined in respect of prothrombin time (PT) and activated partial thromboplastin time (APTT).

Hematology, blood smears

During Week 52, 78 and 104 of treatment, blood smear preparations were prepared from tail vein samples obtained without the use of anaesthesia from all surviving animals assigned to the Carcinogenicity phase that were not used for routine blood sampling. The smears of groups 1 and 4 were examined for: differential leukocyte count, abnormal morphology and unusual cell types, including normoblasts.

Blood chemistry

During weeks 26 and 52 of treatment, further blood samples were collected from the 10 males and 10 females from each group with the highest animal numbers assigned to the Toxicity phase. During week 78 and 104 of treatment blood samples were collected from the 10 males and 10 females from each group with the highest animal numbers assigned to the Carcinogenicity phase. Samples were taken after overnight starvation from the retro-orbital sinus under light anaesthesia induced by isofluorane and were collected into tubes containing lithium heparin as anticoagulant. After separation the plasma was analysed for: alkaline phosphatase (ALP), alanine amino-transferase (ALT), aspartate amino-transferase (AST), gamma glutamyl transpeptidase (gGT), creatine phosphokinase (CPK), glucose concentration (Gluc), total bilirubin concentration (Bili.), total cholesterol concentration (Chol.), creatinine concentration (Creat), urea concentration, total protein concentration (Total Prot), albumin (Alb), albumin/globulin ratio (A/G Ratio), sodium (Na), potassium (K) and chloride (Cl), calcium concentration (Ca Total) and inorganic phosphorus (Phos).

Urinalysis

During Week 12, 25 and 51 of treatment overnight urine samples were collected from the 10 males and 10 females from each group with the highest animal numbers assigned to the Toxicity phase. During Weeks 77 and 103 of treatment, samples were collected from the 10 males and 10 females from each group with the highest animal numbers assigned to the carcinogenicity phase. The individual samples were examined for the following characteristics: appearance (App), volume (Vol), pH, specific gravity (SG), sodium (Na), potassium (K) and chloride (Cl), glucose (Gluc), ketones (Keto), bile pigments (Bili), blood. The sediment from centrifugation was examined microscopically for epithelial cells (Epi), leukocytes (Leuc), erythrocytes (RBC), crystals (Cryst), casts, spermatozoa and precursors (Sperm) or other abnormalities (Abn).

Necropsy and histology

Animals killed during the study and those surviving until the end of the scheduled Toxicity or Carcinogenicity phase were killed by carbon dioxide asphyxiation.
All animals were subjected to a detailed necrospy. After a review of the history of each animal, a full macroscopic examination of the tissues was performed. The requisite organs were weighed.

Organ weights – The following organs, taken from each animal killed after 52 or 104 weeks of treatment, were weighed: adrenals, brain, epididymides, heart, kidneys, liver, ovaries, spleen, testes, thymus, thyroid with parathyroids, uterus with cervix.

Histology – For those animals specified in the Pathology section, the relevant tissues were subjected to histological processing. Tissues and samples were dehydrated, embedded in paraffin wax, sectioned at approximately four to five micron thickness and stained with haematoxylin and eosin. Those tissues subject to histological processing included the following regions: adrenals (cortex and medulla) – brain (cerebellum, cerebrum and midbrain) – femur with joint (bone marrow, epiphysal plate and longitudinal section including articular surface) – heart (auricular and ventricular regions) – kidneys (cortex, medulla and papilla regions) – liver (section from all main lobes) – lungs (section from two major lobes, to include bronchi) – spinal cords (transverse and longitudinal section at the cervical, lumbar and thoracic levels) – sternum (included bone marrow) – stomach (included keratinised, glandular and antrum in sections) – thyroid (included parathyroids in section, where possible) – uterus (uterus section separate from cervix section).

Pathology

All tissues preserved for examination were examined for all Toxicity and Carcinogenicity phase animals of Groups 1 and 4 sacrificed on completion of the scheduled 52 or 104 weeks treatment period and for all animals killed or dying during these periods. The kidneys, liver and lungs were examined for all Toxicity and Carcinogenicity phase animals and the thyroid (with parathyroid) was examined for all Toxicity phase males of groups 2 and 3 sacrificed on completion of the scheduled 52-week treatment period. Tissues reported at macroscopic examination as being grossly abnormal were examined for all animals.

RESULTS AND DISCUSSION

Formulation chemistry:

The test material was found to be homogeneous and stable in the diet matrix at a concentration of 30000 ppm for up to 22 days at room temperature.

The mean concentrations of IR5885 in test formulations analysed during the study were generally within ± 9% of nominal concentrations. Three deviations from acceptable limits (-15% / +10%) occurred in Week 65 when the values for Group 2F and Group 3M and 3F were 10.2, 11.0 and 11.2% above the nominal concentration, respectively. The small isolated variations were not considered to indicate any inherent problems in the diet preparation procedure. Consequently, these results confirmed the accuracy of formulation.

Mortality

A total of six animals assigned to the Toxicity phase died or were killed during the 52-week treatment period. There were no treatment-related findings observed at necropsy or at histopathology examination. These deaths were considered incidental to treatment.

A total of 44 males and 55 females assigned to the Carcinogenicity phase died or were killed during the 104-week treatment period. The group distribution of deaths was unaffected by treatment.

Table 3.9.1.1-02 Premature deaths

Group/sex	1M	2M	3M	4M	1F	2F	3F	4F
Dosage (mg/kg bw/day)	**0**	**15**	**150**	**1000**	**0**	**15**	**150**	**1000**
Toxicity phase								
Group size	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**
Total number of death	1	1	0	0	0	0	1	3
% Survival	95	95	100	100	100	100	95	85
Carcinogenicity phase								
Group size	**50**	**50**	**50**	**50**	**50**	**50**	**50**	**50**
Total number of death	17	7	9	11	13	16	11	15
% Survival	66	86	82	78	74	68	78	70

Detailed physical and arena observations

A slightly increased incidence of yellow staining in the perigenital area was observed in females receiving 1000 mg/kg bw/day.
Brown staining on the cage paper was observed between Weeks 4 and 23 in males receiving 1000 mg/kg bw/day. The staining was identified as being dark between Week 4 and 8. From Week 9 it was described as light brown. Females receiving 1000 mg/kg bw/day had dark brown staining of the cage tray paper in Weeks 4 and 5 only. There were occasional observations of light brown staining on the cage tray paper of low and intermediate dosage animals between Weeks 13 and 23.
Arena observations were unaffected by treatment.

- **Palpable swelling**

Palpable swelling was unaffected by treatment.

- **Motor Activity – Sensory reactivity - Grip strength**

Animals receiving 1000 mg/kg bw/day and, to a lesser extent, those receiving 150 mg/kg bw/day, showed slightly increased motor activity scores during Week 50, when compared to the Controls. Sensory reactivity and grip strength were unaffected by treatment.

Bodyweight

The overall bodyweight gain (Weeks 0 to 104) was low for Carcinogenicty phase males receiving 150 or 1000 mg/kg bw/day (92 and 91% of Controls, respectively), though only the difference at the highest dosage attained statistical significance ($p<0.05$). Female bodyweights were unaffected by the treatment. There was no clear effect of treatment on the Toxicity phase animals.

Food consumption and food conversion efficiency

Food consumption was slightly low, when compared to the control values, during the first two weeks of treatment in males receiving 1000 mg/kg bw/day. However, overall food consumption values (Weeks 1 to 104) were similar for Control and treated animals.
Food conversion efficiency during the first 16 weeks of treatment was unaffected by the treatment.

Achieved dosages and water consumption

The overall achieved dosages were within ±5% of the nominal dosage and were therefore considered satisfactory. The weekly values were generally within ±10% of the target dosages and were also considered satisfactory. Occasional excursions outside this range occurred but in view of their isolated nature they were not considered significant. Water consumption investigation, during Week 24 did not reveal any effect of treatment.

Ophthalmic examination

There were no ophthalmic findings related to treatment.

Hematology

Hematological investigations indicated persistently low hemoglobin concentration in males receiving 1000 mg/kg bw/day. In Week 13 and 26 this finding was associated with reduced erythrocytes counts and mean cell hemoglobin concentration. Hematocrit, hemoglobin concentration and erythrocyte counts were slightly low during Weeks 13, 26 and 52, respectively, also in females receiving 1000 mg/kg bw/day, although these differences were less consistent than in males. Other parameters were found to be slightly different from their respective control values. However, due to their isolated occurrence and/or persisting in time as well as due to a lack of any-dose relationship, these findings, although sometimes statistically significant were considered not to be related to the treatment.

Hematology, blood smears

Examination of tail vein smears prepared in Week 52, 78 or 104 of treatment from all Carcinogenicity phase animals did not reveal any changes considered to be related to treatment.

Blood chemistry

There were no toxicological significant changes in the biochemical composition of the blood plasma. Reduction in plasma alkaline phosphatase as well as plasma glucose concentration in females or slight increase in plasma cholesterol concentration in males occurred throughout the study but, in view of the small and inconsistent nature of these differences and/or the absence of a clear trend with dosage, they could not be attributed to the treatment.

Urinalysis

Urinalysis revealed, when compared to the Controls, a generally slightly high urine volume, low specific gravity and low protein concentration for females receiving 1000 and (to a lesser extent) 150 mg/kg bw/day during investigations performed in Week 12, 25 and 51. Conversely, specific gravity and protein concentration were slightly high in males receiving 1000 mg/kg bw/day during Week 25 (and also Week 51 for protein concentration, only). A slight increase in ketones in the urine, when compared to the Controls, was observed during Weeks 25 and 51 in males receiving 1000 mg/kg bw/day.
The appearance and composition of urine during Weeks 77 and 103 were unaffected by treatment.

Organ weights

Analysis of organ weights in animals killed after 52 weeks of treatment revealed, when compared to controls, slightly high absolute and bodyweight-relative liver weights in males and females receiving 1000 mg/kg bw/day. Bodyweight-relative kidney weights were also marginally higher than those of controls for males at the highest dosage.
After 104 weeks of treatment, slightly high absolute and bodyweight-relative liver weights, when compared to the Controls, were observed in males and females, which received 1000 mg/kg bw/day. However, these differences were minimal, with only the bodyweight-relative values attaining statistical significance.
All other inter-group differences were minor, lacked dosage-relationship and were therefore attributed to normal biological variation.

Table 3.9.1.1-03 Liver and kidney absolute and bodyweight-relative weights

	Group		1M	2M	3M	4M	1F	2F	3F	4F
	Dosage (mg/kg/day)		**0**	**15**	**150**	**1000**	**0**	**15**	**150**	**1000**
Toxicity Phase (Week 52)	**Liver**									
		Absolute	14.04	14.37	14.50	16.05*	8.54	8.66	9.00	9.53*
		bw-relat.	2.890	3.093	3.095	3.441**	3.196	3.178	3.301	3.587**
	Kidney									
		Absolute	2.28	2.28	2.28	2.35	1.64	1.62	1.63	1.58
		bw-relat.	0.472	0.490	0.490	0.508*	0.617	0.599	0.598	0.595
Carcinogen. Phase (Week 104)	**Liver**									
		Absolute	16.27	15.46	15.55	16.71	11.08	10.90	10.76	11.90
		bw-relat.	2.876	2.805	2.906	3.161**	3.203	3.071	3.083	3.446**
	Kidney									
		Absolute	2.78	2.76	2.70	2.72	1.98	2.03	1.96	2.03
		bw-relat.	0.491	0.490	0.517	0.519	0.577	0.574	0.563	0.592

* p<0.05; ** p<0.01 when compared to control groups

Macroscopic pathology

Macroscopic examination at necrospy in animals killed after 52 and 104 weeks of treatment did not reveal any findings that were considered to be related to treatment with IR5885. All findings were minor, confined to one sex or lacked dosage-relationship and were therefore attributed to normal biological variation.
After 52 weeks of treatment, a high incidence of thin optic nerves was apparent among treated males. For all animals affected the finding was observed in the right eye only and was, therefore, attributed to the retro-orbital sinus blood sampling procedure.

Microscopic pathology

Toxicity phase – treatment related changes were confined to the thyroids where there was an increased incidence of follicular cell hypertrophy in male rats, which received IR5885 at 1000 mg/kg bw/day, as shown in table 5.5.2-04

Table 3.9.1.1-04 Group incidence of changes in the thyroids (Toxicity phase)

Group/sex		1M	2M	3M	4M
Dosage (mg/kg bw/day)		**0**	**15**	**150**	**1000**
Follicular cell hypertrophy	**Total**	**3**	**2**	**5**	**11***
	Slight	3	2	5	10
	Moderate	0	0	0	1
Number of animal examined		20	20	20	20

* p < 0.05 when compared to Group 1M

Follicular cell carcinoma was seen in the thyroids of a single male rat at 1000 mg/kg bw/day and follicular cell adenoma was seen in the thyroids of a single male rat at 150 mg/kg bw/day. In the absence of any increase in the incidence of these tumours in the Carcinogenicity phase of this study, they were considered fortuitous.

Carcinogenicity phase :

Neoplastic findings – There were no treatment-related changes. There were no statistically significant differences from Control with respect to tumour incidences in treated groups.

Non-neoplastic findings – There was an increased incidence of pelvic/papillary epithelial hyperplasia in the kidney of females at 1000 mg/kg bw/day (table 3.9.1.1-05).

Table 3.9.1.1-05 Group incidence of changes in the kidney (Carcinogenicity phase)

Group/sex		**1F**	**2F**	**3F**	**4F**
Dosage (mg/kg bw/day)		**0**	**15**	**150**	**1000**
Pelvic/papillary epithelial hyperplasia	**Total**	**9**	**10**	**5**	**25****
	Slight	7	7	4	17
	Moderate	2	3	1	7
	Marked	0	0	0	1
Number of animal examined		50	50	50	50

** $p<0.01$ when compared to Group 1F

All other changes observed in the Carcinogenicity phase of this study were of the type normally encountered in Wistar rats at the performing laboratories.

CONCLUSIONS

The oral administration of IR5885, a fungicide, via the diet to Han Wistar rats for up to 104 weeks at concentrations designed to achieve dosages of up to 1000 mg/kg bw/day was generally well tolerated, with no evidence of significant toxicity or any increase in tumour incidence. The thyroid and kidney were identified as target organs.

This investigation met all the requirements of a satisfactory carcinogenicity study. Survival, which was not affected by treatment with IR5885, was good, with at least 66% of males and 68% of females surviving in each group up to 104 weeks. The slightly low overall bodyweight gain for males at the two highest dosages suggested slight non-specific toxicity.

The changes observed in this study indicated that the only finding of possible toxicological significance was the slight anemia observed at the highest dosage in males, though as it is considered a secondary change to an effect upon thyroid hormone and therefore probably not relevant to man. In addition, changes in the kidney function were attributed to the excretion of IR5885.

Based on these findings, the NOAEL in this study was considered to be 150 mg/kg bw/day in males and 1000 mg/kg bw/day in females.

3.9.1.2 Carcinogenicity study by dietary administration to mouse – IR5885

Reference Number:	KII- Section 3, Volume 7-8, Annex IIA, point 5.5.3/01
Report:	See annex conf. 52
Guidelines:	OECD 451 – US OPPTS 870.4200
Deviation:	Weight of two animals at commencement of treatment was marginally outside the 20% of mean weight for each sex. This minor deviation of a maximum 0.6 g from the 20% bodyweight range for two out of 400 animals did not have any effect on the integrity of the study.
GLP:	Yes (Certified laboratory)

Executive summary:

The carcinogenic potential of the fungicide IR5885 to Crl: CD-1™ (ICR) BR mice by dietary administration was assessed over a period of 78 weeks. Three groups of 50 male and 50 female mice received IR5885 orally, via the diet, at concentrations of 150, 850 and 5000 ppm. A similarly constituted Control group received the vehicle, untreated diet, throughout the treatment period. During the study, clinical condition, bodyweight, food consumption, hematology, organ weight, macroscopic and microscopic pathology investigations were undertaken.

Mortality was not affected by treatment with the test item as well as the appearance and behaviour of treated animals.
Overall bodyweight gain was low and food consumption was high in males receiving 5000 ppm; bodyweight was unaffected in females.
Overall achieved dosages at 150, 850 and 5000 ppm were 16.8, 97.2 and 657 mg/kg bw/day in males and 21.6, 124 and 756 mg/kg bw/day in females, respectively.
Examination of tail vein smears revealed no changes attributable to treatment.
Liver weights were high in animals that received 850 and 5000 ppm and in males treated at 150 ppm.
Kidney weights were slightly high in females receiving 5000 ppm.
Macroscopic examination at necrospy revealed high incidence of liver masses, pale areas and accentuated lobular pattern on the liver in males that received 850 and 5000 ppm and a slightly high incidence of dark areas on the liver in females receiving the highest dose.
An increased incidence of hepatocellular adenoma was observed in animals treated at 850 or 5000 ppm and hepatocellular carcinoma in males receiving 5000 ppm.
Non-neoplastic histological changes in the liver comprised increased centrilobular and/or generalised hepatocyte hypertrophy in males receiving 150 ppm or above and in females receiving 850 ppm or above; increased cytoplasmic eosinophilia and increased pigment in macrophages and hepatocytes in animals receiving the highest dosage; increased eosinophilic foci and increased incidence of choleliths in the gall bladder in females treated at 5000 ppm; centrilobular vacuolation in males, which received 850 ppm or above.
It is concluded that oral administration of IR5885, via the diet, to CD-1 mice for 78 weeks at dietary concentrations of 150, 850 and 5000 ppm was generally well tolerated. The liver was identified as the target organ. There was an increased incidence of hepatocellular tumours in animals receiving 850 or 5000 ppm, which were considered secondary to adaptive metabolic changes. Such findings are observed commonly in mice and are generally considered of no significance for man. There were no toxicological findings in animals receiving 150 ppm and, consequently, this dietary concentration is set as the NOAEL for this study (equivalent to 16.8 mg/kg bw/day in males and 21.6 mg/kg bw/day in females).

MATERIALS AND METHODS

MATERIALS:

Test Item: IR5885
Description: White powder
Lot/Batch #: T 025/02
Purity: 99.56%
CAS #: 283159-90-0
Stability of test item: Stable in the dark at room temperature

Vehicle: None

Test animals –
Species: Mice
Strain: Crl:CD-1™ (ICR) BR
Age: 26-30 days at arrival; 40-44 at beginning of dosing
Weight: 28.9-41.3 males; 19.8-29.9 females
Source: Charles River Ltd, Margate, England (UK)
Acclimation period: 14 days
Diet: Rat and mouse No.1 Maintenance Diet, *ad libitum*
Water: Tap water from the municipal water main system, *ad libitum*
Housing: Two per sex/cage. Cages were made of stainless steel body with a stainless steel mesh lid and floor.

Environmental conditions -
Temperature: 21 °C ± 2
Humidity: 55% ± 15
Air changes: Periodically (frequency not specified)
Photoperiod: 12-hour light : 12-hour dark cycle

STUDY DESIGN AND METHODS:

In life dates: April 03, 2002 to April 16, 2004
October 22, 2003 (necropsy completed)

Animal assignment and treatment

IR5885 was administered to mice over a period of 78 consecutive weeks. The necropsy procedure was completed in eight days, during which time treatment continued and serial observations were recorded at appropriate intervals. The duration of treatment is reported as 78 weeks.

Table 3.9.1.2-01 Study design

Test group	Dosage (ppm)	No. of animals	
		Females	Females
1	0	50	50
2	150	50	50
3	850	50	50
4	5000	50	50

Test item formulate preparation and analysis

IR5885 was incorporated into the diet to provide the required concentration by initial preparation of a premix at a concentration of 40000 ppm. The required amount of IR5885 to prepare the premix was added to an equal amount of sieved diet and stirred. The doubling-up process was repeated until approximately half the premix diet was added. At this stage mixture was ground with a coffee grinder. The mixture was made up to the final weight of the premix with coarse diet and then was mixed using a Turbula mixer for 100 revolutions. The premix was diluted with further quantities of coarse diet to prepare the highest concentration test mix (5000 ppm) and a second premix (4000 ppm). The second premix was used to prepare the intermediate and lowest concentration test mixes (850 and 150 ppm). All formulations were prepared freshly each week and stored at ambient temperature. The unused residue at the end of each week was discarded for all groups.
The suitability of the proposed mixing procedure and the homogeneity and stability of the test material in the diet matrix as well as the linearity of response, precision of inspection, limit of detection, method accuracy and precision were already assessed at concentrations of 50 and 20000 ppm in an associated validation study (IGA 012/013038). It was demonstrated that the test item was homogenously distributed in diet mixes and it was stable during frozen storage for up to 21 days and at ambient temperature storage for 22 days.
Sample of each formulation prepared for administration in Weeks 1, 13, 26, 39, 52, 65 and 77 of treatment were analysed for achieved concentration of the test item. Each sample was analysed in duplicate according to analytical procedure. The accuracy of formulation was expected to be within +10%/-15% of nominal concentrations and the precision of duplicate samples was expected to be within ±5%.

Statistics

All statistical analyses were carried out separately for males and females and considering the individual animal as the basic experimental unit. For categorical data, including pathological findings, the proportion of animals was analysed using Fisher Exact test for each treated group versus control.
For continuous data, Bartlett's test was first applied to test the homogeneity of variance among the groups. Using tests dependent on the outcome of Bartlett's test, treated groups were then compared to the control group, incorporating adjustment for multiple comparisons where necessary. For bodyweight gains and organ weights, whenever Bartlett's test was found to be statistically significant, a Behrens Fisher test was used to perform pairwise comparisons, otherwise a Dunnett's test was used.
Significant differences between control and treated groups were expressed at the 5% ($p<0.05$), 1% ($p<0.01$) or 0.1% ($p<0.01$) level. The following statistical ciphers were used throughout the report:

* – $p<0.05$; ** – $p<0.01$ – using categorical or parametric tests
\# – $p<0.05$; ## – $p<0.01$ – using non-parametric tests

Inter-group differences in mortality and tumour incidence were performed using the approach of Peto *et al*. (British J. Cancer 29 (1974) pp. 101-105).

METHODS:

Mortality and clinical observations

Animals were inspected visually at least twice a day for evidence of ill-health or reaction to treatment. Cages were inspected daily for evidence of any abnormality. Before treatment commenced and weekly thereafter a detailed physical examination, which included palpation, was performed on all animals to monitor general health.
Debilitated animals were observed carefully and, where necessary, isolated to prevent cannibalism. Animals judged *in extremis* were killed.

Bodyweight

The weight of each mouse was recorded on the day treatment commenced (Week 0), at weekly intervals for the first 14 weeks of treatment, and once every four weeks thereafter (in coincidence with the end of a food consumption measurement period) and before necropsy.

Food consumption

The weight of food supplied to each cage, that remaining and an estimate of any spilled was recorded weekly for the first 14 weeks and every four weeks throughout the remaining treatment period. From these records the mean weekly consumption per animal (g/mouse/week) was calculated for each cage.

Haematology, blood smears

During Weeks 52 and 78 of treatment, blood smear preparations were prepared from tail vein samples obtained without the use of anaesthesia from all surviving animals. The smears were fixed and stained using Romanowsky stain. The smears of groups 1 and 4 were examined for differential leukocyte count, abnormal morphology and unusual cell types.

Necropsy and histology

Animals killed during the study and those surviving until the end of the scheduled treatment period were killed by carbon dioxide asphyxiation.
All animals were subjected to a detailed necrospy. After a review of the history of each animal, a full macroscopic examination was performed. The requisite organs were weighed.

Organ weights – The following organs, taken from each animal killed after 78 weeks of treatment, were weighed: adrenals, brain, epididymides, heart, kidneys, liver, ovaries, spleen, testes, uterus with cervix.

Histology – For those animals specified in the Pathology section, the relevant tissues were subjected to histological processing. Tissues and samples were dehydrated, embedded in paraffin wax, sectioned at approximately four to five micron thickness and stained with haematoxylin and eosin. Those tissues subject to histological processing included the following regions: adrenals (cortex and medulla) – brain (cerebellum, cerebrum and midbrain) – femur with joint (bone marrow, epiphysal plate and longitudinal section including articular surface) – heart (auricular and ventricular regions) – kidneys (cortex, medulla and papilla regions) – liver (section from all main lobes) – lungs (section from two major lobes, to include bronchi) – spinal cords (transverse and longitudinal section at the cervical, lumbar and thoracic levels) – sternum (including bone marrow) – stomach (including keratinised, glandular and antrum in sections) – thyroid (including parathyroids in section, where possible) – uterus (uterus section separate from cervix section).

Pathology

All tissues preserved for examination were examined for all animals of Groups 1 and 4 sacrificed on completion of the scheduled 78 weeks treatment period and for all animals killed or dying during this period. The kidneys, liver and lungs were examined for all animals of Groups 2 and 3 sacrificed on completion of the scheduled 78-week treatment period. Tissues reported at macroscopic examination as being grossly abnormal were examined for all animals.

RESULTS AND DISCUSSION

Formulation chemistry

The mean concentrations of IR5885 in diet preparations for Weeks 1, 13, 26, 39, 52, 65 and 77 were between nominal and 10.0% above nominal values, which were within applied limits +10%/-15%, confirming the accuracy of formulation. The precision of individual analyses for each sample was <5%, with the exception of one minor deviation.

Mortality and clinical observations

A total of 66 males and 50 females died or were killed during the 78-week treatment period. Statistical analysis of mortality revealed no significant differences between Control and treated animals.
There were no macroscopic findings at necropsy, which were considered to be related to treatment.

Table 3.9.1.2-02 Premature deaths

Group/sex	1M	2M	3M	4M	1F	2F	3F	4F
Dosage (ppm)	**0**	**150**	**850**	**5000**	**0**	**150**	**850**	**5000**
Group size	50	50	50	50	50	50	50	50
Total number of death	15	20	13	18	11	10	14	15
% Survival	70	60	74	64	78	80	72	70

The appearance and behaviour of animals were unaffected by treatment.
The group distribution, multiplicity and mean onset time of palpable swelling were unaffected by treatment.

Bodyweight

The overall bodyweight gain (Weeks 0 to 78) was low in males receiving 5000 ppm (78% of the Control value; $p<0.05$). The difference from control values was a consequence of a particularly poor rate of growth from Week 14, where these animals gained only 65% of the weight of Control animals.
Bodyweight gain in animals receiving 150 or 850 ppm and in females receiving 5000 ppm was unaffected by treatment.

Food consumption, food conversion efficiency and achieved dosages

Food consumption in males receiving 5000 ppm was higher than that of controls (110% of control animals value, overall).
Food consumption in animals receiving 150 or 850 ppm and in females receiving 5000 ppm was unaffected by treatment.
Overall food conversion efficiency in males receiving 5000 ppm was slightly lower than that of Control for the first 14 weeks of treatment.
Food conversion efficiency in animals receiving 150 or 850 ppm and in females receiving 5000 ppm was unaffected by treatment.
Group mean achieved dosages for the treatment period were as shown in the following table.

Table 3.9.1.2-03 Achieved dosages

Test group	Test item concentration in the diet (ppm)	Achieved dosages (mg/kg bw/day)	
		Males	Females
1	0	0	0
2	150	16.8	21.6
3	850	97.2	124
4	5000	657	756

Hematology, tail vein smears

Examination of tail vein smears taken during Weeks 52 and 78 revealed no clear treatment-related changes.
Lymphocytes counts were slightly low in Week 52 in females receiving 5000 ppm and slightly high in males receiving the same dosage during Week 78. Neutrophil counts were slightly low in Week 78 in males

receiving the highest dosage. In each case, however, the inter-group difference was very small and inconsistent between the sexes and occasions and, therefore, was considered to reflect normal biological variation.

Organ weights

Analysis of organ weights in animals killed after 78 weeks of treatment revealed, when compared to Controls, high absolute and bodyweight-relative liver weights in males and females receiving 850 and 5000 ppm and in males receiving 150 ppm. Males were affected to a greater extent than females. Absolute and bodyweight-relative kidney weights were also marginally higher than those of the Control in females at the highest dosage. All other inter-group differences attaining statistical significance were present in one sex only, lacked dosage-relationship and were therefore attributed to normal biological variation.

Table 3.9.1.2-04 Liver and kidney absolute and bodyweight-relative weights

Group		**1M**	**2M**	**3M**	**4M**	**1F**	**2F**	**3F**	**4F**
Dosage (mg/kg/day)		**0**	**15**	**150**	**1000**	**0**	**15**	**150**	**1000**
Liver									
	Absolute	2.772	3.258	3.568*	4.851**	1.974	2.100	2.434	2.407**
	bw-relat.	4.8072	5.5221	6.1833**	9.4698**	4.6851	4.4816	5.4149	5.7675**
Kidney									
	Absolute	0.985	1.047	0.945	0.956	0.522	0.521	0.589	0.580**
	bw-relat.	1.7414	1.7639	1.6404	1.8604	1.2597	1.1384*	1.3718	1.4094*

* p<0.05; ** p<0.01 when compared to control groups

Macroscopic pathology

When all animals were considered together, macroscopic examination at necropsy revealed a high incidence of liver masses, pale areas and accentuated lobular pattern on the liver in males receiving 850 or 5000 ppm. A slightly high incidence of dark areas on the liver was observed in females receiving the highest dosage.
All other findings were of the type encountered normally in CD-1 mice at the performing laboratories.

Microscopic pathology:

Treatment-related findings

Neoplastic findings – An increased incidence of hepatocellular tumours was observed in males and females given IR5885 at 850 or 5000 ppm.

Table 3.9.1.2-05 Incidence of hepatocellular tumours and respective historical control data ranges

Group/sex		**1M**	**2M**	**3M**	**4M**	**Historic. Range (%)**	**1F**	**2F**	**3F**	**4F**	**Historic. Range (%)**
Dosage (ppm)		**0**	**150**	**850**	**5000**		**0**	**150**	**850**	**5000**	
Liver											
	Hepatocell. adenoma	7	2	14	16*	7.8 – 21.2	0	0	2	5*	0.0 – 1.9
	Hepatocell. carcinoma	2	4	4	10*	1.9 – 8.0	0	1	0	0	0.0
Number of animals examined		50	50	50	50	307	50	50	50	50	307

* p < 0.05 when compared to Group 1

Hepatocellular tumours are relatively common in male CD-1 mice, however the incidence of these tumours in males and females given 850 and 5000 ppm exceeded the background range seen in similarly performed studies at the performing laboratories. The incidence of adenoma was 28 and 8%, respectively, in males given 850 and 5000 ppm. The incidence of carcinoma was 32 and 20%, respectively, in males receiving 850 and 5000 ppm. In females, the incidence of adenoma was 4 and 10% at 850 and 5000 ppm, respectively. The singular incidence of a hepatocellular carcinoma in a female given 150 ppm falls outside the range of historical data. However, a singular incidence of this tumour has been reported in a control group from a study previously performed. This indicates that this is a rare tumour, which can however occur spontaneously in CD-1 mice.

Non-neoplastic findings – Findings considered to be related to treatment were seen in the liver and gall bladder.
Liver findings are shown in the table below. The apparent reduction in the incidence of centrilobular hepatocyte hyperthrophy in males given 850 ppm or above is related to the increase in the incidence of generalised hepatocyte hypertrophy in the same groups.
Gall bladder findings attaining statistical significance were limited to an increased incidence of choleliths in females given 5000 ppm. This finding was considered to be secondary to the changes seen in the livers of these animals.

Table 3.9.1.2-06 Group distribution and severity of treatment related liver findings

Group/sex		**1M**	**2M**	**3M**	**4M**	**1F**	**2F**	**3F**	**4F**
Dosage (ppm)		**0**	**150**	**850**	**5000**	**0**	**150**	**850**	**5000**
Centrilobular hepatocyte hypertrophy	**Total**	**21**	**34***	**26**	**8****	**8**	**9**	**12**	**25****
	Slight	19	21	18	5	8	8	12	22
	Moderate	2	13	8	2	0	1	0	3
	Marked	0	0	0	1	0	0	0	0
Generalised hepatocyte hypertrophy	**Total**	**3**	**6**	**13***	**29****	**2**	**2**	**7**	**5**
	Slight	3	5	10	18	2	2	6	5
	Moderate	0	1	3	11	0	0	1	0
Centrilobular hepatocyte vacuolation	**Total**	**11**	**14**	**33****	**32****	**2**	**8**	**8**	**2**
	Minimal	3	4	2	0	2	1	4	1
	Slight	7	7	20	11	0	7	4	0
	Moderate	1	3	11	20	0	0	0	1
	Marked	0	0	0	1	0	0	0	0
Increased hepatocytic cytoplasmic eosinophilia		**0**	**1**	**1**	**29****	**1**	**0**	**0**	**6**
Pigment in macrophages		**1**	**2**	**4**	**12****	**12**	**20**	**13**	**31****

Group/sex		1M	2M	3M	4M	1F	2F	3F	4F
Pigment in hepatocytes		**1**	**0**	**0**	**18****	**0**	**0**	**3**	**13****
Eosinophilic foci		**1**	**0**	**1**	**1**	**0**	**1**	**1**	**4**
Number of animal examined		**50**	**50**	**50**	**50**	**50**	**50**	**50**	**50**

* p<0.05; ** p<0.01 when compared to Group 1(Fisher's Exact Test)

Findings of uncertain relationship to treatment

Neoplastic findings – The incidence of bronchioloalveolar adenoma of the lungs was reduced in males given 5000 ppm and slightly increased in females at the same dosage group. The incidence in females (24%) was above the background range (5.8-19.2%). The statistical analysis also indicated significant trend tests when all groups are included. However, considering there was a reduction in the incidence of these tumours in males given 5000 ppm and no other evidence of toxicity in the lung, this finding was considered to be arisen by chance.
An increased incidence of histiocytic sarcoma was seen in female group given 850 ppm. The incidence of 14% was outside the background range (0.0-3.8%) and the pairwise comparison with the Control group revealed a statistical significance (p<0.01). However, given the lack of any dosage-relationship, the absence of this tumour in any other female group makes this unlikely to be related to treatment.

Non-neoplastic findings – An increased incidence of inflammation, attaining statistical significance in the highest dosage group, was seen in the urinary bladder of males. However, this is a relative common finding in mice and is considered to have probably arisen by chance.
An increased incidence of interstitial cell hyperplasia, which achieved statistical significance, was recorded in the testes of mice given 5000 ppm.
A number of other findings showing statistically significantly differences from control values are presented in the following table.

Table 3.9.1.2-07 Findings of uncertain relationship to treatment

Group/sex		1M	2M	3M	4M	1F	2F	3F	4F
Dosage (ppm)		**0**	**150**	**850**	**5000**	**0**	**150**	**850**	**5000**
Adrenals									
	Focusal fusiform cell hyperplasia	**23**	**15**	**8**	**12***	**48**	**12**	**20**	**42**
	No. of animals examined	50	30	24	49	50	16	23	50
Lungs									
	Alveolar epithelial hyperplasia	**9**	**1***	**5**	**1***	**2**	**3**	**2**	**2**
	Prominent numbers of alveolar macrophages	**6**	**3**	**2**	**0***	**8**	**4**	**0****	**3**
	No. of animals examined	50	50	50	50	50	50	50	50
Pancreas									
	Islet cell hyperplasia	**13**	**11**	**12**	**4***	**2**	**3**	**6**	**4**
	No. of animals examined	50	33	34	49	50	31	36	50

[*] p<0.05
[**] p<0.01 when compared to Group 1 (Fisher's Exact Test)

CONCLUSIONS

This investigation met all the requirements of a satisfactory carcinogenicity study. Survival, which was not affected by treatment with IR5885, was good, with at least 60% of males and 70% of females surviving in each group up to 78 weeks. Dietary concentrations of up to 5000 ppm were generally well tolerated. Histopathological examination identified the liver as the target organ with neoplastic changes at 850 and 5000 ppm. Reduced bodyweight gain (22% less than that of control values) and reduced food conversion efficiency in males receiving 5000 ppm is indicative of non-specific toxicity and indicates that the Maximum Tolerated Dosage (MTD) had been reached. Females were not clearly affected and, therefore, the MTD for this sex could be considered to be greater than 7000 ppm.

Increased incidence of hepatocellular tumours was evident both in males and females, which received 850 or 5000 ppm. However, this finding was considered secondary to adaptive metabolic changes. Such findings are observed commonly in mice and are generally considered of no significance for man. There were no findings considered to be of toxicological significance among animals receiving 150 ppm and, consequently, this dietary concentration is considered to be the NOAEL in the study (equivalent to 16.8 mg/kg bw/day and 21.6 mg/kg bw/day in females).

3.9.2 Human data

No human data available on carcinogenicity

3.9.3 *In vitro* data (e.g. in vitro germ cell and somatic cell mutagenicity studies, cell transformation assays, gap junction intercellular communication tests)

No other data available.

3.9.4 Other data (e.g. studies on mechanism of action)

3.9.4.1 Mechanistic Study in the Male CD-1 Mouse -IR5885

Reference Number: KII- Section 3, Volume 2, Annex IIA, point 5.10/01

Report:	K. Broich, E.Rached, E. Fernanàndez and K. Weber (2015): IR5885: 14-Day Oral (Feeding) Mechanistic Study in the Male CD-1 Mouse. Harlan Laboratoris Ltd., Zelgliweg 1, CH 4452 Itingen, Switzerland; unpublished report D 64478, date 17. June 2015; dates of experimental work: October 17, 2012 to June 15, 2015.

Guidelines: none

Deviation: Not applicable

GLP: Yes (Certified laboratory)

Executive summary:

This mechanistic oral toxicity study was to assess the mechanism of possible liver toxicity of IR5885 when administered daily to CD-1 mice. They were fed with IR5885 admixed to their diet at concentrations of 0, 150, 1750 and 7000 ppm for 3 or 14 days. A fifth group of animals was treated similarly with 850 ppm of phenobarbitone (positive control) admixed to their diet. Each group comprised 18 animals, of which 6 animals

were sacrificed after 3 days (alloc. A), and the remaining 12 animals were sacrificed after 14 days (alloc. B and C) of treatment.

The following parameters were investigated during the study: mortality/viability, daily general (cage-side) clinical signs, body weights, and food consumption. For determination of cell proliferation, BrdU (10 mg/mL) was administered via osmotic minipumps to alloc. A and B for one week prior to sacrifice. All animals were necropsied and the weights of lungs, livers and brains were recorded at scheduled termination. Blood was collected from the 6 animals/group without BrdU (alloc. C) at scheduled termination for investigation of parameters indicative for oxidative stress (TBARS). Livers and duodenum from allocation A and B animals were collected and processed for histopathologic examination (including BrdU immunohistochemistry). Livers from allocation C animals were used for determination of CYP enzymes (activity and/or mRNA expression), determination of peroxisomal β-oxidation and for investigation of parameters indicative for oxidative stress (TBARS, reduced and oxidized glutathione) and catalase activity staining.

The content of the feed for dose groups 150 and 1750 ppm ranged from 70% to 78%, for the 7000 ppm group between 83 and 86 %. Homogeneity was demonstrated.

There was no mortality and no clinical signs.

Treatment with IR5885 had no effect on food consumption and body weight, while there was a slight increase in the positive control group. The achieved intakes of test compound were 0, 21, 249 and 1050 mg/kg/day, and of phenobarbitone 130.mg/kg/day.

Treatment of male mice with IR5885 at 1750 and 7000 ppm caused a moderate and dose dependent induction of the hepatic cytochrome P450 system. The induction profile of IR5885 was characterized primarily by a strong induction of P450 isoenzymes of subfamily Cyp4a1 accompanied by a moderate induction of peroxisomal β-oxidation. Treatment with IR5885 only caused minor or minimal effects on cytochrome P450 isoenzymes of the subfamilies Cyp1a, Cyp2b, Cyp3a and Cyp2e. The data identified IR5885 as moderate and dose dependent liver enzyme inducer of the peroxisomal-proliferator type with a no effect level at 150 ppm.

IR5885 had no effect on the glutathione status of the liver and did not increase TBARS in liver and serum.

Increased liver weights (absolute and relative) observed in animals fed with 7000 ppm for 3 or 14 days. An increased relative liver weight was also observed in animals fed with 1750 ppm. No effects on liver weights were observed in animals fed with 150 ppm. Increased mean absolute and relative liver weights were also observed in the positive controls after 3 and 14 days of treatment.

No IR5885-related macroscopic findings were noted. Liver findings in two positive control animals were considered to be treatment-related (tan foci corresponding to necrosis and enlarged liver corresponding to hypertrophy).

The treatment with IR 5885 resulted in increased centrilobular to diffuse hepatocellular hypertrophy in a dose-dependent manner in the groups treated with 1750 or 7000 ppm and in dose-dependently increased cell proliferation in the liver of all IR5885-treated groups (150 - 7000 ppm) after 3 and 14 days.

In the positive control animals, hepatocellular hypertrophy and a significant increase in liver cell (hepatocyte) proliferation were observed after 3 and 14 days of treatment. In addition, there was slight focal necrosis in the liver of one animal.

After 3 and 14 days, the values for proliferation in the liver were always the highest in the group treated with phenobarbitone. The proliferation index was in general decreased after 14 days compared to the data after 3 days.

Catalase histochemistry showed a dose related increase in the ratio total catalase area : total nuclear area, incdicating increased peroxysomes per hepatocyte

In conclusion, IR5885 appears as moderate and dose dependent liver enzyme inducer of the peroxisomal-proliferator type.

Similarly, a mode of action of IR5885 as a liver enzyme inducer of the polycyclic aromatic hydrocarbon-, steroid-, or phenobarbitone-type can be excluded.

In addition, IR5885 had no effect on the glutathione status of the liver and did not increase TBARS in liver and serum. Therefore, an induction of oxidative stress can be excluded as mode of action of IR5885.

Materials and Methods

Test Item:	IR5885
Description:	White solid, fine
Lot/Batch #:	G005/07
Purity:	97.83% (HPLC)
Stability of test item:	Stability of the test item in pelleted rodent feed was investigated during a previous Preliminary Reproduction Toxicity Study in the Rat, RCC Study No. 842841. Stability was demonstrated for a period of 21 days.
. Vehicle and/or positive control:	Phenobarbitone sodium salt, PHEUR
Description:	White or almost white, crystalline powder
Lot/Batch #:	2012.10.0179
Purity:	≥99%
Salt/Base correction factor:	1.0948
Stability of test item:	Given the expiration date of Mar-2014 the compound is considered to be stable

Test animals –

Species:	Mouse
Strain:	Crl:CD-1 (ICR) BR
Age at delivery:	7 weeks
Weight at dosing:	25.8 – 34.2 g (males)
Source:	Charles River Germany, Sandhoferweg 7, D-97633 Sulzfeld / Germany
Acclimation period:	9 days
Diet:	Pelleted standard Harlan Teklad 2914C (batch no. 29/12) rodent maintenance diet (Provimi Kliba AG, 4303 Kaiseraugst / Switzerland) was available *ad libitum*.
Water:	Tap water from the Itingen community water main system in bottles *ad libitum*
Housing:	Individually in Makrolon type-2 cages with wire mesh tops and standardized softwood bedding (‘Lignocel’ J. Rettenmaier & Söhne GmbH & Co KG, 73494 Rosenberg / Germany, imported by Provimi Kliba SA, 4303 Kaiseraugst / Switzerland) including paper enrichment (ISO-BLOX from Harlan Laboratories B.V. / Netherlands).

Environmental conditions -

Temperature:	21 – 23 °C
Humidity:	40 – 60 %
Air changes:	10- 15 cycles per hour
Photoperiod:	12-hour light : 12-hour dark cycle

Study Design and Methods

In life dates: October 17, 2012 – November 09, 2012

Animal assignment and treatment

18 males dose group were used. Animals were non-selectively allocated to cages and treatment groups. Treatments were applied as outlined in Table 3.9.4.1-01, study design.

Table 3.9.4.1-01 Study design

Allocation and Dietary Concentration (ppm)	Group 1 Control* 0	Group 2 150	Group 3 1750	Group 4 7000	Group 5 (Positive Control) 850**
Males A	1 - 6	19 - 24	37 - 42	55 - 60	73 - 78
B	7 - 12	25 - 30	43 - 48	61 - 66	79 - 84
C	13 - 18	31 - 36	49 - 54	67 - 72	85 - 90

* Control animals received identical feed without the test item.
** Animals of group 5 received identical feed without test item but with 850 ppm of phenobarbitone
A Animals for investigation of cell proliferation (BrdU), necropsy after 3 days of treatment
B Animals for investigation of cell Proliferation (BrdU), necropsy after 14 days of treatment
C Animals for investigation of liver enzymes and oxidative stress, necropsy after 14 days of treatment

Diet preparation and analysis

Fresh batches of feed pellets for this study were prepared once.

Dietary admixtures for groups 2-4 were prepared with the test item IR5885 as supplied, i.e. no correction factor was applied. A correction factor of 1.0948 was applied for the dietary admixture with phenobarbitone (positive control item, group 5).

Test item or positive control item were weighed into a tared glass baker on a suitable precision balance and mixed with microgranulated feed separately for each dose group. An appropriate amount of water (1 L per 5 kg of feed) was added to aid pelleting. The pellets were then dried with air for approximately 48 hours before storage.

Control feed for the animals of group 1 was prepared similarly, but without test item.

Feed preparations were stored at room temperature (20 ± 5 °C) in disposable paper bags until use.

Stability of the test item in pelleted rodent feed was investigated during a previous Preliminary Reproduction Toxicity Study in the Rat, RCC Study No. 842841. Stability was demonstrated for a period of 21 days.

Concentration and homogeneity of IR5885 in the feed was determined in the feed preparations for groups 1 to 4. Analyses were performed according to an HPLC-method coupled to a UV detector developed at RCC under Study Number 842841. The test item was used as analytical standard.

The results of analysis are summarized below in Table 3.9.4.1-02:

Table 3.9.4.1-02 Homogeneity and stability of test material

Dose group	Sample taken from/after	Data of analysis	Nominal concentration [ppm]	Actual concentration [ppm]	Recovery [%]	Mean Recovery [%]	Maximum variation from Mean [%]
Date of preparation 25 Oct 2012							
1	blank feed	29-Oct-12	0	0.0	---	---	---
1[1]	spike	29-Oct-12	160	153.9	96.2	---	---
	top	29-Oct-12	150	104.7	69.8	73.9	6.0
	middle	29-Oct-12	150	110.3	73.6		
	bottom	29-Oct-12	150	117.5	78.3		
3[1]	top	29-Oct-12	1750	1304	74.5	75.1	1.7
	middle	29-Oct-12	1750	1302	74.4		
	bottom	29-Oct-12	1750	1337	76.4		
4	spike	29-Oct-12	6973	6604	94.7	---	---
	top	29-Oct-12	7000	5804	82.9	83.9	1.9
	middle	29-Oct-12	7000	5983	85.5		
	bottom	29-Oct-12	7000	5831	83.3		

[1] Due to a result out of the accepted range, the sample were reanalyzed; since the repeated analyses confirmed the first result, only the original result is presented

The absence of the test item in the control diet was confirmed.

The homogeneous distribution of IR5885 in the diet preparations was approved because single results did not deviate more than 6% (acceptance criterion: <15%) from the corresponding mean. The content of the feed prepared for dose groups 2 and 3 ranged from 69.8% to 78.3% and, thus, did not meet the required limit of ±20% of the nominal. This was confirmed by reanalysis of the back up diet samples. However, the diet samples of the feed prepared for group 4 were found to contain IR5885 in the range of 82.9% to 85.5% and, thus, the required content limit of ±20% with reference to the nominal concentration was met.

BrdU Administration

Cell replication was assessed by incorporation of synthetic nucleotide bromodeoxyuridine (BrdU) into DNA during the S-phase of cell replication.

All allocation A animals were treated with BrdU from day 6 (22-Oct-2012) of the acclimatization period and all allocation B animals were treated with BrdU from day 8 (02-Nov-2012) of the treatment period. Continuous dosing with BrdU (10 mg/mL at a nominal flow rate of 1.0 µL per hour) was achieved by subcutaneous implantation of osmotic mini-pumps (size 200 µL, Alzet®, no. 2001). The implantation was performed under isoflurane anaesthesia according to the instructions provided by the supplier (Alzet®) of the mini-pumps.

Identification:	Bromodeoxyuridine (BrdU)
Supplier:	BD Pharmingen
Batch Number:	25681
Expiry Date:	31-Jul-2014
Storage Conditions:	-80 ± 10 °C
Safety Precautions:	Routine hygienic procedures (gloves, goggles, face mask)

Activities and observations

Viability/mortality, clinical signs

Animals were inspected at least twice daily for viability/mortality and once daily for signs of ill health.

Severity grades for clinical signs were generally assigned as follows: 0 = not present, 1 = present / slight, 2 = moderate, 3 = marked.

Body weight and food consumption

Food Consumption:

AcclimatizationPeriod	Once before and once after implantation of minipumps
Treatment Period	Twice weekly

Body Weights:

AcclimatizationPeriod	At delivery, once before and once after implantation of minipumps
Treatment Period	Twice weekly

Determination of liver enzymes and parameters for oxidative stress

Sampling

Terminal blood samples were collected by heart puncture from allocation C animals of groups 1 to 5 on the scheduled day of necropsy for investigation of parameters indicative for oxidative stress (TBARS). Serum was prepared immediately and two aliquots were prepared. Serum samples were immediately frozen in liquid nitrogen, transferred on dry ice to the analytical laboratory and stored there at -80 ± 10 °C until analysis.

For liver enzyme determination (enzyme activity and mRNA expression) and for investigation of parameters indicative for oxidative stress, the liver of all allocation C animals was weighed and divided into the following samples (minced):

- mRNA Expression: preserved in RNA*later* RNA Stabilization Reagent (Qiagen; see below).
- Glutathione status: immediately frozen in liquid nitrogen to prevent oxidation
- TBARS
- Enzyme activities, Oxidative Stress: remaining liver

All samples except the samples for mRNA expression were rinsed with 0.9% saline solution, immediately frozen in liquid nitrogen, transferred on dry ice to the analytical laboratory and stored there at -80 ± 10 °C until analysis.

Liver samples for mRNA expression were submerged in an appropriate volume of RNAl*ater* RNA Stabilization Reagent from Qiagen (approx. 2 mL) immediately after sampling. The liver pieces in RNA*later* were kept in the refrigerator (5 ± 3 °C) for 20 to 24 hours to allow the RNA*later* solution to penetrate the tissue. After this step, the tubes with the samples were frozen at -80 ± 10 °C in the dark. Transfer to the analytical laboratory was performed on dry ice and the samples were stored there at -80 ± 10 °C until analysis.

Determination of liver enzyme activities and parameters for oxdative stress

The frozen liver samples were thawed, homogenized in ice cold 10 mM Tris/HCl buffer, pH 7.5, containing 250 mM sucrose to give a 10% (w/v) homogenate and centrifuged for 15 min at 100 *g* at 4 °C. Aliquots of the supernatant were frozen in liquid nitrogen and stored at -80 ± 10 °C for possible additional investigations upon request of the Sponsor.

Microsomal and cytosolic liver fractions were obtained by centrifugation for 60 min at 100’000 *g* at 4 °C.

The following enzyme activities were determined:

- Protein content of subcellular fractions
- Cyp1a2 by determination of microsomal 7-methoxyresorufin O-dealkylation
- Cyp2b1 by determination of microsomal 7-pentoxy-resorufin O-dealkylation
- Cyp3a by determination of microsomal testosterone 6β-hydroxylation

- Cyp4a1 by determination of microsomal lauric acid 12-hydroxylation
- Cyp2e1 by determination of microsomal lauric acid 11-hydroxylation
- Peroxismal β-oxidation by determination of palmitoyl CoA oxidation

The following parameters for oxidative stress were determined:

- TBARS in liver homogenate and serum using a commercially available kit (TBARS Parameter Assay Kit, R&D Systems, KGE013)
- Reduced and oxidized glutathione in liver homogenate using a commercially available kit (Glutathione Assay Kit, Cayman Chemical, 703002)

Determination of cytochrome-P-450 (CYP) mRNA Expression

RNA was isolated from liver using NucleoSpin RNA L (Macherey Nagel) according to the supplier´s recommendations. The amount of RNA was estimated by the absorbance ratio at 260/280 nm.

Isolated RNA was reverse transcribed using Taqman reverse transcription kit. Relative CYP mRNA levels (1A1,1A2, 2B10 and 3A11) were evaluated using Taqman® quantification assays (Applied Biosystems). The Ct values were normalized using the endogenous gene β-actin. Real time reactions were conducted in a Real Time thermocycler CFX-96 (Biorad). Relative quantification of the target genes was made using the ΔΔCt method.

Sacrifice and pathology

Necropsy

All animals were weighed and necropsied. Descriptions of all macroscopic abnormalities were recorded. All animals were anesthetized by intraperitoneal injection of pentobarbitone and killed by exsanguination.

Sacrifice:

After 3 Days: allocation A

After 14 Days: allocation B and C

Terminal blood samples were collected by heart puncture from allocation C animals of groups 1 to 5 on the day of necropsy and serum was prepared for investigation of parameters indicative for oxidative stress.

Lungs, brain, liver and all gross lesions were collected from all animals at necropsy. The liver and duodenum from all Allocation A and B animals were collected and fixed in neutral phosphate buffered 4% formaldehyde solution. Additionally, all remaining carcasses were retained fixed in neutral phosphate buffered 4% formaldehyde solution for possible further investigations.

For liver enzyme determination (enzyme activity and mRNA expression) and for investigation of parameters indicative for oxidative stress, the liver of all allocation C animals was weighed and divided into several samples (minced) as described above.

Organ weights

The weights of the lungs, livers and brains from all animals were recorded on the scheduled dates of necropsy and the organ to terminal body weight ratios as well as organ to brain weight ratios were determined.

Histotechnique

Liver and duodenum from all Allocation A and B animals were processed, embedded, cut at an approximate thickness of 2 - 4 micrometers and stained with hematoxylin and eosin (HE).

In addition, one section was prepared with a detection kit (NovoLink™ Max Polymer Detection System) for BrdU (Anti-Bromo-d-Oxyuridine monoclonal, mouse, Clone IIB5).

Histopathology

The HE-stained sections were evaluated for histopathological findings. Immunohistochemically treated sections were evaluated for proliferation by image analysis, i.e. 2 areas per liver section were analyzed for the amount of BrdU incorporation and calculated by the 'CELL Analysis' system. Image analysis (of stained particles) was performed non-GLP.

Additionally, slides stained for catalase-antibodies were evaluated histopathologically. The catalase area was evaluated and evaluated in relation to the total nuclear area.

Statistics

The following statistical methods were used to analyze food consumption, body weight, organ weights and ratios as well as macroscopic findings:

- The Dunnett-test (many to one t-test) based on a pooled variance estimate was applied if the variables could be assumed to follow a normal distribution for the comparison of the treated groups and the control groups for each sex.
- The Steel-test (many-one rank test) was applied instead of the Dunnett-test when the data could not be assumed to follow a normal distribution.
- Fisher's exact-test.

RESULTS AND DISCUSSION

Analysis of feed preparation

The content of the feed for dose groups 150 and 1750 ppm ranged from 70% to 78%, for the 7000 ppm group between 83 and 86 %. Homogeneity was demonstrated.

Viability/mortality, clinical signs

All animals survived their scheduled treatment period and no test item-related clinical signs were observed.

In several allocation A and B animals, clinical signs were observed at or near the implantation site of the osmotic minipumps, such as erythema, scabs, or bleeding areas. In addition, fissures at the ears were observed in a few animals of these allocations. These findings were considered to be related to the surgery. However, fissures in the ears were also noted in few animals of allocation C (groups 3 and 5). This finding was considered to be incidental.

Food consumption and test material intake

Food consumption was unaffected by the treatment with IR5885.

In group 5 (850 ppm phenobarbitone), absolute and relative food consumption were slightly increased, with the differences to the control values frequently attaining statistical significance. On average, over the 14-day treatment period, absolute and relative food consumption were +9.4% and +5.0%, respectively, higher than in the control group.

The following mean absolute and relative food consumption was recorded:

Table 3.9.4.1-03 Achieved intakes

	Group 1 0 ppm	**Group 2 150 ppm**	**Group 3 1750 ppm**	**Group 4 7000 ppm**	**Group 5 850 ppm***
Abs. food consumption** g/animal/day	5.3	5.2	5.2	5.6	5.8
Rel. Food consumption** g/kg bw/day	145.2	137.7	142.3	149.9	152.4
Test item intake (mg/kg/bw)	--	20.7	249.0	1049.5	129.6

* = Phenobarbitone
** = mean of means over treatment

Body weights

Treatment with IR5885 had no effect on body weights and body weight gain. Any differences to the control group were considered to be incidental, even when they attained statistical significance, as they were only slight and not consistent over time and/or there was no dose-response relationship.

In group 5 (850 ppm phenobarbitone), mean body weights and body weight gain were slightly higher compared to the control group, with the differences generally attaining statistical significance for mean body weight gain and occasionally for mean body weights. This is consistent with the slightly increased food consumption and the increased liver weights observed in this group.

The following mean body weights and body weight gains were recorded:

Table 3.9.4.1-04 Body weights and bodyweight gains

	Group 1 0 ppm	**Group 2 150 ppm**	**Group 3 1750 ppm**	**Group 4 7000 ppm**	**Group 5 850 ppm***
Mean body weight day 14 (g)	38.1	39.1	38.3	38.9	39.7
Mean body weight gain day 14, (%)	10.3	12.7	10.0	12.0	15.5

* = Phenobarbitone

Determinations of liver enzymes and parameters for oxidative stress

Determination of liver enzymes

Treatment with IR5885 or with phenobarbitone had no effect on the protein content of microsomal fractions.

The following effects on activities of hepatic cytochrome P450 enzymes (in percent of control results) were found after 14 days of treatment:

Table 3.9.4.1-05 Hepatic cytochrome P450 activities

		Dose Level			
Enzyme	**Test System**	**150 ppm IR5885**	**1750 ppm IR5885**	**7000 ppm IR5885**	**850 ppm Phenobarbitone**
CYP1a-2	7-methoxyresorufin O-demethylation (MROD)	---	---	---	312%**
CYP2b	O-Depentylation of 7-pentoxyresorufin (PROD)	---	165%**	266%**	1916%**
CYP3a	Hydroxylation of testosterone at the 6β-position	---	150%**	267%**	420%**
Cyp2e-1	Lauric acid 11-hydroxylation	---	206%	384%**	370%**
CYP4a-1	Lauric acid 12-hydroxylase	---	408%*	1106%**	214%**
Peroxisome proliferation	Peroxisomal β-oxidation	---	208%**	308%**	30%**

*: $p<0.05$; **: $p<0.01$

Liver enzyme gene expression

Treatment with phenobarbitone (850 ppm) very strongly increased the mRNA levels of CYP 2B10 (223 fold over the control group). Levels of CYP3A11 also showed a significant increase (12.12 fold over control) and a slight increase was seen in the levels of CYP1A1 (3.58 fold) and CYP1A2 (2.96 fold).

Animals fed with IR5885 showed a similar induction pattern in the mRNA expression levels as those fed with phenobarbitone, although not very pronounced: an increase of CYP2B10 in a dose-dependent manner and a slight increase in the levels of CYP3A11. No increase in the levels of CYP1A1 and CYP1A2 could be observed.

Treatment with 1750 and 7000 ppm of IR5885 increased the levels of CYP2B10, 6.20 and 20.13 fold over the control, respectively. No increase could be observed at 150 ppm IR5885.

CYP3A11 increased 6.08 fold and 9.54 fold over the control in animals fed with 1750 and 7000 ppm of IR5885, respectively.

Table 3.9.4.1-06 Hepatic cytochrome P450 activities

Group	Treatment	CYPs			
		CYP1A1	CYP1A2	CYP2B10	CYP3A11
1	0 ppm	1.0	1.0	1.0	1.0
2	150 ppm	0.81	0.68	1.57	1.09
3	1750 ppm	1.22	0.83	6.20	6.08
4	7000 ppm	1.19	0.31	20.13	9.54
5	PB, 850 ppm	3.58	2.96	223.0	12.12

Determination of parameters for oxidative stress

Reduced glutathione is a representative of water soluble cellular antioxidants, and participates in detoxification at different levels. GSH is easily oxidized to the disulfide dimer GSSG. It may scavenge free radicals induced by oxidative stress, reduce peroxides or be conjugated with electrophilic compounds. The measurement of the oxidative status (GSH/GSSG) therefore is an indicator for oxidative stress.

Following treatment with IR5885 at a dose level of 7000 ppm a slight increase of GSH to 124% of control was observed. This minor increase was accompanied by a decrease of GSSG to 75.8% of control ($p < 0.01$). This decrease was only marginal and is therefore concluded to be of no biological significance. Treatment with phenobarbitone increased hepatic GSH slightly to 128% and reduced hepatic GSSG slightly to 81.0% of control.

Lipid peroxidation, induced by oxidative stress, ultimately results in the oxidative fragmentation of polyunsaturated fatty acids to volatile alkanes and various aldehydes, such as MDA which can be measured as Thiobarbituric Acid Reactive Substances (TBARS). Treatment with IR5885 or phenobarbitone had no effect on liver and serum TBAR levels

Table 3.9.4.1-07 Hepatic GSH and GSSG content, TBARS in liver and serum, % of controls

Group	Treatment	GSH	GSSG	TBARS	
		µmol/g liver	µmol/g liver	nmol/g liver	nmol/mL serum
1	0 ppm	100	100	100	100
2	150 ppm	111	100	89	87
3	1750 ppm	106	95	85	79
4	7000 ppm	124	75.8**	112	63
5	PB, 850 ppm	128	81.0*	105	57

*/** Significantly different (Dunnett's t-test) from control, *: p <0.05; **: p <0.01

Pathology

Organ weights

Increased liver weights observed in animals fed with IR5885 at 1750 or 7000 ppm were considered to be test item-related. In animals at 7000 ppm, higher liver weights (absolute and relative) were recorded both after 3 and 14 days of treatment. In animals at 1750 ppm, the liver-to-body weight ratio was statistically significantly increased after 14 days of treatment. No effect on liver weights was observed in animals fed with 150 ppm of IR5885.

Increased mean absolute and relative liver weights were also observed in group 5 (850 ppm phenobarbitone) after 3 and 14 days of treatment.

Table 3.9.4.1-08 Liver Weights after 3 and 14 Days (Mean Values and Percent Differences Relative to Control Value)

Group	**1**	**2**	**3**	**4**	**5**
Concentration	**0 ppm**	**150 ppm**	**1750 ppm**	**7000 ppm**	**850 ppm**
Test Item/Positive Control item	**---**	**IR5885**	**IR5885**	**IR5885**	**Phenobarbitone**
After 3 days					
Liver weight (absolute)	2.08	2.15 (+3%)	2.31 (+11%)	2.88** (+38%)	3.34** (+61%)
Liver weight (rel. to body weight)	5.86	6.13 (+5%)	6.44 (+10%)	7.85** (+34%)	9.07** (+55%)
Liver weight (rel. to brain weight)	435.02	425.51 (-2%)	457.69 (+5%)	554.28** (+27%)	671.55** (+54%)
After 14 days					
Liver weight (absolute)	2.36	2.44 (+3%)	2.67 (+13%)	3.25** (+38%)	3.37** (+43%)
Liver weight (rel. to body weight)	6.22	6.36 (+2%)	7.03** (+13%)	8.37** (+35%)	8.51** (+37%)
Liver weight (rel. to brain weight)	463.98	468.89 (+1%)	519.40 (+12%)	628.26** (+35%)	678.52** (+46%)

**: p<0.01

After 3 days of treatment, mean absolute brain weights of IR5885-treated animals were slightly higher compared to the control group. This was considered to be incidental as there was no clear dose-response relationship and no similar finding was noted in relative brain weights or after 14 days. The slightly higher lung-to-body weight ratio recorded in animals at 150 ppm was also regarded to be incidental as there was no dose-response relationship and this was an isolated finding.

Macroscopic findings

No gross lesions were observed in control and IR5885-treated animals.

Enlarged liver and tan foci on the liver, respectively, observed in two animals of group 5 (850 ppm phenobarbitone) were considered to be treatment-related.

All other findings in animals of this dose group (sores at the ears) were considered to be related to the surgery (implantation of osmotic minipumps).

Microscopic findings

Liver

Centrilobular to diffuse hepatocellular hypertrophy was recorded in a dose-dependent manner in groups fed with 1750 and 7000 ppm IR5885 as well as in the phenobarbitone group. Hepatocellular hypertrophy was present in the same groups after 14 days, however, the severity increased in phenobarbitone-treated animals when compared with the results obtained after 3 days of treatment. In one animal of the phenobarbitone group, there was a slight focal necrosis after 14 days (corresponding to the macroscopic finding of tan foci). No further induced lesion was recorded in the liver (see Table 3.9.4.1-09).

Table 3.9.4.1-09 Incidence and Mean Severity of Main Findings in Liver (Total affected/mean severity)

Finding / Groups	**0 ppm**	**150 ppm**	**1750 ppm**	**7000 ppm**	**PB 850 ppm**
Total affected/Mean severity	**(6) M**	**(6) M**	**(6) M**	**(6) M**	**(6) M**
Necropsy after 3 Days					
Hepatocellular hypertrophy	0	0	3/1.0	6/1.3	6/1.5
Necropsy after 14 Days					
Hepatocellular hypertrophy	2/1.0	2/1.0	4/1.3	6/1.5	6/3.5
Necrosis, focal	0	0	0	0	1/2.0

At sacrifice after 3 days, the mean number of proliferating cells in the liver - indicated by BrdU-positive nuclei - increased dose-dependently in all IR5885-treated groups, but was significantly higher in phenobarbitone-treated animals.

At the terminal sacrifice after 14 days, there were still higher numbers (dose-dependent) of proliferating cells in all groups treated with IR5885, and, again, the values for proliferation were highest in animals at 850 ppm phenobarbitone. However, compared with the data 3 days after treatment start, the proliferation index generally decreased.

Table 3.9.4.1-10 Descriptive Statistics on Proliferating Hepatocytes: Necropsy after 3 and 14 Days

Group	Mean 3 days	Mean 14 days
0 ppm IR5885	18	15
150 ppm IR5885	34	37
1750 ppm IR5885	65	51
7000 ppm IR5885	69	60
850 ppm Phenobarbitone	164	80

Duodenum

The duodenum served as labelling control (proliferation) that worked for each animal

Liver Catalase-Immunohistochemistry

The mean area per lobe showed the highest value in the 150 ppm group and declined in the higher concentrations. The values for phenobarbitone were lowest. This applied for both lobes. A similar trend was also recorded for the nuclear area values. Due to the hepatocellular hypertrophy, the relation catalase area: nuclear area, i.e. catalase area per hepatocyte was deemed more appropriate. The calculated values revealed a dose-related increase of catalase compared to nucleus (i.e. hepatocyte) in the groups treated with IR5885. The values for the phenobarbitone treated animals were below control levels.

Table 3.9.4.1-11 Mean catalase Total areas and nuclear areas (both liver lobes summarized)

Finding / Groups	0 ppm	150 ppm	1750 ppm	7000 ppm	PB 850 ppm
Total catalase area values Mean (SD)	46.67 (11.59)	51.18 (10.41)	48.05 (9.79)	45.67 (9.85)	29.43 (12.42)
Total uclear area values Mean (SD)	10.14 (2.94)	10.49 (2.05)	9.36 (2.35)	8.54 (1.60)	7.62 (1.84)
Quotient catalase area:total nuclear area	4.603	4.879	5.134	5.348	3.862

Other findings

The remainder of findings recorded was within the range of normal background lesions which may be recorded in animals of this strain and age.

CONCLUSIONS

Treatment of male mice with IR5885 at 1750 and 7000 ppm caused a moderate and dose dependent induction of the hepatic cytochrome P450 system. The induction profile of IR5885 was characterized primarily by a strong induction of P450 isoenzymes of subfamily Cyp4a1 accompanied by a moderate induction of peroxisomal β-oxidation. Treatment with IR5885 only caused minor or minimal effects on cytochrome P450 isoenzymes of the subfamilies Cyp1a, Cyp2b, Cyp3a and Cyp2e. The data identified IR5885 as moderate and dose dependent liver enzyme inducer of the peroxisomal-proliferator type with a no effect level at 150 ppm.

IR5885 had no effect on the glutathione status of the liver and did not increase TBARS in liver and serum.

Increased liver weights (absolute and relative) observed in animals fed with 7000 ppm for 3 or 14 days. An increased relative liver weight was also observed in animals fed with 1750 ppm. No effects on liver weights were observed in animals fed with 150 ppm. Increased mean absolute and relative liver weights were also observed in the positive controls after 3 and 14 days of treatment.

No IR5885-related macroscopic findings were noted. Liver findings in two positive control animals were considered to be treatment-related (tan foci corresponding to necrosis and enlarged liver corresponding to hypertrophy).

The treatment with IR 5885 resulted in increased centrilobular to diffuse hepatocellular hypertrophy in a dose-dependent manner in the groups treated with 1750 or 7000 ppm and in dose-dependently increased cell proliferation in the liver of all IR5885-treated groups (150 - 7000 ppm) after 3 and 14 days.

In the positive control animals, hepatocellular hypertrophy and a significant increase in liver cell (hepatocyte) proliferation were observed after 3 and 14 days of treatment. In addition, there was slight focal necrosis in the liver of one animal.

After 3 and 14 days, the values for proliferation in the liver were always the highest in the group treated with phenobarbitone. The proliferation index was in general decreased after 14 days compared to the data after 3 days.

In conclusion, IR5885 appears as moderate and dose dependent liver enzyme inducer of the peroxisomal-proliferator type.

Similarly, a mode of action of IR5885 as a liver enzyme inducer of the polycyclic aromatic hydrocarbon-, steroid-, or phenobarbitone-type can be excluded.
In addition, IR5885 had no effect on the glutathione status of the liver and did not increase TBARS in liver and serum. Therefore, an induction of oxidative stress can be excluded as mode of action of IR5885.

3.9.4.2 Bridging study to compare the response to Valifenalate in male CD-1 and C57BL/6 mice

Reference Number: KII- Section 3, Volume 2, Annex IIA, point 5.10/01

Report:	A. Vardy (2015a): Bridging study to compare the response to Valifenalate in male CD-1 and C57BL/6 mice. CXR Biosciences Ltd, 2 James Lindsay Place, Dundee Technopole, Dundee, UK; unpublished report CXR1419, date 26 May 2015; dates of experimental work: not stated.

Guidelines: none

Deviation: Not applicable

GLP: No

Executive summary:

The aim of this bridging study was to compare the effect of Valifenalate on Pparα in CD-1 and C57BL/6 wild-type mice to investigate any mouse strain differences ahead of a Pparα knock-out mouse study.

7000 ppm Valifenalate was administered in the diet to both strains for seven days, alongside appropriate controls. The diet was deemed to be homogeneous and achieved 106% of the target concentration. The food consumption was consistent across all groups other than the C57BL/6 treated group, where the consumption was considerably higher, resulting in this strain receiving a greater dose of Valifenalate.

The presence of Valifenalate was assessed in terminal plasma samples by LC-MS/MS but could only be detected in one sample at a low level.

Valifenalate caused no impact on bodyweight, but increased the liver weight in the CD-1 mouse and the liver:bodyweight ratio in both strains. The clinical chemistry markers ALT, AST and ALP were essentially unchanged. Valifenalate caused a 1.6-fold and a 1.9-fold increase in PCoA oxidation in the CD-1 mice and C57BL/6 mice respectively. Valifenalate caused a 2.1-fold and a 3.4-fold increase in hepatic pentoxyresorufin-O-depentylation (PROD) activity in the CD-1 and C57BL/6 mouse strains respectively. Valifenalate caused a 4.9-fold and a 7.1-fold increase in hepatic 12-hydroxylauric acid formation in the CD-1 and C57BL/6 mouse strains respectively. The increased biochemical responses observed in the C57BL/6 mice may be due to the increased food consumption and therefore greater Valifenalate dose received.

In the parameters measured, both strains responded approximately equally to Valifenalate. Therefore, the C57BL/6 mouse strain is an appropriate background strain for further investigations using the Pparα knockout mouse.

Materials and Methods

Test Item:	Valifenalate
Description:	Not stated
Lot/Batch #:	P/13/024
Purity:	99.68% (HPLC)
Stability of test item:	Not stated
. Diet:	RM1 powdered diet (Special Diet Services Ltd., Stepfield, Witham, Essex, UK)
Test animals –	
Species:	Mouse
Strain:	CD-1 (WT) and C57BL/6 (WT)
Sex:	Male
Age at delivery:	7 - 8 weeks
Weight at dosing:	23.2 - 36.7 g
Source:	Harlan UK Limited, Shaw's Farm, Blackthorn, Bicester, Oxon, England, OX25 1TP.
Acclimation period:	At least 5 days
Test diet:	RM1 powdered diet containing 0 or 7000 ppm Valifenalate was available *ad libitum*.
Water:	Tap water from the local public supply, in bottles, *ad libitum*
Housing:	In groups of five per cage on sawdust in solid-bottom, polypropylene cages. Environment enhancing materials were used during this study.
Environmental conditions -	
Temperature:	19 - 23 °C
Humidity:	40 - 70 %
Air changes:	14 - 15 cycles per hour
Photoperiod:	12-hour light : 12-hour dark cycle

Study Design and Methods

In life dates: 16 June 2014 to 23 June 2014

Animal assignment and treatment

4 groups (2 CD-1 and 2 C57BL/6) of 5 males per dose group were used. Animals were randomly allocated to cages and treatment groups. Treatments were applied as outlined in Table 3.9.4.2-01, study design.

Table 3.9.4.2-01 Study design

Group	Strain	Treatment	Sex
1	CD-1	Control*	Male
	C57BL/6		
2	CD-1	Valifenalate 7000 ppm	
	C57BL/6		

* Control animals received identical feed without the test item.

Diet preparation and analysis

Valifenalate 7000 ppm diet: 7 g Valifenalate and 993 g of powdered RM1 diet were weighed out. The Valifenalate was mixed into a small amount of diet using a pestle and mortar. This mixture was transferred to a K-beater mixer; the pestle and mortar was washed with powdered RM1 diet several times, with the diet transferred to the mixer each time until all 993 g of diet was transferred. The Valifenalate diet was then covered and mixed for 1 hour.

Valifenalate diet was stored at ambient temperature throughout the study and prepared without purity correction.

The diet was analysed by reverse phase HPLC followed by UV detection. Nine individual samples of test diet (3 from the top, 3 from the middle and 3 from the bottom of the diet container) were analysed at the study start. Recovery was assessed by including spiked samples in the analytical runs.

The results of analysis are summarized in Table 3.9.4.2-02:

Table 3.9.4.2-02 Concentrations at start of study

Sample		**Recovery of spike (%)**	**Actual dose (ppm)**	**% deviation**	**Mean dose**	**% of target dose**
7000 ppm spiked	Spiked diet	96.38				
7000 ppm test diet	Top 1		7473.9	6.3	7112.4	101.6
	Top 2		6983.4	-0.2		
	Top 3		6879.9	-1.7		
	Middle 1		7750.8	9.7	7420.2	106.0
	Middle 2		7157.3	2.2		
	Middle 3		7352.6	4.8		
	Bottom 1		8088.8	13.5	7801.0	111.4
	Bottom 2		7554.7	7.3		
	Bottom 3		7759.5	9.8		
Total			7444.5 ± 394.3			106.4

Activities and observations

Body Weights: The bodyweight of each mouse was recorded at the start of the study and prior to termination.

Clinical Observations: Prior to the start of the study, all mice were observed to ensure they were physically normal and exhibited normal activity. Each mouse was observed at least once daily during the study.

Food Consumption: Food consumption was measured at the end of the study.

Sampling

On the day of termination the mice were weighed, then transferred to the post mortem room. Individual body weights were recorded manually. The mice were killed by exposure to a rising concentration of CO_2. Food was not withdrawn prior to termination.

Venous blood was taken post mortem *via* cardiac puncture and dispensed into lithium/heparin-coated tubes. The tubes were mixed on a roller for 10 min then cooled on ice. Red blood cells were removed by centrifugation (2,000 rpm for 10 min at 8 – 10 °C), then the supernatant (plasma) was transferred to a second tube and stored at approximately -70 ºC.

The gall bladder was removed from each animal prior to removing the liver. Each liver was weighed and the individual liver weights were recorded. The liver was scissor-minced in ice-cold 1.15% (w/v) KCl prior to homogenisation and subcellular fractionation to heavy pellets and microsomes. Heavy pellets were isolated and stored at approximately -70°C until required for the peroxisome proliferation assay. Microsomes were stored at approximately -70ºC for possible future analysis.

Analysis for Valifenalate

Terminal plasma samples were analysed for Valifenalate by LC-MS/MS.

Biochemistry

Clinical chemistry

For all groups, three markers of liver damage (ALT, AST and ALP) were measured in all plasma samples using the COBAS Integra 400+ (Roche). Any remaining plasma was stored at approximately -70°C for possible future analysis.

Protein determination

The protein concentrations of the liver heavy pellets were determined in aqueous solutions using a modified version of the method of Lowry et al., (1951), with bovine serum albumin standards.

Peroxisome proliferation

Peroxisome CN^- -insensitive acyl CoA oxidation was determined spectrophotometrically in liver heavy pellets, using palmitoyl CoA as a substrate, as described by Bronfmann *et al.*, (1979).

Pentoxyresorufin-O-depentylation (PROD)

The activity of Cyp2b in liver microsomes was determined spectrofluorometrically by the formation of resorufin from pentoxyresorufin, as described by Burke *et al.*, (1985).

12-hydroxylauric acid formation (LAH)

The activity of Cyp4a in liver microsomes was measured as the formation of 12-hydroxylauric acid from lauric acid using LC-MS/MS detection.

Statistics

Statistical comparisons between Valifenalate treated mice and their respective control groups were undertaken for all numerical data sets using a 2-tailed Student's t-test.

RESULTS AND DISCUSSION

Analysis of feed preparation

The diet was deemed to be homogeneous and the achieved concentration was 7444.5 ppm (106% of target).

Plasma analysis for Valifenalate

No Valifenalate was detected in any of the terminal plasma samples analysed other than from mouse #13, which had a low measurement of 6.4 ng/mL Valifenalate.

Food consumption

The CD-1 and C57BL/6 mice consumed a similar level of control diet when corrected for the individual mouse bodyweights (see Table 3.9.4.2-03). However, the C57BL/6 mice consumed a considerable amount more test diet compared to their respective controls and also the CD-1 mice. This increased consumption resulted in the C57BL/6 mice receiving an average dose of 2078.9 mg Valifenalate / kg mouse / day compared to the CD-1 mice that received an average dose of 1249.1 mg Valifenalate / kg mouse / day.

Table 3.9.4.2-03 Food consumption in male CD-1 mice and C57BL/6 mice fed control diet and diet containing 7000 ppm Valifenalate

	CD-1		**C57BL/6**	
Parameter	Control (0 ppm)	Valifenalate (7000 ppm)	Control (0 ppm)	Valifenalate (7000 ppm)
Diet consumed g/mouse/day (%)	5.76 (100.00)	6.14 (106.60)	4.61 (100.00)	5.17 (109.94)
Diet consumed g/kg bwt/day (%)	155.57 (100.00)	167.80 (107.86)	177.30 (100.00)	221.95 (107.67)

Values are mean from each cage. Values in parenthesis are mean % control for the appropriate strain (n=5 per group).

Body weights, body weight gains, liver weights and liver/body weight ratios

The administration of Valifenalate at 7000 ppm did not significantly affect the body weight gain of either strain of mouse (see Table 3.9.4.2-04).

However, 7000 ppm Valifenalate in the diet did cause a significant increase of 19.5% in the liver weights of the CD-1 mouse. This increase was reflected in the liver to bodyweight ratio, which increased by 21%. Although the liver weights of the C57BL/6 mice also increased by 14% when administered the Valifenalate diet, the escalation did not reach statistical significance. However, the rise in liver weights in the C57BL/6 mice did result in a statistically significant increase in the liver to body weight ratio (16%).

Table 3.9.4.2-04 Bodyweights, bodyweight gains, liver weights and liver/bodyweight ratios in CD-1 mice and C57BL/6 mice fed control diet and diet containing 7000 ppm Valifenalate

Strain	**CD-1**		**C57BL/6**	
Parameter	**Control (0 ppm)**	**Valifenalate (7000 ppm)**	**Control (0 ppm)**	**Valifenalate (7000 ppm)**
Day 1 Body weight (g)	34.86 ± 0.69[a] (100.00 ± 1.97)	34.12 ± 1.53 (97.88 ± 4.39)	24.88 ± 0.95 (100.00 ± 3.84)	24.12 ± 0.76 (96.95 ± 3.04)
Terminal Body weight (g)	37.00 ± 1.19 (100.00 ± 3.21)	36.58 ± 1.59 (98.86 ± 4.30)	25.98 ± 0.73 (100.00 ± 2.81)	25.50 ± 0.85 (98.15 ± 3.25)
Body weight gain (g)	2.14 ± 0.82 (100.00 ± 38.19)	2.46 ± 0.50 (114.95 ± 23.27)	1.10 ± 0.62 (100.00 ± 56.04)	1.38 ± 0.40 (125.45 ± 36.59)
Liver Weight (g)	2.06 ± 0.09 (100.00 ± 4.18)	2.47 ± 0.15*** (119.49 ± 7.03)	1.45 ± 0.21 (100.00 ± 14.72)	1.65 ± 0.12 (113.96 ± 8.02)
Liver/Body weight ratio	5.58 ± 0.25 (100.00 ± 4.46)	6.75 ± 0.51*** (121.00 ± 9.20)	5.55 ± 0.74 (100.00 ± 13.39)	6.45 ± 0.30* (116.19 ± 5.33)

[a] Values are Mean ± SD. Values in parenthesis are mean % control ± SD for the appropriate strain or control (n=5 per group). A Student's t-test was performed on the results; * statistically different from control p<0.05; ** p<0.01; *** p<0.001

Clinical Chemistry

Plasma alanine transaminase (ALT) and aspartate aminotransferase (AST) increased by 1.6-fold and 1.5-fold respectively in the C57BL/6 mice following administration of Valifenalate (see Table 3.9.4.2-05). Although these increases reached statistical significance, the changes were not deemed to be clinically relevant. Plasma alkaline phosphatase (ALP) levels increased marginally in the C57BL/6 mice following exposure to Valifenalate. In the CD-1 mice, the levels of all three markers were essentially unchanged.

Table 3.9.4.2-05 Clinical chemistry parameters in plasma from male CD-1 mice and C57BL/6 mice fed control diet and diet containing 7000 ppm Valifenalate.

Strain	**CD-1**		**C57BL/6**	
Parameter	**Control (0 ppm)**	**Valifenalate (7000 ppm)**	**Control (0 ppm)**	**Valifenalate (7000 ppm)**
ALT (U/L)	50.38 ± 24.04[a] (100.00 ± 47.71)	53.50 ± 20.72 (106.19 ± 41.12)	44.10 ± 6.85 (100.00 ± 15.53)	69.24 ± 20.29* (157.01 ± 46.01)
AST (U/L)	77.72 ± 25.63 (100.00 ± 32.97)	88.26 ± 19.50 (113.56 ± 25.09)	94.12 ± 14.81 (100.00 ± 15.73)	144.26 ± 29.38** (153.27 ± 31.21)
ALP (U/L)	130.22 ± 13.99 (100.00 ± 10.74)	126.72 ± 32.08 (97.31 ± 24.64)	111.66 ± 17.42 (100.00 ± 15.60)	138.68 ± 24.20 (124.20 ± 21.67)

[a] Values are Mean ± SD. Values in parenthesis are mean % control ± SD for the appropriate strain or control (n=5 per group). A Student's t-test was performed on the results; * statistically different from control p<0.05; ** p<0.01

Biochemical measurements

Peroxisomal β-Oxidation

Valifenalate caused a 1.6-fold and a 1.9-fold increase in PCoA oxidation in the CD-1 and C57BL/6 mice respectively.

Pentoxyresorufin-O-depentylation (PROD)

Valifenalate caused a 2.1-fold and a 3.4-fold increase in PROD activity in the CD-1 and C57BL/6 mice respectively.

12-hydroxylauric acid formation (12-OH LA)

Valifenalate caused a 4.9-fold and a 7.1-fold increase in 12-OH LA formation in the CD-1 and C57BL/6 mice respectively.

Table 3.9.4.2-06 Biochemical measurements comparing control and Valifenalate treated CD-1 and C57BL/6 mice.

Strain	**CD-1**		**C57BL/6**	
Parameter	**Control (0 ppm)**	**Valifenalate (7000 ppm)**	**Control (0 ppm)**	**Valifenalate (7000 ppm)**
PCoA nmol NAD+ reduced/min/mg protein	13.19 ± 3.68[a] (100.00 ± 27.90)	21.11 ± 2.76** (159.99 ± 20.91)	23.94 ± 1.39 (100.00 ± 5.81)	44.68 ± 1.76*** (186.66 ± 7.37)
PROD pmols resorufin formed/min/mg protein	1.74 ± 0.27 (100.00 ± 15.54)	3.73 ± 0.70*** (213.87 ± 40.19)	1.93 ± 0.34 (100.00 ± 17.41)	6.54 ± 2.32** (383.33 ± 119.98)
LAH nmols 12-OH formed / 10min / mg protein	2.44 ± 0.32 (100.00 ± 12.93)	11.98 ± 3.65*** (490.19 ± 149.32)	2.99 ± 0.48 (100.00 ± 15.97)	21.16 ± 3.19*** (708.35 ± 106.64)

[a] Values are Mean ± SD. Values in parenthesis are mean % control ± SD for the appropriate strain or control (n=5 per group). A Student's t-test was performed on the results; * statistically different from control $p<0.05$; ** $p<0.01$

CONCLUSIONS

The aim of the study was to assess the response in male CD-1 and C57BL/6 mice to 7000 ppm Valifenalate administered *via* the diet. The C57BL/6 strain demonstrated slightly higher biochemical responses as measured by hepatic PCoA oxidation, PROD activity and 12-hydroxylauric acid formation compared to the CD-1 strain, but these findings may be due to the increased Valifenalate dose received by this strain as a result of the increased food consumption. In general, both strains responded approximately equally to Valifenalate with respect to increased liver weights, increased liver / bodyweight ratio and clinical chemistry. Therefore, the C57BL/6 mouse strain is an appropriate background strain for further investigations using the Pparα knockout mouse.

3.9.4.3 Dietary study with Valifenalate in male C57BL/6 and PPARα knockout mice study

Reference Number: KII- Section 3, Volume 2, Annex IIA, point 5.10/01

Report:	A. Vardy (2015b): A 7 and 14 day dietary study with Valifenalate in male C57BL/6 and PPARα knockout mice: hepatic mode of action investigation. CXR Biosciences Ltd, 2 James Lindsay Place, Dundee Technopole, Dundee, UK; unpublished report CXR1398, date 22 June 2015; dates of experimental work: not stated.

Guidelines: none

Deviation: Not applicable

GLP: No

Executive summary:

The aim of this study was to investigate a Pparα-mediated mode of action for Valifenalate comparing the responses in Pparα knockout mice with the equivalent wild type controls.

7000 ppm Valifenalate was administered in the diet to both strains for seven and fourteen days, alongside appropriate controls. The diet was deemed to be homogeneous and achieved 102% and 96% of the target concentration at the start and end of the study, respectively. The food consumption was essentially consistent across all groups other than the Pparα KO mice where the treated group consumed 24% more diet during the second week of the 14 day time-point compared to Pparα KO mice receiving control diet. At all time-points examined, the WT mice received more Valifenalate compared to the Pparα KO equivalents. However, the difference was small with the largest variation being 11.5% during the second week of the 14 day time-point. The bodyweight gain was higher in the WT mice receiving Valifenalate for 7 days and slightly lower in the Pparα KO mice receiving Valifenalate for 14 days, but otherwise there was no impact on bodyweight. The liver weights and liver/bodyweight ratios increased in both strains of mice at both time-points, but to a greater extent in the WT mice.

The clinical chemistry markers ALT, AST and ALP were essentially unchanged. The increase in S-phase was higher in the WT mice compared to the Pparα KO mice; 8.2-fold vs. 5.4 fold at day 7 and 3.5-fold vs. 1.9-fold at day 14, showing the response was returning to baseline by the 14 day time-point.

WT mice treated with Valifenalate for 7 days exhibited minimal to mild centrilobular hypertrophy. This finding was not apparent in Pparα KO mice administered Valifenalate for 7 days, where only 2 mice exhibited minimal centrilobular hypertrophy. When treated with Valifenalate for 14 days, all WT mice presented with moderate centrilobular hypertrophy accompanied by increased mitosis in 6/10 WT mice. In contrast, centrilobular hypertrophy and increased mitoses were not observed in the livers of Pparα KO mice treated with Valifenalate for 14 days.

Peroxisome proliferation was investigated by assessing PCoA oxidation and Acox1 mRNA levels. Following 14 days administration, Valifenalate caused a 2.0-fold and a 1.3-fold increase in PCoA oxidation in the WT mice and Pparα KO mice respectively. Similarly the Acox1 mRNA levels were increased at this time-point by 1.8-fold and 1.3-fold in the WT mice and Pparα KO mice respectively.

Pparα activation was investigated by assessing Cyp4a mRNA levels and 12-hydroxylauric acid formation. Cyp4a10 and Cyp4a14 mRNA levels were increased in both the WT and Pparα KO mice following Valifenalate administration and although the absolute levels were low, the fold-changes were higher in the KO model compared to the WT mice, which was unexpected. 12-OH LA levels were increased in the WT mice by 7.7-fold following Valifenalate administration for 14 days, but were only increased by 4.0-fold in the Pparα KO mice following the same Valifenalate administration.

In addition to Pparα, Car activation was investigated by assessing Cyp2b10 mRNA levels and activity by PROD measurements. The Cyp2b10 mRNA levels were increased by approximately 50-fold in both strains of mouse. PROD activity was elevated by 6.0-fold and 7.1-fold in the WT and Pparα KO mice respectively following 14 days of Valifenalate administration.

Finally, Pxr activation was also assessed by investigating Cyp3a11 mRNA levels and activity by BQ measurements. The Cyp3a11 mRNA levels were increased by approximately 6.3-fold and 8.5-fold in the WT and Pparα KO mice respectively following 14 days of Valifenalate administration. BQ activities were increased by 2.4-fold in both strains of mouse.

These data suggest that although the Pparα pathway is responsible for a portion of the hepatic response to Valifenalate, additional mechanisms mediated by Car and Pxr activation are also involved.

Materials and Methods

Test Item: Valifenalate
Description: Not stated

Lot/Batch #: P/13/024
Purity: 99.68% (HPLC)
Stability of test item: Not stated
Diet: RM1 powdered diet (Special Diet Services Ltd., Stepfield, Witham, Essex, UK)

Test animals –
Species: Mouse
Strain: C57BL/6 (WT) and Pparα KO on a C57BL/6 background
Sex: Male
Age at delivery: 8-9 weeks
Weight at dosing: 18.9 - 26.0 g
Source: C57BL/6: Harlan UK Limited, Shaw's Farm, Blackthorn, Bicester, Oxon, England, OX25 1TP. Pparα KO: Taconic Farms, 273 Hover Avenue, Germantown, NY 12526 USA.
Acclimation period: At least 5 days
Test diet: RM1 powdered diet containing 0 or 7000 ppm Valifenalate was available *ad libitum*.
Water: Tap water from the local public supply, in bottles, *ad libitum*
Housing: In groups of five per cage on sawdust in solid-bottom, polypropylene cages. Environment enhancing materials were used during this study.

Environmental conditions -
Temperature: 19 - 23 °C
Humidity: 40 - 70 %
Air changes: 14 - 15 cycles per hour
Photoperiod: 12-hour light : 12-hour dark cycle

Study Design and Methods

In life dates: Not stated

Animal assignment and treatment

8 groups (4 WT and 4 Pparα KO) of 10 males per dose group were used. Animals were randomly allocated to cages and treatment groups. Treatments were applied as outlined in Table 3.9.4.3-01, study design.

Table 3.9.4.3-01 Study design

Group	Treatment	Termination	Sex	Strain
1	Control*	Day 8	Male	WT
2	Valifenalate 7000 ppm			Pparα KO
3	Control*	Day 15		WT
4	Valifenalate 7000 ppm			Pparα KO

* Control animals received identical feed without the test item.

Diet preparation and analysis

Valifenalate 7000 ppm diet: 7 g Valifenalate and 993 g of powdered RM1 diet were weighed out. The Valifenalate was mixed into a small amount of diet using a pestle and mortar. This mixture was transferred to a K-beater mixer; the pestle and mortar was washed with powdered RM1 diet several times, with the diet transferred to the mixer each time until all 993 g of diet was transferred. The Valifenalate diet was then covered and mixed for 1 hour.

Valifenalate diet was stored at ambient temperature throughout the study and prepared without purity correction.

The diet was analysed by reverse phase HPLC followed by UV detection. Nine individual samples of test diet (3 from the top, 3 from the middle and 3 from the bottom of the diet container) were analysed at the study start and end. Recovery was assessed by including spiked samples in the analytical runs.

The results of analysis are summarized below in Tables 3.9.4.3-02 and 3.9.4.3-03:

Table 3.9.4.3-02 Concentrations at start of study

Sample		Recovery of spike (%)	Actual dose (ppm)	% deviation	Mean dose	% of target dose
5020 ppm spiked	Spiked diet	102.6				
7000 ppm test diet	Top 1		7007.57	0.11	7086.5	101.2
	Top 2		7029.27	0.42		
	Top 3		7222.59	3.18		
	Middle 1		6884.91	-1.64	7009.5	100.1
	Middle 2		6974.91	-0.36		
	Middle 3		7168.70	2.41		
	Bottom 1		7475.98	6.80	7324.3	104.6
	Bottom 2		7034.16	0.49		
	Bottom 3		7462.89	6.61		
Total			7140.1 ± 211.6			102.0

Table 3.9.4.3-03 Concentrations at end of study

Sample		Recovery of spike (%)	Actual dose (ppm)	% deviation	Mean dose	% of target dose
7460 ppm spiked	Spiked diet	103.1				
7000 ppm test diet	Top 1		7460.0	-4.5	6615.9	94.5
	Top 2		6683.0	-7.1		
	Top 3		6502.5	-4.8		
	Middle 1		6662.1	2.3	6983.6	99.8
	Middle 2		7158.3	2.4		
	Middle 3		7169.8	-5.4		
	Bottom 1		6622.7	-8.5	6625.6	94.7
	Bottom 2		6403.0	-5.7		
	Bottom 3		6871.8	-1.8		
Total			6741.7 ± 271.4			

BrdU Administration

Two groups (1 and 2) were terminated on day 8. These animals were implanted with Alzet osmotic pumps (Alzet 2001 supplied by Charles River UK Ltd, Manston Road, Margate, England CT9 4LT) containing approximately 200 µL bromodeoxyuridine solution (BrdU, Sigma B-5002, 15 mg/mL in phosphate buffered saline [PBS], pH 7.4) on day 1 of the study. Two further groups (3 and 4) were terminated on day 15. These animals were implanted with BrdU filled Alzet osmotic pumps on day 8. The osmotic pumps were implanted subcutaneously to the dorsal region under isoflurane anesthesia.

Activities and observations

Body Weights:	For groups 1 and 2, the bodyweight of each mouse was recorded at the start of the study and on day 8 prior to termination. For groups 3 and 4, the bodyweight of each mouse was recorded at the beginning of the study, on day 8 and finally on day 15 prior to termination.
Clinical Observations:	Prior to the start of the study, all mice were observed to ensure that they were physically normal and that they exhibited normal activity. Each mouse was observed at least once daily during the study.
Food Consumption:	For groups 1 and 2, food consumption was measured on day 8. For groups 3 and 4, food consumption was measured on day 8 and day 15.

Sampling

Groups 1 and 2 were terminated on study day 8 and groups 3 and 4 were terminated on study day 15.

Venous blood was taken post mortem *via* cardiac puncture and dispensed into lithium/heparin-coated tubes. The tubes were mixed on a roller for 10 min then cooled on ice. Red blood cells were removed by centrifugation (2,000 rpm for 10 min at 8 – 10 °C), then the supernatant (plasma) was transferred to a second tube and stored at approximately -70 ºC until required for clinical chemistry analysis.

The gall bladder was removed from each animal prior to removing the liver. Each liver was weighed; two pieces of liver (5 mm^3) were removed from the left lobe for TaqMan® analysis. These liver pieces were placed in the same cryovial, flash frozen in liquid nitrogen and then stored at approximately -70 ºC until required. Additionally, two further pieces of liver (5 mm^3) were removed and stored at approximately -70 ºC for possible future gene analysis.

Two samples of liver (approximately 3 mm strips) were taken; one from the left lobe and one from the median lobe. These were placed in a 60 mL plastic sample pot containing approximately 20 mL of 10% neutral buffered formalin (NBF) for subsequent immunohistochemical and histological analysis.

For groups 1 and 2, the remaining liver was weighed and placed in a cryovial for possible future processing to microsomes and heavy pellet. For groups 3 and 4, the remaining liver was weighed and scissor-minced in ice-cold 1.15% (w/v) KCl prior to processing. Heavy pellets were isolated and stored at approximately -70°C until required for protein determination and peroxisome proliferation assessment. Microsomes were isolated and stored at approximately -70°C until required for any subsequent analysis.

As a positive immunohistochemical control, a 0.5 cm section of the proximal small intestine (duodenum) was removed from all animals and placed in the container of 10% NBF with the corresponding liver sample.

Pathology

Hepatocellular labelling index

For all groups, liver and duodenum samples were fixed for approximately 36 - 48 hrs prior to processing the tissues into paraffin blocks according to LMS Pat-005-6 and Pat-011-12. Each wax block containing liver and duodenum (as a positive control) had 4 - 6 µm sections cut. The sections were dried overnight at 37°C, prior to antigen retrieval using 1 mM EDTA buffer. Sections underwent acid hydrolysis to denature the DNA, followed by exposure to anti-BrdU primary antibody. The sections were subsequently exposed to a horseradish peroxidase conjugated secondary antibody and detected using 3,3'diaminobenzidine (DAB). Sections were counterstained with Gills haematoxylin, dehydrated and mounted. Following qualitative assessment, slides were quantitatively evaluated using Improvision Acquisition and Volocity 4 Analysis image analysis software and the results were calculated as a labelling index expressing the replicating cell number as a percentage of the total cell number.

Haematoxylin and eosin (H&E) histopathology

For all groups, paraffin-embedded liver samples were sectioned and stained with H&E.

Biochemistry

Clinical chemistry

For all groups, three markers of liver damage (ALT, AST and ALP) were measured in all plasma samples using the COBAS Integra 400+ (Roche). Any remaining plasma was stored at approximately -70°C for possible future analysis.

Protein determination

For groups 3 and 4, the protein concentrations of the liver heavy pellets were determined in aqueous solutions using a modified version of the method of Lowry et al., (1951), with bovine serum albumin standards. The liver microsomes prepared from mice 41 – 80 also had protein quantification carried out using the same method.

Peroxisome proliferation

For groups 3 and 4, CN^- -insensitive acyl CoA oxidation was determined spectrophotometrically in liver heavy pellets, using palmitoyl CoA as a substrate, as described by Bronfmann *et al.*, (1979).

12-hydroxylauric acid formation (LAH)

The activity of Cyp4a in liver microsomes was measured as the formation of 12-hydroxylauric acid from lauric acid using LC-MS/MS detection. Only liver microsomes prepared from mice 41 – 80 were assessed.

Pentoxyresorufin-O-depentylation (PROD)

The activity of Cyp2b in liver microsomes was determined spectrofluorometrically by the formation of resorufin from pentoxyresorufin, as described by Burke *et al.*, (1985). Only liver microsomes prepared from mice 41 – 80 were assessed.

Benzyloxyquinoline-O-debenzylation (BQ)

The activity of Cyp3a in liver microsomes was determined spectrofluorometrically by the formation of 7-hydroxyquinoline from benzyloxyquinoline, as described by the Corning Gentest HTS technical bulletin. Only liver microsomes prepared from mice 41 – 80 were assessed.

mRNA expression (qRT PCR, Taqman®)

For groups 3 and 4, RNA was extracted from mouse liver according to LMS DNAt-008. cDNA was synthesised from all available RNA samples. TaqMan® analysis was performed on all available samples using primers specific for Cyp4a10, Cyp4a14 and Acox1 (Assay-on-demand kits, Applied Biosystems). WT Pparα and disrupted Pparα were also assessed. Finally, Cyp2b10 and Cyp3a11 mRNAs were assessed in addition to those listed above). Mouse β-actin was used as the internal standard (Assay-on-demand kit, Applied Biosystems). Data was analysed by generation of threshold cycle (CT) and delta CT values for all genes.

Statistics

Statistical comparisons between Valifenalate treated mice and their respective control groups were undertaken for all numerical data sets using a 2-tailed Student's t-test.

RESULTS AND DISCUSSION

Analysis of feed preparation

At the start of the study, diet was deemed to be homogeneous and the achieved concentration was 7140 ppm (102% of target). At the end of the study, the diet was still deemed to be homogeneous and the achieved concentration was 6742 ppm (96% of target).

Food consumption and test material intake

The WT mice consumed similar levels of control and test diet when corrected for the individual mouse bodyweights (see Table 3.9.4.3-04) with the variation reaching no more than 10%. The Pparα KO mice

receiving the test diet consumed more in all groups compared to those receiving the control diet with the variation reaching a maximum of 24%.

Taking into account the Valifenalate dietary analysis, in all groups the WT mice received slightly more Valifenalate compared to the Pparα KO equivalents. However, the difference was small with the largest variation being 11.5% during the second week of the 14 day time-point (1448 mg/kg/day vs. 1636 mg/kg/day).

Table 3.9.4.3-04 Food consumption data from male wild-type and Pparα KO mice fed control diet and diet containing 7,000 ppm Valifenalate

Parameter	Day	WT mice Control (0 ppm)	WT mice Valifenalate (7000 ppm)	Pparα KO mice Control (0 ppm)	Pparα KO mice Valifenalate (7000 ppm)
Diet consumed g/mouse/day (%)	7	5.87 (100.00)	5.66 (96.44)	4.71 (100.00)	5.17 (109.94)
Diet consumed g/kg bwt/day (%)		244.11 (100.00)	228.22 (93.49)	206.14 (100.00)	221.95 (107.67)
mg/kg dosed/day (%)		N/A	1629.50 (100)	N/A	1584.72 (97.25)
Diet consumed g/mouse/day (%)	14 (week 1)	5.40 (100.00)	4.63 (85.88)	3.87 (100.00)	4.37 (113.00)
Diet consumed g/kg bwt/day (%)		222.11 (100.00)	201.69 (90.80)	166.73 (100.00)	185.50 (111.26)
mg/kg dosed/day (%)		N/A	1440.04 (100.00)	N/A	1324.48 (91.98)
Diet consumed g/mouse/day (%)	14 (week 2)	5.33 (100.00)	5.59 (104.92)	3.99 (100.00)	5.02 (126.10)
Diet consumed g/kg bwt/day (%)		214.15 (100.00)	229.17 (107.01)	163.56 (100.00)	202.81 (123.99)
mg/kg dosed/day (%)		N/A	1636.26 (100.00)	N/A	1448.07 (88.50)

Values are mean from each cage. Values in parenthesis are mean % control for the appropriate strain (n=10 per group).

Body weights, body weight gains, liver weights and liver/body weight ratios

The bodyweights at the beginning of the study were lower in the WT mice receiving test diet compared to their respective controls for the 14 day time point. Also at the study start, the bodyweights of the Pparα KO mice receiving test diet were higher compared to their respective controls for the 14 day time-point. However the variation in both cases was less than 8% (see Table 3.9.4.3-05). The WT mice receiving Valifenalate for 7 days gained more weight compared to their control diet counterparts, whereas the Pparα KO mice receiving Valifenalate for 14 days gained less weight compared to their control diet counterparts. However, although statistically significant, the differences were not marked.

7000 ppm Valifenalate in the diet did cause significant increases of 21% and 17% in the liver weights of the WT mice at 7 and 14 days respectively (see Table 3.9.4.3-05). Although Valifenalate administered *via* the diet also caused an increase in the liver weights of the Pparα KO mice (9% and 12% at 7 and 14 days respectively), the increases were lower than those observed in the WT mice. These liver weight increases were also reflected in the liver to bodyweight ratios in both strains at both time-points.

Table 3.9.4.3-05 Bodyweights, bodyweight gains, liver weights and liver/bodyweight ratios from male wild-type and Pparα KO mice fed control diet and diet containing 7,000 ppm Valifenalate for 14 days

Parameter	WT mice Control (0 ppm)	WT mice Valifenalate (7000 ppm)	Pparα KO mice Control (0 ppm)	Pparα KO mice Valifenalate (7000 ppm)
Day 1 Body weight (g)	23.18 ± 0.65[a] (100.00 ± 2.81)	22.28 ± 0.59** (96.12 ± 2.53)	21.71 ± 1.47 (100.00 ± 6.77)	23.37 ± 1.74* (107.65 ± 8.01)
Terminal Body weight (g)	24.83 ± 1.11 (100.00 ± 4.46)	24.42 ± 1.20 (98.32 ± 4.84)	24.38 ± 1.55 (100.00 ± 6.35)	24.80 ± 1.60 (101.73 ± 6.57)
Body weight gain (g)	1.65 ± 1.01 (100.00 ± 60.95)	2.14 ± 0.89 (129.32 ± 54.03)	2.67 ± 1.37 (100.00 ± 51.36)	1.43 ± 0.81* (53.61 ± 30.37)
Liver Weight (g)	1.32 ± 0.06 (100.00 ± 4.54)	1.55 ± 0.15*** (116.95 ± 11.22)	1.29 ± 0.08 (100.00 ± 5.90)	1.45 ± 0.11** (112.42 ± 8.67)
Liver/Body weight ratio	5.33 ± 0.20 (100.00 ± 3.75)	6.33 ± 0.36*** (118.70 ± 6.73)	5.28 ± 0.15 (100.00 ± 2.90)	5.83 ± 0.21*** (110.42 ± 3.95)

[a] Values are Mean ± SD. Values in parenthesis are mean % control ± SD for the appropriate strain or control (n=10 per group). A Student's t-test was performed on the results; * statistically different from control p<0.05; ** p<0.01; *** p<0.001

Clinical Chemistry

Plasma samples were assessed for ALT, AST and ALP, however there was very little change in any of the three markers. The only changes that reached statistical significance were a decrease in the AST level and an increase in the ALP in WT mice following 14 days of Valifenalate administration (see Table 3.9.4.3-06). Note that AST levels from samples 53 (Pparα KO control) and 63 (WT treated) were considered outliers and removed from the analysis.

Table 3.9.4.3-06 Clinical chemistry parameters in terminal plasma from male WT and Pparα KO mice administered Valifenalate for 7 and 14 days.

Parameter	Day	WT mice Control (0 ppm)	WT mice Valifenalate (7000 ppm)	Pparα KO mice Control (0 ppm)	Pparα KO mice Valifenalate (7000 ppm)
ALT (U/L)	7	31.84 ± 5.77[a] (100.00 ± 18.11)	30.92 ± 2.97 (97.11 ± 9.33)	34.56 ± 5.53 (100.00 ± 15.99)	38.29 ± 4.16 (110.79 ± 12.04)
	14	39.29 ± 2.34 (100.00 ± 5.95)	37.05 ± 5.87 (94.30 ± 14.95)	38.41 ± 4.39 (100.00 ± 11.42)	39.36 ± 4.66 (102.47 ± 12.13)
AST (U/L)	7	141.00 ± 53.15 (100.00 ± 37.69)	101.32 ± 34.88 (71.86 ± 24.74)	139.14 ± 48.82 (100.00 ± 35.09)	126.71 ± 46.29 (91.07 ± 33.27)
	14	123.99 ± 28.68 (100.00 ± 23.13)	86.23 ± 18.35**# (69.55 ± 14.80)	149.00 ± 51.71# (100.00 ± 34.71)	122.27 ± 38.87 (82.06 ± 226.09)
ALP (U/L)	7	94.80 ± 30.77 (100.00 ± 36.29)	104.99 ± 18.01 (123.81 ± 21.24)	74.86 ± 12.41 (100.00 ± 16.57)	65.24 ± 13.03 (87.15 ± 17.41)
	14	73.34 ± 9.71 (100.00 ± 13.24)	89.06 ± 14.60* (121.43 ± 19.91)	61.29 ± 13.09 (100.00 ± 21.36)	62.31 ± 13.03 (101.66 ± 21.26)

[a] Values are Mean ± SD. Values in parenthesis are mean % control ± SD for the appropriate strain or control (n=10 per group). A Student's t-test was performed on the results; * statistically different from control p<0.05; ** p<0.01; *** p<0.001

Hepatocellular labelling index

The nuclear incorporation of BrdU was utilised as a measure of cell proliferation and the hepatocellular labelling index (S-phase, percentage of labelled cells) was determined. In the WT mice, the labelling index increased by 8.2-fold at day 7, but was returning to baseline by day 14 where the increase was only 3.5-fold. The labelling index increased to a lesser extent in the Pparα KO mice reaching 5.4-fold at day 7. This response was also returning to baseline by day 14 where the increase was only 1.9-fold (Table 3.9.4.3-07).

Table 3.9.4.3-07 Replicative DNA synthesis in male WT and Pparα KO mice administered Valifenalate *via* the diet for 14 days.

Strain	Day	Control (0 ppm)	Valifenalate (7000 ppm)
WT	7	1.90 ± 0.30[a] (100.00 ± 15.59)	15.46 ± 2.85***[#] (815.4 ± 150.3)
Pparα KO		0.79 ± 0.15 (100.00 ± 18.66)	4.24 ± 2.18*** (536.83 ± 276.28)
WT	14	2.66 ± 1.08 (100.00 ± 40.58)	9.42 ± 3.57*** (354.72 ± 134.53)
Pparα KO		1.68 ± 0.67 (100.00 ± 39.88)	3.14 ± 0.93*** (186.89 ± 55.35)

[a] Values are Mean ± SD. Values in parenthesis are mean % control ± SD for the appropriate strain (n=10 per group; [#] n=9 as mouse #29 was considered an outlier). A Student's t-test was performed on the results; *** statistically different from control p<0.001

H&E Histopathology

A histopathology evaluation of the livers from male wild type C57Bl/6 and Pparα KO mice given Valifenalate for seven or fourteen days, at a dose level of 7000 ppm in the diet, showed a differential response between the two mouse strains at both time points.

At the seven day time period, 9/10 of the wild type C57Bl/6 mice showed a mild to moderate decrease in glycogen and a minimal to mild, centrilobular hepatocellular hypertrophy. A single mouse, number 25, had a normal liver. In comparison, the Pparα KO mouse also showed a decrease in glycogen of similar grade to that showed by the wild type mice in 7/10 mice following Valifenalate administration but minimal hepatocellular hypertrophy was only observed in 2/10 mice.

At the fourteen day time period, 7/10 of the wild type C57Bl/6 mice showed a minimal to mild decrease in glycogen and all ten mice showed a moderate, centrilobular hepatocellular hypertrophy. This was accompanied, in 6/10 mice by a minimal to moderate increase in mitotic figures in the livers.

In comparison, at the fourteen day time period, the Pparα KO mouse showed only a decrease in glycogen of mild to moderate severity in 8/10 mice in the absence of the other hepatic changes seen in the wild type mice.

Table 3.9.4.3-08 Liver histopathology comparison (centrilobular hypertrophy and mitosis) between male WT and Pparα KO mice administered Valifenalate for 7 and 14 days.

Strain, treatment	**Day 8**		**Day 15**	
	Incidence of lesion (grade)		Incidence of lesion (grade)	
	Centrilobular hypertrophy	Increased mitosis	Centrilobular hypertrophy	Increased mitosis
WT, control	1/10 (2)	0/10	0/10	0/10
WT, Valifenalate	8/10 (1.2 ± 0.7)[a]	0/10	10/10 (3)	6/10 (1.2 ± 1.1)[b]
KO, control	0/10	0/10	0/10	0/10
KO, Valifenalate	2/10 (0.2 ± 0.4)[c]	0/10	0/10	0/10

a, b, c, Mean ± SD

Biochemical measurements

Peroxisomal β-Oxidation

Valifenalate caused a 2.0-fold and a 1.3-fold increase in PCoA oxidation in the WT and Pparα KO mice respectively after 14 days administration via the diet.

Pentoxyresorufin-O-depentylation (PROD)

Valifenalate caused a 6.0-fold and a 7.1-fold increase in PROD activity in the WT and Pparα KO mice respectively after 14 days of dietary administration.

Benzyloxyquinoline-O-debenzylation (BQ)

Valifenalate caused a 2.4-fold increase in both strains of mice following 14 days of dietary administration.

12-hydroxylauric acid formation (LAH)

Valifenalate caused a 7.7-fold and a 4.0-fold increase in LAH formation in the WT and mice following 14 days of dietary administration.

Table 3.9.4.3-09 Gene expression and enzyme induction profiles for male WT and Pparα KO mice administered Valifenalate *via* the diet for 14 days.

Strain	**WT**		**Pparα KO**	
Treatment	Control (0 ppm)	Valifenalate (7000 ppm)	Control (0 ppm)	Valifenalate (7000 ppm)
Gene Expression				
Cyp2b10	1.0	55.59**	1.0	48.09
Cyp3a11	1.0	6.26**	1.0	8.49
Cyp4a10	1.0	5.89**	1.0	68.80
Cyp4a14	1.0	151.59**	1.0	151.59
Acox1	1.0	1.79**	1.0	1.34
Enzyme Activity				
PCoA (Peroxisomal β-oxidation)	17.03 ± 3.36[a] (100.00 ± 19.75)	34.49 ± 2.71*** (202.49 ± 15.91)	11.71 ± 0.86 (100.00 ± 7.38)	15.70 ± 1.76*** (134.12 ± 15.06)
PROD (Cyp2b1)	2.23 ± 0.58 (100.0 ± 25.8)	13.45 ± 3.34*** (603.5 ± 149.9)	2.70 ± 0.37 (100.0 ± 13.8)	19.10 ± 2.59*** (706.7 ± 95.9)
BQ (Cyp 3a)	2.06 ± 0.55 (100.0 ± 26.9)	5.02 ± 0.43*** (244.0 ± 21.0)	2.43 ± 0.33 (100.0 ± 13.4)	5.85 ± 0.52*** (240.6 ± 21.2)
LAH (Cyp4a1)	2.74 ± 0.95 (100.0 ± 34.7)	21.22 ± 3.12*** (774.6 ± 113.8)	1.02 ± 0.14 (100.0 ± 13.6)	4.09 ± 1.24*** (399.9 ± 121.4)

[a] Values are Mean ± SD. Values in parenthesis are mean % control ± SD for the appropriate strain or control (n=10 per group). A Student's t-test was performed on the results; *** statistically different from control p<0.001; ** p<0.01

mRNA Expression (qRT PCR, Taqman®)

In order to confirm the genotype of the mice, Taqman® assays were custom designed to detect the native Pparα mRNA and the disrupted Pparα mRNA. The native Pparα mRNA was only detectable in the WT mice and the disrupted Pparα mRNA was only detectable in the Pparα KO mice, thereby confirming the genotype of the mice (Table 3.9.4.3-10).

The level of Acox1 mRNA was assessed at the 14 day time-point as an indicator of peroxisome proliferation. In the WT and Pparα KO mice, the mRNA increased by 1.8-fold and 1.3-fold respectively, which agrees with the pattern of the hepatic PCoA response (Table 3.9.4.2-10).

The Cyp4a10 and Cyp4a14 mRNA levels were increased in the WT mice (5.9-fold and 15.5-fold respectively) and even more substantially in the Pparα KO mice increasing by 68.8-fold and 151.6-fold respectively. However, the absolute levels of the mRNAs were considerably lower in the Pparα KO mice, with the Valifenalate-mediated induction returning the Cyp4a mRNA levels to those observed under control conditions in the WT mice. For example, the CT values for Cyp4a10 in the WT mice under control conditions ranged from 22.5 to 24.6 and were 23.0 to 25.6 in the treated Pparα KO mice (Table 3.9.4.3-10).

Finally, Valifenalate was found to increase Cyp2b10 and Cyp3a11 mRNA levels by similar margins in both strains of mouse. Cyp2b10 mRNA levels were increased by 56-fold and 48-fold in the WT and Pparα KO mice respectively and Cyp3a11 mRNA levels were increased by 6.3-fold and 8.5-fold in the WT and Pparα KO mice respectively (Table 3.9.4.3-10).

Table 3.9.4.3-10 Taqman® analysis of murine hepatic Cyp2b10, Cyp3a11, Cyp4a10, Cyp4a14, Acox1, native Pparα and disrupted Pparα mRNAs from WT and Pparα KO mice administered Valifenalate *via* the diet for 14 days.

Gene	**Fold change relative to control**			
	WT		**Pparα KO**	
Treatment	Control (0 ppm)	Valifenalate (7000 ppm)	Control (0 ppm)	Valifenalate (7000 ppm)
Cyp2b10	1.00 ± 0.34	55.59 ± 14.55***	1.00 ± 0.50	48.09 ± 14.41***
Cyp3a11	1.00 ± 0.17	6.26 ± 1.73***	1.00 ± 0.29	8.49 ± 1.38***
Cyp4a10	1.00 ± 0.44	5.89 ± 2.01***	1.00 ± 0.41	68.80 ± 33.13***
Cyp4a14	1.00 ± 0.51	15.49 ± 6.17***	1.00 ± 0.42	151.59 ± 82.84***
Acox1	1.00 ± 0.09	1.79 ± 0.35***	1.00 ± 0.15	1.34 ± 0.17***
Wild type PPARα	1.00 ± 0.22	2.06 ± 1.72	Not detected	Not detected
Dispupted PPARα	Not detected	Not detected	1.00 ± 0.15	1.01 ± 0.29

a Values are Mean ± SD (n=10 per group).
Results are expressed as fold change relative to control, where control values were normalised to 1.00. Murine β-actin was employed as the internal control.
A Student's t-test was performed on the results; *** statistically different from control p<0.001

CONCLUSIONS

The aim of this study was to investigate a Pparα-mediated mode of action for Valifenalate comparing the responses in Pparα knockout mice with the equivalent wild type controls. Throughout the study it is clear that the WT mouse responded more to Valifenalate compared to the Pparα KO mouse with respect to increased liver weight, liver:bodyweight ratio, hepatic replicative DNA synthesis (S-phase) and peroxisome proliferation as determined by hepatic PCoA oxidation and Acox1 mRNA analysis. These data demonstrate that Pparα is responsible for a portion of the hepatic response to Valifenalate. However, the Pparα KO mouse also responded to Valifenalate albeit to a lesser extent in the majority of measurements made.

To further understand the portion of Valifenalate-mediated hepatic response still evident in the Pparα KO mice, Car activation was investigated by assessing Cyp2b10 mRNA levels and activity by PROD measurements. Cyp2b10 mRNA levels were increased by approximately 50-fold in both strains of mouse and PROD activity was elevated by 6.0- and 7.1-fold in the WT and Pparα KO mouse respectively, showing mild to moderate activation of Car.

Finally, Pxr activation was investigated by assessing Cyp3a11 mRNA levels and activity by BQ measurements. Cyp3a11 mRNA levels and BQ activities were increased by a similar amount in both strains of mouse, suggesting a mild to moderate activation of Pxr.

One result that was unexpected was the elevated fold-change in Cyp4a10 and Cyp4a14 mRNA in the Pparα KO mice compared to the WT controls. However, the absolute values were much lower in the Pparα KO mouse, with the induction returning the Cyp4a levels to the approximate levels measured in the WT mice under control conditions.

To summarise, these data suggest that although the Pparα pathway is responsible for a portion of the hepatic response to Valifenalate, additional mechanisms mediated by Car and Pxr activation are also involved.

3.9.4.4 Valifenalate - enzyme and DNA-synthesis induction in cultured male wild-type CD-1 mouse hepatocytes

Reference Number: KII- Section 3, Volume 2, Annex IIA, point 5.10/01

Report:	A. Vardy (2015c): Valifenalate - enzyme and DNA-synthesis induction in cultured male wild-type CD-1 mouse hepatocytes. CXR Biosciences Ltd, 2 James Lindsay Place, Dundee Technopole, Dundee, UK; unpublished report CXR1476, date 26 May 2015; dates of experimental work: not stated.

Guidelines: none

Deviation: Not applicable

GLP: No

Executive summary:

The aim of this study was to investigate the potential of Valifenalate to activate Car and/or Pparα nuclear hormone receptors and stimulate cell proliferation in isolated hepatocytes prepared from male CD-1 mice. Phenobarbital (PB) and Wy-14,643 were included as positive controls.

An initial cytotoxicity assay (assessing ATP levels) was performed with Valifenalate. A depletion in ATP levels and therefore cytotoxicity was measured at 300 µM making this a suitable top concentration to be assessed in the main enzyme induction and proliferation study.

Neither PB nor Valifenalate had any impact on replicative DNA synthesis in the male CD-1 mouse hepatocytes. PB can often have a weak effect on mouse hepatocyte proliferation *in vitro*. However, Wy-14,643 increased replicative DNA synthesis by a maximum of 1.7-fold and EGF also produced a robust response, demonstrating that the test system could respond to a proliferative stimulus.

In both the male CD-1 mouse hepatocytes, PB induced Cyp2b10, as measured by Taqman® mRNA analysis and PROD activity, but as expected had little impact on the Cyp4a or peroxisome proliferation markers. Conversely, Wy-14,643 induced Cyp4a and peroxisome proliferation as measured by Taqman® mRNA analysis (Cyp4a10, Cyp4a14 and Acox1), 12-OH LA formation and PCoA oxidation. Valifenalate however had essentially no impact on any of the biochemical markers assessed.

To summarise, these data demonstrate that Valifenalate does not activate either mouse Car or Pparα when assessed *in vitro* as demonstrated by the lack of hypertrophic and hyperplasic responses in the CD-1 mouse hepatocytes. The positive controls PB and Wy-14,643 produced the expected results with the exception of the lack of S-phase increase by PB.

Materials and Methods

Test Item:	Valifenalate
Description:	Not stated
Lot/Batch #:	P/13/024
Purity:	99.68% (HPLC)
Stability of test item:	Not stated
Control Items:	Phenobarbital sodium salt (PB, P-5178), Wy-14643 (C-7801) Epidermal growth factor (EGF, E-9644) were supplied by

	Sigma-Aldrich Company Ltd.
Diet:	RM1 powdered diet (Special Diet Services Ltd., Stepfield, Witham, Essex, UK)
Test animals –	
Species:	Mouse
Strain:	CD-1
Sex:	Male
Age at delivery:	5 - 6 weeks
Source:	Charles River UK Limited, Manston Road, Margate, Kent, CT9 4LT, England.
Acclimation period:	At least 5 days
Test diet:	RM1 powdered was available *ad libitum*.
Water:	Available *ad libitum*
Housing:	In groups on sawdust in solid-bottom, polypropylene cages.
Environmental conditions -	
Temperature:	19 - 23 °C
Humidity:	40 - 70 %
Air changes:	14 - 15 cycles per hour
Photoperiod:	12-hour light : 12-hour dark cycle

EXPERIMENTAL PROCEDURES

Hepatocyte isolation

Mice were terminally anaesthetised using euthatal and hepatocytes isolated by *in situ* perfusion. Viabilities of the hepatocyte preparations, as determined by Trypan Blue exclusion were in excess of 65% and hepatocytes from a minimum of two independent perfusions were pooled.

Hepatocyte Culture

Primary monolayer cultures of hepatocytes were prepared in 25 cm^2 flasks, 96- and 6-well plastic tissue culture plates as described below. In all 96-well plate cultures the outside wells were not used, but filled with sterile phosphate buffered saline.

For the preliminary dose ranging study, hepatocytes were cultured in Leibowitz CL15 medium for 4 hours to allow adherence, the medium changed and the hepatocytes then separately exposed to Valifenalate at 9 concentrations (0.03, 0.1, 0.3, 1, 3, 10, 30, 100 & 300 µM) and a vehicle control [0.1% v/v DMSO]. The media including test item was replenished daily for a further 3 days. There were 6 replicates for each concentration in 96-well plates for cell toxicity (ATP) measurements. ATP assays were run at 96 hours (after 4 daily exposures). The results were used to establish the concentrations to be assessed in the main part of the study (see below).

For the main part of the study, hepatocytes were also cultured in Leibowitz CL15 medium for 4 hours to allow adherence. The medium was then changed and the hepatocytes exposed to phenobarbital sodium salt at 2 concentrations (100 and 1000 µM) and Wy-14,643 at 2 concentrations (50 and 100 µM) as assay controls, to Valifenalate at 4 concentrations (10, 30, 100 and 300 µM) or to a vehicle control (0.1% DMSO).

The media, with test/control substances, was replenished daily for a further 3 days. There were 3 replicates for each concentration in 25 cm^2 flasks for enzyme activity measurements, 5 replicates for each concentration in 6-well plates for replicative DNA synthesis (S-phase) analysis, 3 replicates for each concentration in 6-well

plates for possible Taqman® analysis and 6 replicates for each concentration in 96-well plates for cell toxicity (ATP) measurements.

The test substances were formulated in DMSO and administered such that the final DMSO concentration in all cultures was 0.1% (1 µL DMSO/mL medium).

In addition to hepatocytes exposed to the test and reference items, 5 replicates in 6-well plates were exposed to EGF (25 ng/mL) as a positive control for induction of replicative DNA synthesis (S-phase).

Assays

After 96 hours in culture, hepatocytes were either methanol fixed or harvested by scraping into SET buffer (0.25 M sucrose, 5 mM EDTA, 20 mM Tris-HCl, pH 7.4). The scraped mixtures were sonicated and stored at –70ºC until analysis. Additional cells were harvested into RLT lysis buffer, then vortexed and stored at -70ºC for future Taqman® mRNA analysis. Protein was determined by the method of Lowry et al. (1951).

Adenosine 5'-Triphosphate (ATP)

Cell toxicity was assessed following 96 hours of culture as indicated by ATP depletion. Cellular Adenosine 5'-Triphosphate (ATP) was determined by luminometry.

The bioluminescent determination of ATP released from viable somatic cells was carried out using an assay kit supplied by Promega (CellTitre-Glo luminescent cell viability assay).

Results were expressed as a percentage of the maximum amount of ATP released (*i.e.* the value of control cells).

Replicative DNA Synthesis (S-Phase)

The number of cells undergoing replicative DNA synthesis (S-phase) in any given cell population can be determined by the incorporation of BrdU followed by immunostaining. S-phase was determined immunocytochemically following the incorporation of BrdU into hepatocyte nuclei over the last 3 days of culture. Immunostaining was performed after fixation at 96 hours. The number of hepatocytes undergoing DNA synthesis was recorded as the labelling index [(no. of labelled hepatocytes /total no. of hepatocytes) x 100].

Pentoxyresorufin-O-depentylation (PROD)

The activity of Cyp2b in cultured hepatocytes was determined spectrofluorometrically by the formation of resorufin from pentoxyresorufin, as described by Burke *et al.,* (1985).

12-hydroxylauric acid formation (LAH)

The activity of Cyp4a in cultured hepatocytes was measured as the formation of 12-hydroxylauric acid from lauric acid using LC-MS/MS detection.

Peroxisome proliferation

CN^- -insensitive acyl CoA oxidation was determined spectrophotometrically in cultured hepatocyte sonicates, using palmitoyl CoA as a substrate, as described by Bronfmann *et al.,* (1979).

Taqman® Analysis

RNA was extracted from cultured hepatocytes. cDNA was synthesised from all available RNA samples. TaqMan® analysis was performed on all available samples using primers specific for Cyp2b10, Cyp4a10, Cyp4a14 and Acox1 (Assay-on-demand kits, Applied Biosystems). Mouse specific β-actin was used as the internal standard (Assay-on-demand kits, Applied Biosystems). Data was analysed by generation of CT and delta CT values for all genes.

Statistics

Statistical comparisons between Valifenalate treated hepatocytes and their respective control groups were undertaken for all numerical data sets using a 2-tailed Student's t-test.

RESULTS AND DISCUSSION

Preliminary ATP assay to assess cytotoxicity of Valifenalate

There were small decreases in ATP levels detected at the majority of Valifenalate concentrations assessed. However, 300 µM Valifenalate produced a more robust decrease in ATP levels (28%). Therefore, this concentration of Valifenalate was selected as a suitable top concentration for the main part of the study (see Table 3.9.4.4-01).

Table 3.9.4.4-01 Preliminary ATP assay results for Valifenalate

Concentration	ATP content (luminescence units)
Vehicle control (0.1% [v/v] DMSO)	362763 ± 7399 (100.0 ± 2.0)
0.03 µM	424237 ± 69241 (116.9 ± 19.1)
0.1 µM	319773 ± 10807*** (88.1 ± 3.0)
0.3 µM	320368 ± 7535*** (88.3 ± 2.1)
1.0 µM	333349 ± 14455** (91.9 ± 4.0)
3.0 µM	346489 ± 11311* (95.5 ± 3.1)
10 µM	332221 ± 12478*** (91.6 ± 3.4)
30 µM	343492 ± 16540* (94.7 ± 4.6)
100 µM	369639 ± 20069 (101.9 ± 5.5)
300 µM	268274 ± 38891*** (74.0 ± 10.7)

Values are Mean ± SD. Values in parenthesis are mean % control ± SD; n = 6 per group. A Student's t-test (2-sided) was performed on the results; * statistically different from control p<0.05; ** p<0.01; *** p<0.001.

ATP assay to assess cytotoxicity of Valifenalate and PB during main study

Neither PB nor Wy-14,643 caused any statistically significant impact on ATP levels. Similarly, the ATP levels were unchanged following Valifenalate administration up to and including 100 µM. However, 300 µM Valifenalate caused a 73% decrease in ATP levels demonstrating that a cytotoxic concentration had been reached.

Table 3.9.4.4-02 ATP assay results for PB and Valifenalate from the main study

Test item and concentration	ATP content (luminescence units)
Vehicle control (0.1% [v/v] DMSO)	436918 ± 47416 (100.0 ± 10.9)
PB 100 µM	405658 ± 42447 (92.8 ± 9.7)
PB 1000 µM	432136 ± 27057 (98.9 ± 6.2)
Wy-14643 50 µM	475430 ± 45627 (108.8 ± 10.4)
Wy-14643 100 µM	496961 ± 76154 (113.7 ± 17.4)
Valifenalate 10 µM	461539 ± 35657 (105.6 ± 8.2)
Valifenalate 30 µM	440125 ± 13828 (100.7 ± 3.2)
Valifenalate 100 µM	435432 ± 23177 (99.7 ± 5.3)
Valifenalate 300 µM	116405 ± 48278*** (26.6 ± 11.0)

Values are Mean ± SD. Values in parenthesis are mean % control ± SD; n = 6 per group. A Student's t-test (2-sided) was performed on the results; *** statistically different from control p<0.001.

Biochemical assay results

In CD-1 mouse hepatocytes, PB caused significant elevations in PROD activity in a dose-dependent manner, increasing by 1.8- and 3.4-fold at 100 and 1000 µM respectively. PB caused small increases in 12-OH LA formation and PCoA oxidation but these did not reach statistical significance.

Conversely, Wy-14,643 had no effect on PROD activity in the CD-1 mouse hepatocytes but significantly increased both 12-OH LA formation and PCoA oxidation. At 50 and 100 µM Wy-14,643 12-OH LA formation was increased by 8.1- and 6.9-fold respectively and PCoA oxidation was increased by 4.9- and 5.4-fold respectively.

Valifenalate however, had essentially no impact on any of the biochemical markers assessed. The only statistically significant change was an increase in PCoA oxidation following administration at 100 µM (see Table 3.9.4.4-03).

Please note that sample 16 was omitted from the 12-OH LA formation assessment and samples 9 and 16 and PCoA oxidation assessment as they were considered to be outliers.

Table 3.9.4.4-03 Biochemical measurements for CD-1 mouse primary hepatocytes following PB, Wy-14,643 and Valifenalate administration

Test item and concentration	PROD (pmol resorufin/min/mg)	LAH nmols 12-OH formed / 60min / mg protein	PCoA nmol NAD+ reduced/min/mg protein
Vehicle control (0.1% [v/v] DMSO)	9.49 ± 1.70 (100.0 ± 18.0)	0.83 ± 0.16 (100.0 ± 19.5)	0.25 ± 0.04 (100.0 ± 14.3)
PB 100 µM	17.18 ± 1.70** (181.1 ± 17.9)	0.98 ± 0.25 (118.2 ± 30.2)	0.53 ± 0.18 (212.1 ± 72.1)
PB 1000 µM	31.79 ± 5.59** (335.2 ± 59.0)	1.57 ± 0.82 (189.2 ± 99.2)	0.73# (291.1)
Wy-14643 50 µM	8.75 ± 0.72 (92.3 ± 7.6)	6.71 ± 0.31*** (811.3 ± 37.1)	1.23 ± 0.23** (490.6 ± 91.2)
Wy-14643 100 µM	10.45 ± 1.38 (110.1 ± 14.5)	5.74 ± 1.28*** (694.0 ± 154.4)	1.36 ± 0.34** (541.9 ± 136.9)
Valifenalate 10 µM	13.79 ± 5.61 (145.4 ± 59.2)	0.91# (109.9)	0.51# (205.0)
Valifenalate 30 µM	11.43 ± 0.82 (120.5 ± 8.7)	0.80 ± 0.16 (96.9 ± 19.4)	0.48 ± 0.21 (191.7 ± 82.7)
Valifenalate 100 µM	12.85 ± 1.74 (135.5 ± 18.4)	0.72 ± 0.08 (87.5 ± 9.3)	0.40 ± 0.04** (160.6 ± 17.6)
Valifenalate 300 µM	9.15 ± 1.08 (96.5 ± 11.4)	0.81 ± 0.10 (97.8 ± 12.3)	0.37 ± 0.18 (149.4 ± 70.3)

Values are Mean ± SD. Values in parenthesis are mean % control ± SD; n = 3 per group (# n=2). A Student's t-test (2-sided) was performed on the results; ** statistically different from control p<0.01; *** p<0.001.

Taqman® analysis of mouse Cyp2b10, Cyp4a10, Cyp4a14 and Acox1 mRNA

PB caused 3.8- and 9.1-fold increases in Cyp2b10 mRNA levels at 100 and 1000 µM respectively. PB also caused small, but statistically significant increases in Cyp4a10 and Cyp4a14c mRNA levels. Acox1 mRNA levels were unaffected by this positive control.

Wy-14,643 caused large increases in both Cyp4a10 (>298-fold) and Cyp4a14 (>643-fold) mRNA levels. Wy-14,643 also caused small but significant increases in Cyp2b10 and Acox1 mRNA levels (see Table 3.9.4.4-04).

Valifenalate had no effect on Cyp2b10, Cyp4a10 or Acox1 mRNA levels at any concentration assessed. Small increases in Cy4a14 mRNA were measured at 10 and 30 µM Valifenalate but the top two concentrations assessed had no effect.

Table 3.9.4.4-04 Taqman® analysis of mouse Cyp2b10, Cyp4a10, Cyp4a14 and Acox1 mRNA from CD-1 mouse hepatocyte cultures following PB, Wy-14,643 and Valifenalate administration

Test item and concentration	Cyp2b10 mRNA	Cyp4a10 mRNA	Cyp4a14 mRNA	Acox1
Vehicle control (0.1% [v/v] DMSO)	1.00 ± 0.23	1.00 ± 0.16	1.00 ± 0.05	1.00 ± 0.13
PB 100 µM	3.77 ± 0.87**	1.51 ± 0.27*	1.81 ± 0.50*	0.84 ± 0.09
PB 1000 µM	9.11 ± 5.23	2.29 ± 0.21***	3.70 ± 0.43***	0.83 ± 0.04
Wy-14643 50 µM	2.73 ± 0.41**	391.80 ± 90.41**	762.31 ± 138.72***	2.07 ± 0.44*
Wy-14643 100 µM	2.96 ± 0.73*	297.84 ± 24.63***	643.00 ± 18.05***	2.23 ± 0.24**
Valifenalate 10 µM	0.61 ± 0.18	1.08 ± 0.28	2.32 ± 0.63*	0.74 ± 0.13
Valifenalate 30 µM	0.70 ± 0.15	1.39 ± 0.11*	3.01 ± 0.38***	0.76 ± 0.05*
Valifenalate 100 µM	0.82 ± 0.32	1.29 ± 0.18	1.61 ± 0.53	0.79 ± 0.09
Valifenalate 300 µM	0.54 ± 0.12*	1.01 ± 0.20	0.54 ± 0.29	1.07 ± 0.11

[a] Values are Mean ± SD (n=3 per group). Results are expressed as fold change relative to control, where control values were normalised to 1.00. Mouse β-actin was employed as the internal control. A Student's t-test was performed on the results; *statistically different from control p<0.05; ** p<0.01; ***p<0.001.

Replicative DNA synthesis (S-phase) results

In the male CD-1 mouse hepatocytes, PB had no effect on replicative DNA synthesis at either concentration assessed. However, Wy-14,643 increased replicative DNA synthesis by 1.5- and 1.7-fold at 50 and 100 µM respectively. EGF caused a robust response, increasing replicative DNA synthesis by 4.9-fold (see Table 3.9.4.4-05).

Valifenalate had no effect on replicative DNA synthesis (S-phase) at any concentration examined.

Table 3.9.4.4-05 S-phase labelling index for PB, Wy-14,643, Valifenalate and EGF in male CD-1 mouse hepatocyte primary cultures.

Test Item & Concentration	S-phase labelling index
Vehicle control (0.1% [v/v] DMSO)	1.80 ± 0.55 (100.0 ± 30.3)
PB 100 µM	1.28 ± 0.26 (71.0 ± 14.4)
PB 1000 µM	1.99 ± 0.35 (110.2 ± 19.6)
Wy-14,643 50 µM	2.79 ± 0.56* (154.9 ± 31.2)
Wy-14,643 100 µM	3.05 ± 0.23** (169.2 ± 13.0)
Valifenalate 10 µM	1.56 ± 0.32 (86.5 ± 17.9)
Valifenalate 30 µM	1.70 ± 0.28 (94.5 ± 15.4)
Valifenalate 100 µM	1.62 ± 0.20 (90.0 ± 11.2)
Valifenalate 300 µM	2.02 ± 0.56 (112.0 ± 31.3)
EGF 25 ng/mL	8.90 ± 1.18*** (494.2 ± 65.3)

Values are Mean ± SD. Values in parenthesis are mean % control ± SD; n = 6 per group. A Student's t-test (2-sided) was performed on the results; * statistically different from control p<0.05; ** p<0.01; *** p<0.001.

CONCLUSIONS

Administration of Valifenalate to male CD-1 mouse hepatocytes caused cytotoxicity at ≥300 µM; thus this was the top concentration assessed during the main study.

Neither PB nor Valifenalate had any impact on replicative DNA synthesis in the male CD-1 mouse hepatocytes. PB can often have a weak effect on mouse hepatocyte proliferation *in vitro*. However, Wy-14,643 increased replicative DNA synthesis by a maximum of 1.7-fold and EGF also produced a robust response, demonstrating that the test system could respond to a proliferative stimulus.

In both the male CD-1 mouse hepatocytes, PB induced Cyp2b10, as measured by Taqman® mRNA analysis and PROD activity, but as expected had little impact on the Cyp4a or peroxisome proliferation markers. Conversely, Wy-14,643 induced Cyp4a and peroxisome proliferation as measured by Taqman mRNA analysis (Cyp4a10, Cyp4a14 and Acox1), 12-OH LA formation and PCoA oxidation. Valifenalate had essentially no impact on any of the biochemical markers assessed.

To summarise, these data demonstrate that Valifenalate does not activate either mouse Car or Pparα when assessed *in vitro* as demonstrated by the lack of hypertrophic and hyperplasic responses in the CD-1 mouse hepatocytes. The positive controls PB and Wy-14,643 produced the expected responses in the mouse CD-1 hepatocytes other than the lack of S-phase increase by PB.

3.10 Reproductive toxicity

3.10.1 Animal data

3.10.1.1 Two-generation reproductive toxicity in rat – IR5885

Reference Number:	KII- Section 3, Volume 9-10, Annex IIA, point 5.6.1/02
Report:	See annex conf. 27
Guidelines:	OECD 416 (2001) and OPPTS 870.3800 (1998)
Deviation:	Weighing and preservation of organs and tissues from one randomly selected F1 male and female pup from each litter was erroneously not performed. This error is considered to have no impact on the validity of the study because weighing and preservation of these organs/tissues was performed for F2 pups and revealed no effects or findings of toxicological relevance.
GLP:	Yes (Certified laboratory)

Executive summary:

The purpose of this study was to provide general information concerning the effects of the test item, IR5885, on reproductive function as assessed by gonadal function, oestrus cycle, mating behaviour, conception, parturition, lactation, weaning and growth and development of the offspring. The study also provided information about the effects of the test item on neonatal morbidity, mortality, development and behaviour.

IR5885 was administered in the diet over two generations to males and females rats for 70-120-day pre-pairing period (P and F1 generation, respectively), during pairing period and, for females, during gestation and lactation periods. The study design included behavioural evaluation of the F1 progeny.

Dietary concentrations were set as shown in table 5.6.1-05; dietary concentrations were reduced during lactation period in order to avoid overdosing of lactating females, and maintain a more constant intake throughout the study.

Table 3.10.1.1-01 Dietary concentrations

Test group	Pre-pairing, pairing and gestation period	Lactation period and post-weaning
1	0 ppm (vehicle control)	0 ppm (vehicle control)
2	1250 ppm	850 ppm
3	4300 ppm	2900 ppm
4	15000 ppm	10000 ppm

Mean achieved test item intakes were as shown in table 5.6.1-06:

Table 3.10.1.1-02 Mean achieved test item intakes

		Group 2 (mg/kg bw/day)	Group 3 (mg/kg bw/day)	Group 4 (mg/kg bw/day)
P generation				
Males	Pre-pairing	80.8	277.4	986.3
	After pairing	61.4	216.1	757.9
Females	Pre-pairing	92.7	318.8	1150.3
	Gestation	79.2	273.2	992.8
	Lactation	123.9	408.4	1384.0
F1 generation				
Males	Pre-pairing	83.5	294.2	1024.8
	After pairing	63.8	216.3	763.8
Females	Pre-pairing	93.0	326.1	1145.6
	Gestation	84.1	295.5	1030.8
	Lactation	129.2	429.3	1383.3

Parent animals

Treatment with the test item was generally well tolerated in all P generation animals and F1 generation males. No test item-related mortalities or clinical signs of reaction to treatment were observed in any group, as well as effects on mean food consumption and bodyweights. For four F1 females of group 3 and one F1 female of group 4, total litter loss was noted.

In F1 generation females no signs of discomfort, clinical signs or differences in food consumption attributable to treatment were observed in any group during the pre-pairing or gestation periods. However, during early lactation ruffled fur was noted for four dams at 4300/2900 and 15000/10000 ppm. These findings were considered to be test item-related. Reduced food consumption was also noted in one female each at the two highest dosages.

Enlarged livers were noted in four P generation males at 15000/10000 ppm and in most of F1 males at 4300/2900 and 15000/10000 ppm as well as in one F1 female at the highest dosage.

In addition, reduced kidney weights were also noted in F1 females at the highest dosage. Increase liver weights were also noted in P and F1 males as well as in P females at 4300/2900 and 15000/10000 ppm.

Sperm analysis of P and F1 generation males did not reveal any treatment-related effects on sperm mobility, sperm morphology or on epididymal or testicular sperm count.
There was no indication for test item-related effects on ovarian follicles in the F1 generation.

Histopathological examination findings were limited to hepatocellular hypertrophy, decreased glycogen deposition in the liver (both sexes of P and F1 generation animals, at the two highest doses) and increased grading of tubular hyaline change in the kidneys (P and F1 generation males). However, these findings were considered not to represent adverse effects.
Slightly increased incidence and/or severity of follicular hypertrophy occurred in the thyroid gland in males at 4300/2900 and in both sexes at the highest dosage.

For both generations, the fertility rate was high, resulting in at least 21 litters per group for evaluation of reproduction data. There were no treatment-related effects on estrous cycles, medial precoital time, fertility indices, mean duration of gestation and number of implantations.

In F1 generation, the number of dead pups at first litter check was increased at 4300/2900 ppm. Neonatal pup mortality was increased at 4300/2900 and 15000/10000 ppm and the resulting viability indices were decreased at these dosages.

Offspring

F1 pup weights on days 1 to 21 post partum as well as F2 pups weights at day 1 were unaffected by treatment with the test item at all dosages.
F2 pups weights development during lactation resulted to be decreased at the two highest dosages, if compared to control pups at day 21.

No test item-related effect on sex ratios was noted in either generation at any dietary concentration.

No test item-related effect on sexual maturation and behavioural parameters was noted at all.

The types and frequency of gross lesions noted in F1 and F2 pups gave no indication of test item-related effects.

Spleen weights of F2 pups (both males and females) at 4300/2900 and 15000/10000 ppm were statistically significantly reduced. However, subsequent histopathological examination of spleen revealed no abnormal findings.

F2 pups of either sex showed some decrease in incidence and/or severity of glycogen deposition in the liver at 4300/2900 and 15000/10000 ppm. This finding, however, was considered to be of no adverse character.
Based on these data, it was concluded that 1250 ppm (reduced to 850 ppm during lactation/post weaning, and equivalent to about 80-100 mg/kg bw/day), was the overall NOAEL of IR5885 for all effects in P and F1 generation adults.
The NOAEL for reproductive effects was established at 15000/10000 ppm. *NB. Study report suggests a NOAEL of 1250/850 ppm based on an effect on post-implantation loss and reduced neonatal viability and weaning index at the mid and high dose levels, F1 only. This is due to the inclusion of 3 litters with total litter loss at the mid dose and 1 litter at the high dose. There is no evidence for the total litter losses being treatment-related. Exclusion of these litters from the calculated mean values confirms the lack of effect on the viability and survival of the offspring.*
The NOAEL for effects on pup development was established at 1250/850 ppm.
The NOAEL for effects on pup behaviour was established at 15000/10000 ppm (more than 1000 mg/kg bw/day).

MATERIALS AND METHODS

MATERIALS:

Test Item: IR5885
Description: White powder

Lot/Batch #:	T 025/02
Purity:	99.56%
CAS #:	283159-90-0
Stability of test item:	Stable in the dark at room temperature.
Vehicle and/or positive control:	None
Test animals –	
Species:	Rat
Strain:	HanBrl:WIST (SPF)
Age:	6 weeks at delivery
Weight at dosing:	188 – 232 g males; 131 – 154 g females
Source:	RCC Ltd Biotechnology & Animal Breeding Division Wölferstrasse 4 CH-4414 Füllinsdorf
Acclimation period:	7 days, minimum
Diet:	Pelleted standard Provimi Kliba-Nafag 3433 rat/mouse maintenance diet (Provimi Kliba AG, CH-4303 Kaiseraugst/Switzerland), *ad libitum*
Water:	Tap water from the municipal water main system, *ad libitum*
Housing:	At arrival, animals were caged individually in Makrolon cages (type 3) with wire mesh top and standard granulated softwood bedding. During the pre-pairing period males and females were caged separately one to a cage (cages of males were interspersed among those holding females to promote the development of regular estrus cycles). During the pairing period, the rats were housed one male/one female in Makrolon pairing cages. After mating or at the end of the pairing period the males and females were housed individually, again.
Environmental conditions -	
Temperature:	19 – 25 °C
Humidity:	30 – 70 %
Air changes:	10-15 per hour
Photoperiod:	12-hour light : 12-hour dark cycle. Music was played at low volume during light period

STUDY DESIGN AND METHODS:

In life dates:	October 28, 2002 to February 12, 2004

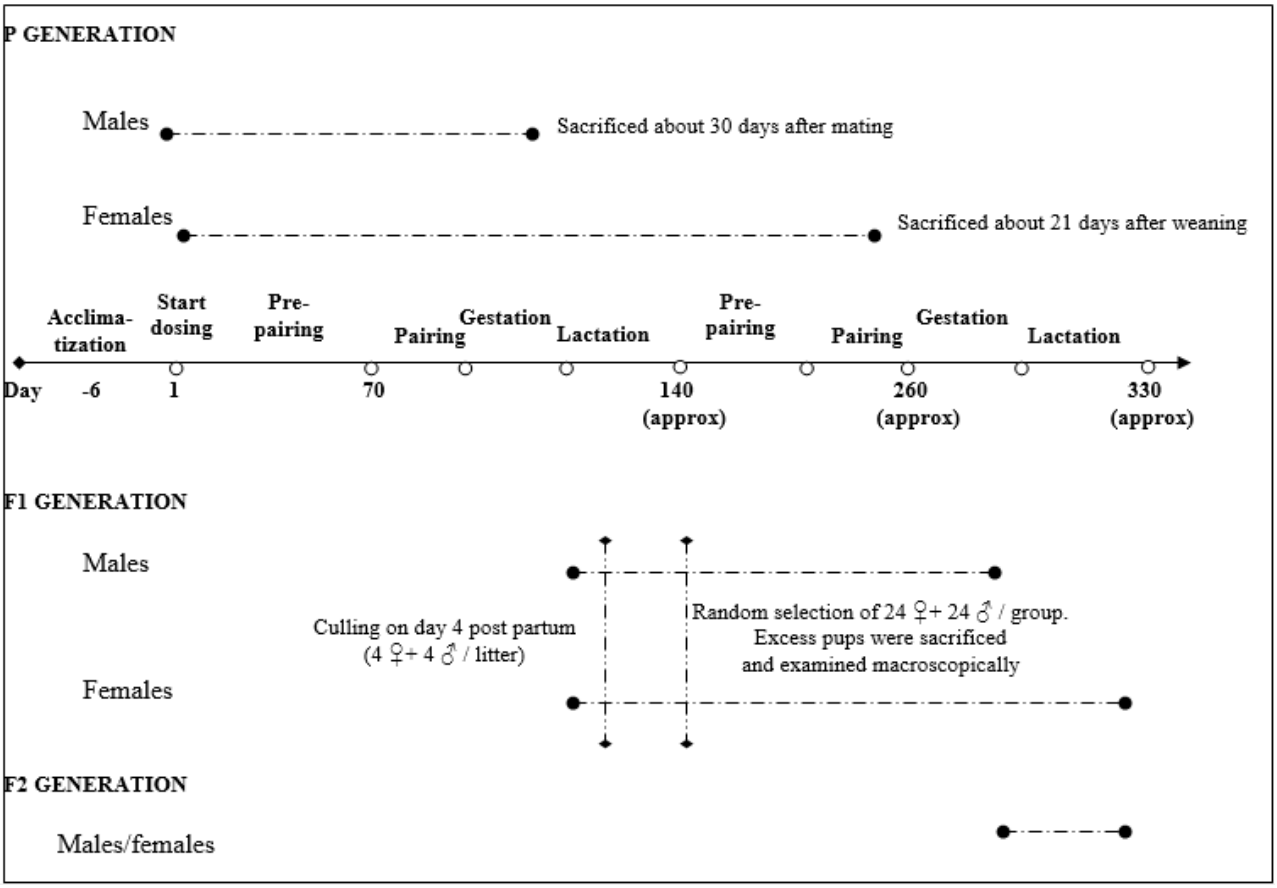
Animal assignment and treatment

24 animals of each sex per dose group were used. The P animals were randomly allocated to cages and treatment groups using a computer-generated algorithm, whereas the F1 animals were randomly selected. IR5885 was administered via the diet to parent animals and F1 offspring pups according to the concentrations and the timetable reported below. A negative control group received untreated diet. Dose levels were based in order to achieve a constant intake in terms of mg/kg bw/day avoiding overdosing of females during lactation period.
The test item was administered during a 70-day pre-pairing period and also during pairing, gestation and lactation periods for breeding of the F1 litters. Following weaning of F1 litters on day 21 post partum, F1 animals were selected for the next generation. The test item was administered during growth of the F1 generation to adulthood (at least 120-day pre-pairing period) and also during pairing, gestation and lactation periods for breeding of F2 litters. (Figure 3.10.1.2-02).

Table 3.10.1.1-03 Study design

Test group	Dietary concentrations in ppm: Pre-pairing, pairing and gestation / lactation and post-weaning	P generation: # of rats per group		F1 generation: # of rats per group	
		males	females	males	females
1	0 ppm (vehicle controls)	24	24	24	24
2	1250 / 850 ppm	24	24	24	24
3	4300 / 2900 ppm	24	24	24	24
4	15000 / 10000 ppm	24	24	24	24

Figure 3.10.1.1-01 - Course of the study



Diet preparation and analysis

The test item was mixed to granulated diet. Water was then added to each feed preparation at a volume/weight ratio of 1:5 to ensure pelleting, after which the pellets were dried with warm air for approximately 48 hours before storage. The feed preparations were stored in disposable paper bags at room temperature. Samples for analysis of test item, the content and homogeneity in the feed were drawn at the start of the pre-pairing periods and at the end of gestation/start of lactation periods (i.e. four occasions). The stability of the test item in the feed (up to 3 weeks) was determined at the start of the pre-pairing period of P generation (i.e. one occasion). For assessment of content and homogeneity samples of every dietary admixture (including controls) were collected from three different locations of the drying shelves. Evaluation of stability was performed by drawing samples of each dietary admixture on the day of preparation; these samples were stored at room temperature for 3 weeks and consequently retained frozen between –24 °C and –16°C prior to analysis.

Statistical analysis

The following statistical methods were used where appropriate to analyse body weights, food consumption, and reproduction data:

- Univariate one-way analysis of variance was used to assess the significance of intergroup differences.
- If the variables could be assumed to follow a normal distribution, the Dunnett t-test was used for intergroup comparisons
- The Steel test was applied when the data could not be assumed to follow a normal distribution
- Fisher exact test for 2x2 tables was applied if the variables could be dichotomised without loss of information.

METHODS:

Mortality and clinical signs

Animals were inspected at least twice daily for any mortality or for signs of reaction to treatment and/or symptoms of ill health. Additionally, a detailed physical examination was conducted on each animal on a weekly basis.

Body weight

Each animal was weighed on the first day of dosing and weekly thereafter, with the exception of the pairing period. After mating, females were weighed on days 1, 7, 14 and 21 post coitum. Dams that littered were weighed on days 1, 4, 7, 14, and 21 post partum and on the day the animals were sacrificed. Bodyweight of males were recorded weekly during the after pairing period until the day of sacrifice (day 29 after mating).

Food consumption, food conversion efficiency and achieve dosage

Food consumption was measured at weekly intervals, together with the recording of body weights, except during the pairing period when no food consumption was measured. During the lactation period, food consumption was recorded only until Day 14 post partum to avoid overestimation due to food consumed by pups. Relative food consumption ratios and intakes of the test item were calculated.

Mating

A record of mating of females was made by daily examination of vaginal smears for spermatozoa and/or appearance of a vaginal plug throughout the pairing period. The day of which evidence of mating was observed was considered to be Day 0 post coitum.

Gestation and parturition

Toward the end of the gestation period, females were examined twice a day for signs of parturition. The duration of gestation was calculated as the time between the day of successful mating and parturition. Pregnancy rates were calculated. Females without litters and female that lost their litter were killed and necropsied with the dams after weaning of the pups. Each uterus was placed in a solution of ammonium sulfide to visualize possible implantation sites.

Lactation and litter data

Day 0 of lactation was the day on which a female has delivered all her pups. As soon as possible**,** litters were examined for their size, live birth, stillbirth and any gross anomalies. The sex ratio of the pups was recorded on Day 0, 4 and 21 of lactation. Litters were caged together with the dam until weaning on Day 21 of lactation. Pups were weighed individually on Days 0 (if possible) and/or 1, 4, 7, 14 and 21 of lactation. To prevent cannibalism immediately after birth, the pups were weighed individually but without tattooing on Day 0 post partum. On Day 1 post partum, pups were tattooed individually with Indian ink. At the onset of hair growth, the pups were identified by colour spots on the fur. The dams and the pups were observed daily for survival, behavioural abnormalities in nesting and nursing. In cases where cannibalism was suspected but not confirmed by remains, the pup was designated as “missing”. When possible, dead young were autopsied and/or preserved

in fixative for possible further examination. On Day 4 post partum, the size of each litter was culled to (as near as possible) 4 males and 4 females per litter. The surplus pups were sacrificed and examined macroscopically.

Sacrifice and pathology

All P and F1 animals selected for breeding were sacrificed and subjected to a macroscopical examination when they were no longer necessary for the assessment of effects on reproduction. F1 pups not selected for the F1 generation pairing and all F2 pups were sacrificed and examined for defects after weaning; special attention was directed to the organs of the reproductive system. Implantation sites were counted for all dams. Each uterus was placed in a solution of ammonium sulfide to visualize possible implantation sites.
From all P and F1 parent animals, weights of the following organs were recorded: brain, liver, kidney, spleen, pituitary, adrenal glands, thyroid, ovaries, uterus, testes, epididymides, prostate and seminal vesicles. The following organs were recorded from one randomly selected male and female pup of each F2 litter (on Day 21 post partum): brain, spleen and thymus.

Histopathology

From all P and F1 animals selected for pairing and from one male and female pup of each F1 and F2 litter, samples of the following organs and tissues were collected at necropsy and fixed in neutral phosphate buffered 4% formaldehyde solution: gross lesions – ovaries – pituitary – adrenal glands – prostate – seminal vesicles with coagulating gland – right testis with epididymidis – uterus and cervix – vagina, kidneys and liver. Full histopathology was performed on the organs listed above for all high dose and control P and F1 animals selected for pairing, and one male and one female pup (selected for organ weight recording) of each F2 litter (high dose and control). Microscopic examination of all tissues showing gross pathological changes and of the reproductive organs of all infertile males and females was made. Because of reduced spleen weight observed in F2 pups, spleen was also examined.

Seminology and spermatid count

Motility: At terminal necropsy, the left testis and epididymidis were removed and weighed. An epididymidal sperm sample was obtained from the left caudal epididymidis of each male and soon after 100 sperm were counted microscopically for determination of percentage progressively motile sperm.
Morphology: A sample from the caudal epididymidis was also used for morphological assessment. 500 sperm per sample were evaluated microscopically and classified with regard to hook and tail (groups 1 and 4 only).
Sperm, spermatid count: The left caudal epididymidis and left testis were taken for determination of homogenization-resistant spermatids and caudal epididymidal sperm reserve (groups 1 and 4 only).

RESULTS AND DISCUSSION

Analytical results

Chemical analysis determined concentration, homogeneity and stability of IR5885 in rodent feed (Table 3.10.1.2-04).

Date of preparation: 30 October, 2002: The mean concentrations of the test item in the feed samples were found to be in the range from 88.8% to 94.3% of the nominal concentrations for the respective doses in groups 2 to 4. The individual concentrations varied in the range from –5% to +3% of the mean concentrations. Therefore, the test item was found to be homogeneously distributed in the feed. IR5885 was found to be stable in the feed (pellets) at room temperature (about 20°C) for at least 21 days.

Date of preparation: 23 January, 2003; 07 March, 2003; 24 July, 2003: The mean concentrations of the test item in the feed samples were found to be in the range from 78.9% to 87.1 % of the nominal concentrations for dose group 2, from 88.0% to 93.2% for dose group 3 and from 87.6% to 96.8% for dose group 4. The individual concentrations varied in the range from –5% to +3% of the mean concentrations. Therefore, the test item was found to be homogeneously distributed in the feed.

Table 3.10.1.1-04 Analytical results

TEST SAMPLES								FORTIFIED SAMPLES	
Dose group	Nominal conc. (ppm)	Date of analysis	A B C S	Concentration found					
				[ppm]	% of nominal	Mean % of nominal	± Dev. in % of mean	Nominal conc. [ppm]	Rec [%]
Date of preparation: 30 October, 2002									
1	0	08-JAN-03	--	0.0	--	---	---	---	---
2	1250	10-JAN-03	A	1121	89.7	88.8	-1 / +1	1309	117.3
			B	1109	88.7				
			C	1098	87.8				
		10-JAN-03	S	1094	87.5			1309	126.1
3	4300	08-JAN-03	A	3962	92.1	92.1	± 0	4425	127.1
			B	3975	92.4				
			C	3944	91.7				
		10-JAN-03	S	3867	89.9				
4	15000	08-JAN-03	A	14565	97.1	94.3	-5 / +3	15167	130.0
			B	14394	96.0				
			C	13479	89.9				
		10-JAN-03	S	14151	94.3				
Date of preparation: 23-January, 2003									
1	0	28-FEB-03	--	0.0	--	---	---	---	---
2	850	28-FEB-03	A	687.4	80.9	78.9	-5 / +3	936.3	104.2
			B	637.2	75.0				
			C	688.6	81.0				
3	2900	28-FEB-03	A	2596	85.9	88.0	-1 / +2	2809	100.7
			B	2545	87.7				
			C	2518	86.8				
4	10000	28-FEB-03	A	8787	87.9	87.6	± 0	9363	104.3
			B	8733	87.3				
			C	8750	87.5				
Date of preparation: 07-March, 2003									
1	0	21-MAR-03	--	0.0	--	---	---	---	---
2	1250	21-MAR-03	A	1083	86.6	87.1	-1 / +1	1275	101.5
			B	1078	86.3				
			C	1104	88.3				
3	4300	21-MAR-03	A	3822	88.9	91.4	-3 / +2	4281	102.5
			B	3997	93.0				
			C	3969	92.3				

TEST SAMPLES								FORTIFIED SAMPLES	
Dose	**Nominal**	**Date of**	**A**	**Concentration found**					
4	15000	21-MAR-03	A	14337	95.8	94.0	-3 / +2	14789	103.3
			B	14189	94.6				
			C	13730	91.5				
Date of preparation: 24-July, 2003									
1	0	20-AUG-03	--	0.0	--	---	---	---	---
2	850	20-AUG-03	A	724.5	85.2	85.2	± 0	912.9	100.8
			B	723.0	85.1				
			C	724.4	85.2				
3	2900	20-AUG-03	A	2687	92.7	93.2	-1 / 0	3140	101.0
			B	2710	93.4				
			C	2715	96.6				
4	10000	20-AUG-03	A	9546	95.5	96.8	-1 / +1	9830	103.4
			B	9733	97.3				
			C	9758	97.6				

A/B/C: Three different locations of the drying shelves
S: Storage stability test over a period of 21 days at room temperature
Rec: Recovery rate as used for correction of test samples

Parent animals – P generation

Mortalities and clinical signs

All animals survived until scheduled necropsy and no signs of discomfort or clinical signs of reaction to treatment were observed in any group. Findings of localised alopecia were noted both for males of group 2 and for females of group 4, but these were considered to be incidental since not dose-related.

Body weights

The test item did not affect body weight gain either during pre-pairing and after pairing periods (in males) or during pre-pairing, gestation and lactation periods in females. The only difference, when compared to respective control values, was the lower mean body weight gain of males of group 3 during pre-pairing period. This difference is considered to reflect normal biological variation since not showing any dose-relationship. Moreover, this difference was no more evident after pairing.

Table 3.10.1.1-05 Body weight gain in P generation (%)

	Group	Pre-pairing	After pairing	Gestation	Lactation
MALES	1	+91.8	+7.5	/	/
	2	+85.7	+7.0	/	/
	3	+78.5	+6.8	/	/
	4	+82.1	+6.3	/	/
FEMALES	1	+65.0	/	+45.4	+11.3
	2	+64.8	/	+47.6	+11.2

	3	+67.1	/	+45.4	+15.0
	4	+64.5	/	+47.9	+17.1

Values that significantly differ from respective control values are printed in bold.

Food consumption

Males – Pre-pairing and after pairing periods – Mean food consumption was similar in all groups during pre-pairing (21.3, 21.2, 20.3 and 21.6 g/animal/day for groups 1 to 4, respectively) and after pairing (21.9, 20.5, 20.1 and 21.0 g/animal/day, for groups 1 to 4, respectively). Relative food consumption in groups 2, 3 and 4 (during pre-pairing) was statistically significantly higher than that of the vehicle control in 6, 3 and 1 out of ten recording intervals, respectively. This difference was considered to be attributable to the incidentally marginally higher body weights noted in the vehicle controls. During the after pairing period, relative food consumption was similar in all groups (50.4, 49.1, 50.3 and 50.5 mg/kg bw/day in ordering of ascending dose level).

Females – Pre-pairing, gestation and lactation periods – During pre-pairing period mean food consumption of females was not affected by treatment with the test item. Overall mean food consumption in groups 1 to 4 was 15.1, 15.2, 15.5 and 15.6 g/animal/day, respectively. The statistically significant increase in food consumption that was noted in the group 3 and 4 during singular recording intervals was considered to be incidental. During gestation (18.3, 18.3, 18.8 and 19.3 g/animal/day for groups 1 to 4, respectively) and lactation (42.4, 41.4, 40.8 and 39.6 g/animal/day for groups 1 to 4, respectively) periods mean food consumption of females was not affected by treatment with the test item. During pre-pairing and gestation periods, the relative food consumption in groups 2 and 3 was similar to that of controls. During pre-pairing and gestation periods statistically significant increased mean relative food consumption was noted in group 4 (76.7 compared to 73.0 g/kg bw/day and 66.2 compared to 62.3 g/kg bw/day in the vehicle control, during pre-pairing and gestation periods, respectively). During lactation, decreased relative food consumption was noted in groups 3 and 4 (140.8 and 138.4 g/kg bw/day, respectively, compared to 150.9 g/kg bw/day in the vehicle control). This parameter was not affected by treatment in group 2 (145.8 g/kg bw/day).

Reproduction data

Estrous cycle – No test item-related effect on estrous cycles was noted. Mean estrous cycle duration was 4.0 days in all groups except for two females in group 4. For one of these females a shortened and for the other a prolonged cycle was noted. These finding were considered to be incidental.

Mating performance and fertility – All females mated during the 14-day pairing period. The median and mean precoital times were unaffected by treatment with the test item. Mean precoital times were 2.9, 3.0, 3.4 and 3.3 days in groups 1 to 4, respectively. Median precoital time was 3 days in all groups.
The fertility rate was generally high and fertility indices were similar in all groups (100.0% in groups 1, 2 and 4 and 95.8% in group 3). The gestation index was 100% in all groups.

Duration of gestation – No test item-related effect was noted. The mean duration of gestation was 21.6, 21.5, 21.7 and 21.5 days in groups 1 to 4, respectively.

Implantation rate and post-implantation loss – In group 3 one female was not pregnant. The mean number of implantations per litter was similar in all experimental groups (12.8, 12.3, 12.3 and 12.5 for groups 1 to 4, respectively). Post-implantation losses as a percentage of total implantations were 1.6, 2.4, 1.8 and 1.3% in order of ascending dose level.

Litter size at first litter check – Mean litter size at first litter check was similar in all groups (12.6, 12.0, 12.0 and 12.3 living pups per dam, respectively).

Postnatal loss (Day 0 - 4 post partum) – Neonatal mortality was generally low and considered to be unaffected by treatment with the test item. The total number of pups loss during the first four days of life were 3, 10, 10 and 2 for groups 1 to 4, corresponding to 1.0, 3.5, 3.6 and 0.7% of living pups, respectively. The higher number in group 2 was mainly due to one dam with total litter loss. Although the number of dead pups was statistically significantly higher in groups 2 and 3 than in vehicle control, the value was still low, did not show any dose dependency and was therefore considered to reflect biological variation. The resulting viability indices were 99.0, 96.5, 96.4 and 99.3% in order of ascending dose level.

Breeding loss – Pups mortality from Day 5 to Day 21 of lactation was unaffected by treatment with the test item. The resulting weaning indices were 100.0, 97.2, 100.0 and 96.4% for groups 1 to 4, respectively.

Terminal findings – P generation

Necropsy findings

Enlarged liver was noted in four males in group 4. This finding was confirmed by increased mean liver weight in this group. The other gross necropsy findings did not reveal an effect of treatment at any dose level.

Organ Weights

Males – Test item-related effects on organ weights were confined to increase absolute and relative liver weights in group 4 and increased liver weight in group 3. Lower absolute mean brain and thyroid weights were noted for males of groups 2 and 3, respectively. However, these differences, although statistically significant, were too small to be considered of toxicological relevance.

Females – Test item-related effects on organ weights were confined to increase absolute and relative liver weights in group 4 and increased absolute liver weight in group 3.

Table 3.10.1.1-06 Selected organ weights

Organ weight	Males				Females			
	Control	Low dose	Mid dose	High dose	Control	Low dose	Mid dose	High dose
Absolute liver weight (g)	12.45	12.32	12.52	14.45**	12.09	12.07	12.84*	13.95**
Relative liver/body weight (%)	2.84	2.90	3.08**	3.42**	4.59	4.53	4.74	5.10**
Absolute right kidney weight (g)	1.150	1.138	1.092	1.128	0.866	0.866	0.897	0.894
Absolute left kidney weight (g)	1.149	1.127	1.098	1.121	0.854	0.844	0.861	0.881
Relative right kidney/body weight (%)	0.262	0.268	0.268	0.268	0.329	0.325	0.331	0.327
Relative left kidney / body weight (%)	0.262	0.265	0.271	0.267	0.325	0.316	0.318	0.322

*/** Dunnett-test based on pooled variance sig. at 5% or 1% level

Sperm analysis data

Sperm motility – No treatment-related effects on sperm motility were noted at any dose level. Motility scores for groups 2 to 4 were similar to the vehicle control and gave no indication for any test item-related effects.

Morphology – Analysis of sperm morphology (performed ingroups 1 and 4 only) gave no indication for any test item-related effects. The percentage of complete sperm with normal hook and tail was 92.9% compared to

93.3% in the vehicle control. Also the percentages of the different categories of morphologically abnormal sperm gave no indication for any test item-related effects.

Sperm Head Count – Determination of homogenisation-resistant spermatids obtained from caudal epididymides or testes tissue samples gave no indication of any test item-related effects (examination performed in groups 1 and 4 only). 664.06 Mio spermatids per gram of epididymal tissue were counted in group 4 (compared to 657.60 in the vehicle control). 132.24 Mio spermatids per gram of testis tissue were counted in group 4 (compared to 137.08 in the vehicle control).

Histopathology findings

Kidneys – Treated males exhibited increased severity of tubular hyaline change in groups 3 and 4. This effect represents a male rat-specific phenomenon with no toxicological relevance for humans.
Liver – Treated males and females of groups 3 and 4 had decreased grading of hepatocellular deposition of glycogen when compared to the corresponding control values. Centrilobular hepatocellular hypertrophy of minimal to moderate severity was observed in 7 out of 24 and 15 out of 24 males of groups 3 and 4, respectively, versus 4 out of 24 males in the vehicle control.

Effects on liver and kidneys were considered not to represent adverse effects.

F1 pups

Abnormal findings at birth and during lactation to weaning – No test item-related findings were noted at first litter check or during lactation.

Sex ratios – Sex ratios at first litter check and on Day 21 post partum were unaffected by treatment with the test item. The proportion of males at first litter check was 49, 48, 44 and 49% in order of ascending dose level. On Day 21 post partum the proportion of males was 49, 48, 47 and 50%.

Pups weight to weaning (Day 21 post partum) – Mean pups weight at Day 0 and Day 1 post partum were unaffected by treatment with the test item. For pups whose weights were recorded at Day 0 post partum, mean body weights were 5.7, 5.8 and 5.6 g in groups 2 to 4 (no Day 0 data are available for the vehicle control). On Day 1 post partum mean pup weights were 5.6, 5.7, 5.7 and 5.8 g in order of ascending dose level.
Mean pup weight development during lactation was unaffected by treatment with the test item. Mean pups weight on Day 21 post partum were 45.8, 45.5, 44.3 and 42.7 g in order of ascending dose level.

Sexual maturation - The age at which vaginal patency or preputial separation occurred in F1 pups was unaffected by treatment with IR5885 at all dose levels. The group mean ages at which male preputial separation occurred were 26.7, 26.1, 26.2 and 26.1 days. Vaginal patency occurred at mean age of 35.1, 34.9, 34.8 and 35.4 days, in order of ascending dose level.

Necropsy findings – The types and frequency of gross lesions noted in F1 pups gave no indication of any test item related effects.

F1 generation behaviour tests

Modified Irwin screen test – During performance of the modified Irwin Screen Test no findings considered to be related to the test item were noted.

Locomotor activity – Locomotor activity of males and female F1 progeny, as assessed quantitatively in terms of low beam counts, was not affected by treatment with the test item. Although mean overall locomotor activity of group 4 females was satistically significantly lower than that of vehicle control (1314 versus 1599 low beam counts), the difference was too small to be an adverse effect.

Parent animals – F1 generation

Mortalities and clinical signs

All animals survived until scheduled necropsy and no signs of discomfort or clinical signs of reaction to treatment were observed in any group.
In groups 3 and 4, ruffled fur was noted in five and four dams, respectively, during early lactation and was considered to be test item-related. Localised alopecia was noted in one male of group 1 and in one female of group 3. Irregular upper incisor growth was noted in one female of group 4. However, these observations were considered to be incidental.

Body weights

Test item did not affect body weight gain either during pre-pairing and after pairing periods in males or during pre-pairing, gestation and lactation periods in females.

Table 3.10.1.1-07 Body weights gain in F1 generation (%)

	Group	Pre-pairing	After pairing	Gestation	Lactation
MALES	1	+321.9	+8.0	/	/
	2	+326.2	+7.4	/	/
	3	+332.3	+9.1	/	/
	4	+307.5	+8.4	/	/
FEMALES	1	+162.9	/	+37.6	+13.1
	2	+165.6	/	+35.4	+16.3
	3	+164.1	/	+38.3	+15.0
	4	+176.1	/	+38.3	+12.7

Food consumption

Males – Pre-pairing and after pairing periods – Mean food consumption was similar to control in group 4 during pre-pairing period. Mean food consumption in groups 2 and 3 was slightly lower (-4.0% and –4.5%, respectively) compared to vehicle control. However, in the absence of an effect on mean food consumption in group 4, this lower food consumption was considered to be incidental. During after pairing period only mean food consumption in group 3 remained marginally lower, while food consumption in groups 2 and 4 was similar to that of vehicle control. Relative food consumption in groups 2 to 4 (during pre-pairing) was slightly lower than that of the vehicle control but without any dose-relationship. During after pairing period, relative food consumption was similar in all groups (50.8, 51.1, 50.3 and 50.9 g/kg bw/day in ordering of ascending dose level).

Females – Pre-pairing, gestation and lactation periods – During pre-pairing period mean food consumption of females was not affected by treatment with the test item. Overall mean food consumption in groups 1 to 4 was 16.6, 15.5, 15.1 and 15.8 g/animal/day, respectively. During gestation (20.3, 20.3, 20.3 and 20.9 g/animal/day for groups 1 to 4, respectively) mean food consumption of females was not affected by treatment with the test item. During lactation period mean food consumption was reduced in groups 3 and 4 (-7.2 and –11.3%, respectively, compared with the vehicle control) while in group 2 this parameter was unaffected by treatment. The relative food consumption in groups 2, 3 and 4 was slightly higher and slightly lower, during pre-pairing and gestation periods, respectively, when compared to control values. In the absence of any consistency and dose dependency these differences were considered to be incidental. During lactation, decreased relative food consumption was noted only in group 4 (138.3 g/kg bw/day compared to 152.2 g/kg bw/day in the vehicle control).

Reproduction data

Estrous cycle – No test item-related effect on estrous cycles was noted. Mean estrous cycle duration was 4.0, 4.0, 4.1 and 4.1 days for groups 1 to 4. Slightly shortened cycles were noted in two females in group 1, one female in group 2 and two females in group 3. Slightly prolonged cycles were noted in one female in groups 1, 3 and 4.

Mating performance and fertility – All females in groups 2 to 4 mated during the 14-day pairing period. In group 1, three females did not mate.
The median and mean precoital times were unaffected by treatment with the test item. Mean precoital times were 2.9, 3.0, 4.0 and 3.8 days in groups 1 to 4, respectively. Median precoital time was 3, 2, 3 and 3 days in groups 1 to 4.
The fertility rate was generally high and fertility indices were higher in groups 2 to 4 than in the vehicle control (87.5% in group 1, 95.8% in groups 2, 3 and 4, respectively). The gestation index was 100% in groups 1, 2 and 4 and 95.7% in group 3.

Duration of gestation – No test item-related effect was noted. The mean duration of gestation was 21.8, 21.6, 21.8 and 21.6 days in groups 1 to 4, respectively.

Implantation rate and post-implantation loss – The mean number of implantations per litter was similar in all experimental groups (12.7, 12.2, 12.0 and 12.6 for groups 1 to 4, respectively). Post-implantation losses as a percentage of total implantations were 8.6, 11.4, 14.2 and 11.4% in order of ascending dose level. The statistically higher value reported for group 3 could also be due to pup loss and cannibalization before first litter check.

Litter size at first litter check – Mean litter size at first litter check was similar in groups 1 to 4 (11.6, 10.8, 10.3 and 11.1 living pups per dam, respectively). In groups 2 and 4, the number of dead pups at first litter check was similar to the vehicle control while in group 3, 19 dead pups in 6 litters were found (compared to 0 dead pups in the vehicle control).

Postnatal loss (Day 0 - 4 post partum) – Neonatal mortality was increased in groups 3 and 4 while in group 2 it was similar to that of the control. The total number of pups loss during the first four days of life were 18, 8, 35 and 39 for groups 1 to 4, corresponding to 7.4, 3.2, 14.8 and 15.2% of living pups, respectively. Total litter loss was noted for three dams in group 3 and one dam in group 4. The resulting viability indices were 92.6, 96.8, 85.2 and 84.8% in order of ascending dose level.

Breeding loss – Pups mortality from Day 5 to Day 21 of lactation was increased in groups 3 and 4. The total number of pups per group dying during this period was 4, 1, 9 and 10 in order of ascending dose level. The resulting weaning indices were 97.5, 99.4, 94.2 and 93.9% for groups 1 to 4, respectively.

N.B. The data for postnatal loss and for breeding loss have been reported including the total litter losses in the calculation of the group mean values. However, the occurrence of the total litter losses is considered to be incidental to treatment given the lack of dose response, the absence of pup death amongst surviving litters and the lack of a similar effect in the P litters. The data are presented including and excluding the animals with total litter loss, 3 in the mid dose group and 1 in the high dose group.

Table 3.10.1.1-08: Summary table of litter data in F1 animals – selected parameters

F1 Group	Control	Low dose	Mid dose	High dose
All dams giving birth	N = 21	N = 23	N = 23	N = 23
Mean no. implantations	12.7	12.2	12.0	12.6
% post-implantation loss	8.6	11.4	14.2	11.4
Mean no. dead pups at 1st check	0.0	0.2	0.8	0.0
Mean no. live pups at 1st check	11.6	10.8	10.3	11.1
% Postnatal loss days 0-4	7.4	3.2	14.8	15.2

% viability index	92.6	96.8*	85.2**	84.8**
All dams weaning young	N = 21	N = 23	N = 19	N = 22
Mean no. implantations	12.7	12.2	11.5	12.4
% post-implantation loss	8.6	11.4	9.1	12.1
Mean no. dead pups at 1st check	0.0	0.2	0.2	0.0
Mean no. live pups at 1st check	11.6	10.8	10.5	10.9
% Postnatal loss days 0-4	7.4	3.2	5.5	9.6
% viability index	92.6	96.8*	94.5	90.4

*/** statistically significant difference from control at 5% /1% level

These data clearly demonstrate that the apparent increase in post-implantation loss / neonatal loss is attributable to the inclusion of the animals with total litter loss. The occurrence of the total litter loss was unrelated to treatment. Therefore, it can be concluded that valifenalate has no adverse effect on pup survival in utero or post partum.

Terminal findings – F1 generation

Necropsy findings

Enlarged liver was noted in 12 and 17 (out of 24, each) males, in groups 3 and 4, respectively, compared to 7 animals in vehicle control. Enlarged liver was also noted in one female in group 4. The other gross necropsy findings were within the range of spontaneous findings that may be recorded in rats of this strain and age and therefore did not reveal an effect of treatment at any dose level.

Organ Weights

Males – Test item-related effects on organ weights were confined to increase absolute and relative liver weights in group 4 and increased relative liver weight in group 3. Lower absolute mean brain as well as mean right epididymides weight and cauda epididymides weight were noted in males of group 4. However, these differences, although statistically significant, were too small to be considered of toxicological significance.

Females – Test item-related effects on organ weights were confined to increase relative liver weight and reduced absolute and relative kidney weights in group 4. Absolute ovarian weights as well as ovary/brain ratios were reduced in group 4. This finding may reflect the tendency to lower content of corpora lutea found in this group, which was considered to be not related to the treatment with the test item because only 3 out 24 females of group 4 had a cycle stage in metestrus, being the other generally in diestrous. This distribution of a functional state, which may be related to group and housing effects, may be the cause of a tendency towards a lower corpora lutea content in the ovaries of group 4 females, since corpora lutea formation occurs during estrus/metestrus.

Table 3.10.1.1-09 Selected organ weights

Organ weight	Males				Females			
	Control	**Low dose**	**Mid dose**	**High dose**	**Control**	**Low dose**	**Mid dose**	**High dose**
Absolute liver weight (g)	14.49	14.65	15.35	16.18**	12.37	12.24	12.27	13.30
Relative liver/body weight (%)	2.97	3.07	3.22**	3.38**	4.48	4.54	4.63	4.92**
Absolute right kidney weight (g)	1.228	1.172	1.182	1.233	0.945	0.906	0.906	0.875*

Absolute left kidney weight (g)	1.205	1.163	1.172	1.223	0.906	0.874	0.879	0.858
Relative right kidney/body weight (%)	0.253	0.246	0.249	0.258	0.342	0.336	0.343	0.323*
Relative left kidney / body weight (%)	0.248	0.244	0.246	0.256	0.328	0.324	0.333	0.318

*/** Dunnett-test based on pooled variance sig. at 5% or 1% level

Sperm analysis data

Sperm motility – No treatment-related effects on sperm motility were noted at any dose level. The proportion of non-motile, stationary and progressively motile sperm in groups 2 to 4 was similar to the vehicle control and gave no indication for any test item-related effects.

Morphology – Analysis of sperm morphology (performed in groups 1 and 4 only) gave no indication for any test item-related effects. The percentage of complete sperm with normal hook and tail was 92.6% compared to 90.9% in the vehicle control.

Sperm Head Count – Determination of homogenisation-resistant spermatids obtained from caudal epididymides or testes tissue samples gave no indication of any test item-related effects (examination performed in groups 1 and 4 only). 573.71 Mio spermatids per gram of epididymal tissue were counted in group 4 (compared to 575.54 in the vehicle control). 113.27 Mio spermatids per gram of testis tissue were counted in group 4 (compared to 115.07 in the vehicle control).

Histopathology findings

Kidneys – Treated males exhibited increased severity of tubular hyaline change in groups 3 and 4. The average grade was 2.2 and 2.3, respectively, versus 1.6 in the vehicle control. This finding represents a male rat-specific phenomenon with no toxicological relevance for humans.

Liver – Treated males of groups 3 and 4 had slightly decreased incidences (group 4 only) and severity of hepatocellular deposition of glycogen when compared to the control group. This finding was considered to reflect the nutritional state of the animals and not to be of any adverse character.
Centrilobular hepatocellular hypertrophy generally slight in degree was observed in 17/24 and 21/24 males of groups 3 and 4, respectively versus 2/24 males in the vehicle control. In the highest dose female group, 21 out of 24 animals showed centrilobular hepatocellular hypertrophy minimal to slight in degree, correlating with increased relative liver weight and with enlarged liver in one animal.

Thyroid gland – Slightly increased incidences and severity of follicular hypertrophy were observed in group 4 animals of either sex. 22/24 (mean grade 2.1) males and 19/24 (mean grade 1.6) females were affected versus 17/24 (mean grade 1.4) males and 10/24 (mean grade 1.1) females of the vehicle control. In males the severity of this finding was also evident in group 3, where an average grade of 1.8 was noted.

Quantitative ovarian histopathology data for F1 females – Counts of ovarian follicles and corpora lutea were performed in ten females each of the vehicle control and group 4. Counts were similar in both groups, indicating no test item-related effect.

F2 pups

Abnormal findings at birth and during lactation to weaning

No test item-related findings were noted at first litter check or during lactation. In group 1 three partially cannibalized pups from one litter were killed for human ethical reason. In group 3, all twelve pups from one litter were dead and partially cannibalized. During lactation, the incidence of the finding “no milk in the

stomach" was increased in groups 3 and 4 between Day 2 and 5 of lactation. Most of the affected pups were found dead or cannibalized. The increased incidence was considered to be test item-related.

Sex ratios

Sex ratios at first litter check and on Day 21 post partum were unaffected by treatment with the test item. The proportion of males at first litter check was 50, 50, 48 and 52% in order of ascending dose level. On Day 21 post partum the proportion of males was 51, 51, 49 and 53%.

Pups weight to weaning (Day 21 post partum)

Mean pups weights at Day 1 post partum were unaffected by treatment with the test item. On Day 1 post partum mean pup weights were 5.7, 5.4, 5.8 and 6.0 g in order of ascending dose level.
Mean pup weight development during lactation was statistically significantly lower than control value in groups 3 and 4 on Day 21 post partum (45.1 g and 45.8 g, respectively, versus 49 g in control animals). Mean pup weight development in group 2 was unaffected by treatment with the test item (46.9 g versus 49 g in vehicle control animals.).

Necropsy findings

The types and frequency of gross lesions noted in F2 pups gave no indication of any test item related effects.

Organ weight data

Mean brain and thymus weights of male and female F2 pups gave no indication of test item related effects. Absolute and relative organ weights were similar in all groups. Statistically significantly reduced mean spleen weights were noted for male and female pups in groups 3 and 4. Histopathological examination of spleen failed to reveal any abnormalities.

Histopathological data

Liver – Males and females of groups 3 and 4 had slightly decreased incidences and severity of hepatocellular deposition of glycogen when compared to the corresponding control groups. However, these finding were considered to reflect the nutritional state of the animals and not to be of any adverse character.

CONCLUSIONS

IR5885 administered in the diet over two generations rats was generally well tolerated without sign of over toxicity. No effects on estrous cycle, medial precoital time, fertility index, mean duration of gestation, number of implantations as well as on sperm analysis were observed in both generations. In F1 only, the number of dead pups at first litter check and viability index were reduced at the two higher dosages (about 300-400 mg/kg bw/day and more than 1000 mg/kg bw/day, respectively).
Histopathological findings were limited to minimal to moderate centrilobular hepatocyte hypertrophy in both parental generations, considered adaptive in nature.
The overall NOAEL was established at 1250/850 ppm (equivalent to about 80-100 mg/kg bw/day).

The NOAEL for reproductive effects was established at 15000/10000 ppm (more than 1000 mg/kg bw/day).

The NOAEL for effects on pup development was established at 1250/850 ppm on the basis of reduced pups' weight recorded at weaning of F2 pups at higher dosages.

The NOAEL for effects on pup behaviour was established at 15000/10000 ppm (more than 1000 mg/kg bw/day), the highest dosage tested, based on the lack of any adverse effects in pups' behaviour in either generation.

3.10.2 Human data

No human data available on Reproductive Toxicity.

3.10.3 Other data (e.g. studies on mechanism of action)

3.10.3.1 Teratogenicity test by the oral route in the rat - IR5885

Reference Number: KII- Section 3, Volume 10, Annex IIA, point 5.6.10/02

Report: See conf. annex 64

Guidelines: OECD 414 (2001)

Deviation: None

GLP: Yes (Certified laboratory)

Executive summary:

The effects of IR5885 given by oral route to presumed pregnant female Crl:CD (SD) BR rats were assessed in this study. Females were treated from the day 6 to day 19 of pregnancy, the day of positive vaginal smear being considered as day 0 of pregnancy.
Dose levels tested were 100, 300 and 1000 mg/kg/day on the basis of the results of a Preliminary Teratogenesis study in rats (RBM Exp. R15030), and considering the prolongation of treatment required by the updated guideline. Control rats received 0.5% methylcellulose aqueous solution (w/v). On day 20 of pregnancy dams were killed, laparotomized and subject to post mortem examination.

No clinical signs, behavioural changes or deaths were observed in any group.

A slightly higher mean bodyweight gain was observed in the treated groups compared to control group being the statistically significant level reached only at 1000 mg/kg/day during the interval days 6-12 of pregnancy.
No embryo-foetotoxic effects were observed up to and including 1,000 mg/kg bw/day.
No compound-related malformed foetuses were found, the only one malformed foetus with agnathia found at 300 mg/kg bw/day was considered incidental since not dose-related and owing to the low incidence.
No effects were noted at the autopsy of the dams.

In conclusion, IR5885, given by the oral route to the pregnant rats during the pregnancy period, induced only a slightly higher mean bodyweight gain, being statistically significant only at 1000 mg/kg bw/day. Neither embryo-fetotoxic nor compound related malformed foetuses were seen up to and including 1000 mg/kg bw/day, therefore, 1000 mg/kg bw/day was set as the NOAEL for dams and as the NOEL for foetuses.

MATERIALS AND METHODS

MATERIALS:

Test Item: IR5885

- **Description:** White powder
- **Lot/Batch #:** FCF/T/180-00 (ex ZI-068)
- **Purity:** 98.9%
- **CAS #:** 283159-90-0
- **Stability of test item:** Stable in the dark at room temperature

Vehicle: 0.5% methylcellulose aqueous solution (w/v)

Test animals –

Species:	Rat
Strain:	Crl:CD (SD) BR
Age:	9-10 weeks at arrival. 11-12 weeks at dosing.
Weight:	200-225 g at arrival. 233-333 g at beginning of dosing.
Source:	Charles River Italia S.p.A. – Lecco (Italy). Shipping slip No. 1EC05138, July 6, 2001.
Acclimation period:	about 2 weeks
Diet:	GLP 4 RF 25 top certificate diet (Charles River Italia) *ad libitum*
Water:	Tap water from the municipal water main system, *ad libitum*
Housing:	Divided by sex and kept in 3D Makrolon cages. Males: 2/cage; females: 2/cage during pre-mating, individually in same type cages after the assessment of pregnancy.
Environmental conditions -	
Temperature:	22 °C ± 2
Humidity:	55% ± 15
Air changes:	15-20 per hour
Photoperiod:	12-hour light : 12-hour dark cycle (7 a.m. – 7 p.m.)

STUDY DESIGN AND METHODS:

In life dates: July 18 to August 23, 2001

Animal assignment and treatment

25 copulated female rats per dose group were used. Animals were non-selectively allocated to cages and treatment groups. IR5885 was administered by gavage from day 6 to 19 of pregnancy, the day of positive vaginal smear being considered as day 0 of pregnancy, at the doses shown in table 5.6.10-02. A negative control group received 0.5% methylcellulose aqueous solution.

Table 3.10.3.1-01 Study design

Test group	Females with positive smear	dosage (mg/kg bw/day)	Volume administered (mL/kg/day)	Test item concentration in vehicle (mg/mL)
1	25	0	10	0
2	25	100	10	10
3	25	300	10	30
4	25	1000	10	100

Test item formulate preparation and analysis

Appropriate amounts of test item were weighed, ground in mortars with some drops of vehicle, transferred into graduated containers, made up to the final volumes with vehicle and magnetically stirred to obtain suspension at the final concentration of 10, 30 and 100 mg/mL. Formulates were kept magnetically stirred until the end of daily administration.
Data concerning the stability of the test item in the vehicle up to 96 hours at room temperature in the concentration's range of 1 and 250 mg/mL were supplied by the Sponsor.
Concentration checks throughout the study were performed by an external GLP certified laboratory (ChemService S.r.l. – Milano - Italy) on formulates prepared by RBM on July 27, 2001. The storage stability of the test item was confirmed at nominal concentrations ranging from 10 to 100 mg/mL. The percentage of test item found, compared to nominal values, ranged from -8.7% to 3.50% (-3.0% for the low, -8.67% for the intermediate and 3.50% for the high dose level, respectively).

Statistics

To compare frequencies, the heterogeneity test (CHI square 2xN) and Fisher's exact test were applied. Then trend test was also applied. All these tests were one-sided.
Group mean values were calculated in two ways: Mean A (calculated on all the surviving females having evident signs of pregnancy including those that presented 100% post-implantation losses) – Mean B (calculated only on those females with viable foetuses at term).

METHODS:

Observations

Animals were observed three times a day for mortality, physical appearance, behavioural and clinical signs. During the treatment period, the animals were observed for any reaction to the treatment.

Body weight

Each animal was weighed on days 0, 6, 8, 10, 12, 14, 16, 18, 19 and 20 of pregnancy.

Food consumption

The leftover amounts of the weighed food offered to each dam were recorded on days 6, 8, 10, 12, 14, 16, 18, 19 and 20 of pregnancy in order to calculate the mean food consumption as g/animal/day.

Sacrifice and pathology

On day 20 of pregnancy**,** the dams were killed by cervical dislocation after preliminary CO_2 anaesthesia, and a gross pathology examination was performed. The following parameters were considered: pregnant uterus weight – number of corpora lutea – number of implantations – number of resorptions: a) early, placenta visible b) late, placenta and embryo visible – number and sex of viable foetuses – number and sex of dead foetuses – individual foetus weight – individual placenta weight.
A preliminary gross external examination was performed on all foetuses immediately. The externally malformed foetuses were fixed in order not to lose the evidence of malformation. Skeletal malformations, anomalies or variants were seen by clearing about half of the foetuses per litter. The observations were classified as follows: malformations: rare and/or usually lethal – anomalies: more frequent and not lethal – variants: common in control populations and often definable only in terms of continuous variable gradients.
The uteri of apparently non-pregnant female were stained according to Salewski and examined for the presence of implantation sites.

RESULTS AND DISCUSSION

Clinical signs and mortality

No clinical signs, behavioural changes or deaths were observed during the study.

Body weight, bodyweight gain and food consumption

A slightly higher mean bodyweight gain was observed in the treated groups in comparison with the control group being statistically significant level reached only at 1000 mg/kg bw/day during the interval days 6-12 of pregnancy.

No statistically significant differences were found on mean daily food consumption of the treated groups and that of control group.

Uterus content: effect on embryo-foetal development

No embryotoxic effects were observed up to and including 1000 mg/kg bw/day.
No effects were noted on mean foetal weight, mean placental weight, mean pregnant uterus weight and on the mean litter weight in any treated group.

Foetal examination

The external examination showed one malformed foetus in the 300 mg/kg bw/day treated group with agnathia. No other malformed foetuses were observed either at the skeletal or at the visceral examinations.
No effects were noted in the frequency per group and in the mean value per litter of skeletal and visceral anomalies and variants in any experimental group. The statistically significantly higher frequency per group of skeletal variants observed at 100 mg/kg bw/day was considered incidental since without any dose-relationship.

Autopsy:

No pathological changes were seen at the schedule killing of dams.

CONCLUSIONS

The test item IR5885 given by oral route to pregnant rats during the pregnancy period, at the doses of 100, 300 and 1000 mg/kg bw/day induced only a slightly higher mean body weight gain in the treated groups in comparison with the control group, being the statistically significant level reached only at 1000 mg/kg bw/day during the interval days 6-12 of pregnancy.
Neither embryo-foetotoxic effects nor compound-related malformed foetuses were seen up to the highest concentration tested. Therefore, 1000 mg/kg bw/day was set as the NOAEL for dams and as the NOEL for foetuses.

3.10.3.2 Teratogenicity test by the oral route in the rabbit – IR5885

Reference Number:	KII- Section 3, Volume 11, Annex IIA, point 5.6.11/02
Report:	See annex conf. 10
Guidelines:	OECD 414 (2001)
Deviation:	None
GLP:	Yes (Certified laboratory)

Executive summary:

The effects of IR5885 given by oral route to presumed pregnant female New Zeeland white rabbits were assessed in this study. Females were treated from the day 6 to day 28 of pregnancy, the day of positive vaginal smear being considered as day 0 of pregnancy.
Dose levels tested were 100, 300 and 1000 mg/kg/day on the basis of the results of the preliminary teratogenesis study in rabbits (RBM Exp. R15050), and considering the longer treatment period according to the updated guideline. Control rabbits received 0.5% methylcellulose aqueous solution (w/v). On day 29 of pregnancy dams were killed, laparotomized and subject to post mortem examination.

No clinical signs, behavioural changes or compound-related deaths were observed during the study.

A slightly higher mean bodyweight gain was observed in the 300 and 1000 mg/kg bw/day treated groups during the treatment period (6-28 of pregnancy) in comparison with the control group, without any dose-relationship.

No embryo-toxic effects were observed up to and including 1000 mg/kg bw/day.
Dead foetuses were present in one litter/group in each treated group without any increase in frequency with increasing the dosages.
The foetal examination showed one foetus with arthrogryposis, one foetus with misshapen sternum and one foetus with scoliosis in two separate litters of the 1000 mg/kg bw/day group, and one foetus with the absence of left testis in the 100 mg/kg bw/day. No malformations were observed at 300 mg/kg bw/day.
An increase of skeletal anomalies and variants was noted in the treated groups in comparison with the control group, but without any dose-relationship.
No pathological changes were seen at the schedule killing of the does.

IR5885, given by the oral route to the pregnant rabbits during the days 6-28 of pregnancy, at the doses of 100, 300 and 1000 mg/kg bw/day induced only slightly higher and not dose-related mean bodyweight gain during the treatment period at 300 and 1000 mg/kg bw/day. No embryo-toxic effects were observed up to the highest dose. Dead foetuses were present in the one litter/group in each treated group but without any increase in frequency, notwithstanding the increased dose level. The foetal examination did not reveal any dose-related increased alteration. Therefore, 1000 mg/kg bw/day was set as the NOAEL for dams and foetuses.

MATERIALS AND METHODS

MATERIALS:

Test Item:	IR5885
Description:	White powder
Lot/Batch #:	FCF/T/180-00 (ex ZI 068)
Purity:	98.9%
CAS #:	283159-90-0
Stability of test item:	Stable in the dark at room temperature
Vehicle:	0.5% methylcellulose aqueous solution (w/v)
Test animals –	
Species:	Rabbit
Strain:	New Zeeland (HY/CR)
Age:	14-18 weeks at arrival. 16-20 weeks at dosing
Weight:	2700-3200 g at arrival. 3410-4980 g at start of dosing
Source:	Charles River Italia S.p.A. – Lecco (Italy). Shipping slip No. 1EC05130, July 6, 2001.
Acclimation period:	about 2 weeks
Diet:	GLP 2 RB 19 certificate diet (Charles River Italia) *ad libitum*
Water:	Tap water from the municipal water main system, *ad libitum*
Housing:	Divided by sex and kept in cages measuring 62.0x47.5x38.5h cm. Males: 2/cage; females: 2/cage during pre-mating, individually in same type cages after the assessment of pregnancy.
Environmental conditions -	
Temperature:	19 °C ± 2
Humidity:	55% ± 15
Air changes:	15-20 per hour
Photoperiod:	12-hour light : 12-hour dark cycle (7 a.m. – 7 p.m.)

STUDY DESIGN AND METHODS:

In life dates: July 25 to October 19, 2001 (beginning of mating - last autopsy day)

Animal assignment and treatment

22 copulated female rabbits per dose group were used. Animals were non-selectively allocated to cages and treatment groups. IR5885 was administered by gavage from day 6 to 28 of pregnancy, the day of positive vaginal smear being considered as day 0 of pregnancy, at the doses shown in table 3.10.3.2-01. A negative control group received 0.5% methylcellulose aqueous solution.

Table 3.10.3.2-01 Study design

Test group	Females with positive smear	dosage (mg/kg bw/day)	Volume administered (mL/kg/day)	Test item concentration in vehicle (mg/mL)
1	22	0	4	0
2	22	100	4	25
3	22	300	4	75
4	22	1000	4	250

Test item formulate preparation and analysis

Appropriate amounts of test item were weighed, ground in mortars with some drops of vehicle, transferred into graduated containers, made up to the final volumes with vehicle and magnetically stirred to obtain suspension at the final concentration of 25, 75 and 250 mg/mL. Formulates were kept magnetically stirred until the end of daily administration.
Data concerning the stability of the test item in the vehicle up to 96 hours at room temperature in the concentration's range of 1 and 250 mg/mL were supplied by the Sponsor.

Concentration checks throughout the study were performed by an external GLP certified laboratory (ChemService S.r.l. – Milano - Italy) on formulates prepared by RBM on August 3, 2001. The storage stability of the test item was confirmed at nominal concentrations ranging from 25 to 250 mg/mL. The percentage of test item found, compared to nominal values, ranged from –18.8% to 13.0% (-18.8% for the low, -25.3% for the intermediate and 13.0% for the high dose level, respectively).

Statistics

To compare frequencies, the heterogeneity test (CHI square 2xN) and Fisher's exact test were applied. Then trend test was also applied. All these tests were one-sided. Group mean values were calculated in two ways: Mean A (calculated on all surviving females having evident signs of pregnancy including those that presented 100% post-implantation losses) – Mean B (calculated only on those females with viable foetuses at term).

METHODS:

Observations

Animals were observed three times a day for mortality, physical appearance, behavioural and clinical signs. During the treatment period, the animals were observed for any reaction to treatment.

Mortality

The female rabbits found dead were subjected to autopsy to detect the cause of death. Corpora lutea and implantations were counted, wherever possible.

The organs with gross alterations were fixed in formalin for histological examination, if necessary.

Body weight

Each animal was weighed on days 0, 6, 8, 10, 12, 14, 16, 18, 22, 26, 28 and 29 of pregnancy.

Food consumption

The leftover amounts of the weighed food allocated to each cage were recorded every two days during pregnancy.

Sacrifice and pathology

On day 29 of pregnancy**,** the does were killed by bleeding after i.v. anaesthesia with 5% sodium pentobarbital solution (1 mL/kg). They were necropsied and the following parameters were recorded: pregnant uterus weight – number of corpora lutea – number of implantations – number of resorptions: a) early, placenta visible b) late, placenta and embryo visible – number and sex of viable foetuses – number and sex of dead foetuses – individual foetus weight – individual placenta weight.
A gross external examination was performed on all foetuses immediately. Each foetus was eviscerated and its internal organs macroscopically examined. One third of the foetuses per litter were decapitated and the heads fixed in Bouin's fluid for examination by Wilson's technique. The rest of the carcass of these foetuses and the whole carcass of all the other foetuses were kept in ethanol, until cleared, for skeletal examination. Skeletal malformations, anomalies or variants were seen by clearing about half of the foetuses per litter. The observations were classified as follows: malformations: rare and/or usually lethal – anomalies: more frequent and not lethal – variants: common in control populations and often definable only in terms of continuous variable gradients.

RESULTS AND DISCUSSION

Clinical signs and mortality

One female aborted during the study. Female No. 43 of the 100 mg/kg bw/day group aborted on Day 20 of pregnancy. No foetuses were found inside the uterus at the scheduled killing.

No compound-related deaths were noted during the study. Some accidental deaths were observed in all the experimental groups as listed in table 3.10.3.2-02. The cause of death for these animals was incidental pneumonia.

Table 3.10.3.2-02 Premature deaths

Test group	Females with positive smear	dosage (mg/kg bw/day)	schedule deaths on Day 29	Premature death(s) / day(s)
1	22	0	19	**3** / 20, 21, 25
2	22	100	18	**4** / 19, 20, 22, 28
3	22	300	21	**1** / 14
4	22	1000	19	**3** / 16, 18, 23

Body weight, body weight gain and food consumption

A slightly higher mean bodyweight gain was observed in the 300 and 1000 mg/kg bw/day treated groups during the treatment period (6-28 of pregnancy) in comparison with the control without any dose-relationship.

No statistically significant differences were found in the mean daily food consumption among the different experimental groups.

Uterus content - effect on reproduction and on embryo-foetal development

The frequency per group and the mean value of resorptions per litter were similar in all the experimental groups.
A statistically significant higher frequency per group of dead foetuses was observed in all treated groups in comparison with the control group: no dose-relationship was observed, and dead foetuses were present in only one litter/group in each treated group. A statistically significant lower frequency per group of live foetuses was noted in the 100 mg/kg bw/day group in comparison with the control, as mean A (calculated on all surviving females having evident signs of pregnancy including those that presented 100% post-implantation losses). This finding was considered incidental since no dose-relationship was present and when this parameter was considered as mean B (calculated only on those females with viable foetuses at term), statistical level was not reached.
No important effects were noted on mean foetal weight, mean placental weight and on the mean litter weight among the different experimental groups.

Foetal examination

The external and autoptic examination on foetuses showed one malformed foetus with arthrogryposis in litter No. 76 of the 1000 mg/kg bw/day treated group and one foetus with absence of left testis in No. 42 of the 100 mg/kg bw/day group. No external or autoptic malformations were observed either at 300 mg/kg bw/day.
The skeletal examinations showed two malformed foetuses in two separate litters of the 1000 mg/kg bw/day, one with misshapen sternum in litter No. 87 and one with scoliosis in litter No. 76.
The statistically significant higher frequency per group of skeletal anomalies and variants in the 300 and 1000 mg/kg bw/day treated groups in comparison with the control group were considered without any biological significance since devoid of any dose-relationship.
No visceral malformations, anomalies and variants were noted during head's examination of 1/3 of foetuses per litter.

Autopsy

No pathological changes were seen at the schedule killing of the does.

CONCLUSIONS

IR5885 given by oral route to pregnant rabbits from day 6 to day 28 of pregnancy, at the doses of 100, 300 and 1000 mg/kg bw/day induced a slightly higher, not dose-related, body weight gain in the 300 and 1000 mg/kg bw/day treated groups during the treatment period.
No embryo toxic effects were seen up to the highest concentration tested. Dead foetuses were present in the one litter/group in each treated group but without any increase in frequency notwithstanding the increasing dose level.
The foetal examination did not reveal any dose-related increased alteration.
Therefore, 1000 mg/kg bw/day was set as the NOAEL for dams and foetuses.

3.11 Specific target organ toxicity – single exposure

3.11.1 Animal data

All clinical signs observed in the acute toxicity studies via the oral, dermal and inhalation routes were considered to be non-specific signs of general acute toxicity.

3.11.1.1 Acute oral neurotoxicity study in rats – Valifenalate technical

Report:	Herberth M. (2015) Valifenalate Technical : Acute oral (gavage) neurotoxocity study in rats. WIL Research, Ashland, Ohio, USA, unpublished report No. WIL-105146, July 14, 2015, dates of experimental work: August 05 to August 29, 2014 (pretest evaluations - last autopsy day)

Guidelines: OECD 424 (1997)

Deviation: None

GLP: Yes (Certified laboratory)

Executive Summary:

The test substance, Valifenalate Technical, in the vehicle (0.5% [w/v] methylcellulose in deionized water) was administered orally by gavage as a single dose to 3 groups (Groups 2-4) of Crl:CD(SD) rats. Dose levels were 500, 1000, and 2000 mg/kg. A concurrent control group (Group 1) received the vehicle on a comparable regimen. Animals were approximately 6 weeks old at the initiation of dose administration. Each group consisted of 10 rats/sex. The dose volume was 10 mL/kg for all groups.

All animals were observed twice daily for mortality and moribundity. Clinical examinations were performed daily, except on days of Functional Observational Battery (FOB) assessments. Individual body weights were recorded weekly. FOB and motor activity data were recorded for all animals prior to the initiation of dose administration, at the time of peak effect (approximately 2 hours following dose administration) on study day 0, and on study days 7 and 14. All animals were deeply anesthetized on study day 15 and perfused *in situ*; brain weights and brain dimensions (excluding olfactory bulbs) were recorded. A neuropathological evaluation of selected tissues from the central and peripheral nervous systems was performed on all animals in all groups.

All animals survived to the scheduled euthanasia (study day 15). There were no test substance-related clinical findings during the daily observations. Mean body weights and body weight gains were unaffected by Valifenalate Technical administration.

No test substance-related effects were apparent when FOB evaluations were performed at the time of peak effect (2 hours post-dosing) on study day 0 or on study days 7 and 14. The FOB evaluations collectively involved home cage, handling, open field, sensorimotor, neuromuscular, and physiological parameters.

No test substance-related effects were apparent in mean ambulatory and total motor activity counts at the time of peak effect (2 hours post-dosing) on study day 0 or on study days 7 and 14. No remarkable shifts in the pattern of habituation occurred in any of the test substance-treated groups when the animals were evaluated on study days 0, 7, and 14.

There were no test substance-related gross observations or alterations in brain weight, brain length, or brain width.

Histologic changes of note were limited to a seemingly slight increase in the incidence of axonal degeneration in multiple nerves when considered in combination [with particular emphasis on the lumbar spinal nerve (females), lumbar dorsal root fibers (males), and sciatic nerve] in the 2000 mg/kg group animals; however, the number of animals in each dose group with axonal degeneration of 1 or more nerves did not show a clear dose-response.

Based on the results of the FOB and motor activity evaluations, none of the functional domains described by Moser (1991) were affected by a single dose of Valifenalate Technical at 500, 1000, or 2000 mg/kg. Histologic changes of note were limited to a seemingly slight increase in the incidence of axonal degeneration in multiple nerves when considered in combination [with particular emphasis on the lumbar spinal nerve (females), lumbar dorsal root fibers (males), and sciatic nerve] in the 2000 mg/kg group animals; however, the number of animals in each dose group with axonal degeneration of 1 or more nerves did not show a clear

dose-response. Based on the results of this study following a single oral dose of Valifenalate Technical to rats, the no-observed-adverse-effect level (NOAEL) was 2000 mg/kg.

MATERIALS AND METHODS

MATERIALS:

Test Material: Valifenalate Technical
Description: White powder
Lot/Batch: PL14-0060
Purity: 98.9 %
CAS: 283159-90-0
Stability of test compound: Stable in 0.5% MC (400 cps) in DI at room temperature.
Control Materials: Vehicle: 0.5% methylcellulose (MC)
Test animals:
Species: Rat
Strain: Crl:CD(SD)
Age: 6 weeks at the start of dosing
Weight at dosing: 185 – 267 g (males); 136 – 194 g (females)
Source: Charles River, Laboratories, Inc., Raleigh, NC
Acclimation period: 13 days
Diet: Certified diet PMI Nutrition International, LLC Certified Rodent LabDiet® 5002, *ad libitum*
Water: Reverse osmosis-treated (on-site) water, *ad libitum*
Housing: all animals were housed individually in clean, stainless steel, wire-mesh cages suspended above cage-board.
Environmental conditions –
Temperature: 19 - 25°C (Actual 21.4°C - 22.0°C)
Humidity: 30 - 70 % (Actual 41.5% - 48.9%)
Air changes: Minimum 10 changes per hour
Photoperiod: Alternating 12-hour light and dark cycles (6 am- 6 pm)
Test compound concentrations: 0, 500, 1000 and 2000 mg/kg

STUDY DESIGN:

In life dates: 29 July, 2014 to 29 August, 2014

Animal assignment and treatment

On 04-Aug-2014 (7 days prior to the initiation of dose administration), all available rats were weighed and examined in detail for physical abnormalities. These data were collected using WTDMS™ and reviewed by the Study Director. The animals judged suitable for assignment to the study were selected for use in a computerized randomization procedure. A printout containing the animal numbers, corresponding body weights, and individual group assignments was generated based on body weight stratification in a block design. The animals were then arranged into groups according to the printout. Individual body weights at randomization were within ± 20% of the mean for each sex. Each group (Groups 1-4) consisted of 10 males and 10 females. These animals were then randomized into 4 study replicates to allow for the reasonable conduct of the functional observational battery and motor activity assessments. Each dose group and sex was approximately equally represented within each study replicate. Animals not assigned to study were euthanized by carbon dioxide inhalation and discarded. The selected animals were approximately 6 weeks old at the initiation of dose administration; individual body weights ranged from 185 g to 267 g for males and from 136 g to 194 g for females.

Diet preparation and analysis

The vehicle suspension was prepared once for administration to the control group (Group 1) and for preparation of the test substance formulations; aliquots were prepared for dispensation to the control group and stored at room temperature. The vehicle was mixed throughout the preparation, sampling, and dose administration procedures. The test substance formulations used for dose administration were prepared once as single formulations for each dose level, divided into aliquots for dispensation, and stored at room temperature. The test substance formulations were stirred continuously throughout the preparation, sampling, and dose administration procedures.

The test substance dosing formulations were visually inspected by the Study Director and were found to be visibly homogeneous and acceptable for administration.

Analyses to demonstrate the homogeneity, stability (following at least 7 days of room temperature storage), and resuspension homogeneity of the test substance in the vehicle were conducted as part of a previous study (Farris, 2014, WIL-105144; FMC Tracking No. 2014TOX-VAL1371). Samples for homogeneity and concentration analysis were collected from the top, middle, and bottom strata of the dosing formulation (including the control group). One set of samples from each collection was subjected to the appropriate analyses. The remaining samples were stored refrigerated as back-up. All analyses were conducted by the WIL Research Analytical Chemistry Department using a validated high performance liquid chromatography method with ultraviolet absorbance detection (Farris, 2014, WIL-105144; FMC Tracking No. 2014TOX-VAL1371).

Table 3.11.1.1-01 Study Group Assignment

Group Number	Treatment	Dose Level (mg/kg)	Dose Volume (mL/kg)	Number of Animals	
				Males	Females
1	Vehicle control	0	10	10	10
2	Valifenalate Technical	500	10	10	10
3	Valifenalate Technical	1000	10	10	10
4	Valifenalate Technical	2000	10	10	10

Statistics

Statistics (Conducted by WIL Research)

All statistical tests were performed using WTDMS™ unless otherwise noted. Analyses were conducted using two-tailed tests (except as noted otherwise) for minimum significance levels of 1% and 5%, comparing each test substance-treated group to the control group by sex.

Body weight, body weight change, continuous FOB, and brain weight and measurement data were subjected to a parametric one-way ANOVA (Snedecor and Cochran, 1980) to determine intergroup differences. If the ANOVA revealed significant ($p<0.05$) intergroup variance, Dunnett's test (Dunnett, 1964) was used to compare the test substance-treated groups to the control group. FOB parameters that yielded scalar or descriptive data were analyzed using Fisher's Exact Test (Steel and Torrie, 1980).

Statistics Conducted by BioSTAT Consultants, Inc.

All statistical analyses were conducted using SAS® version 9.2 (SAS Institute, Inc., 2002-2008), or higher.

Motor activity was analyzed using a repeated measures analysis of variance (RMANOVA) (based on a modification from the methods described by Holson *et al.*, 2008). Both total and ambulatory counts were considered, and analyses were conducted for each sex separately. The observation period lasted 1 hour, and activity counts (total and ambulatory) from each 10 minute time interval within the observation period were used as the response variables. Factors in the model included treatment group (TRT), time interval (10 min)(TIME), and the interaction of time interval and treatment group (TRT*TIME). The interaction term assesses the degree to which potential differences among treatment groups were consistent over time. Because

observations from the same animal made over time are not independent, an additional term for animal (ANIMAL) was included as a random effect. The SAS® procedure PROC MIXED was used for analysis, and the covariance structure across time was selected based on Akaike's Information Criterion (AIC).

The monotonic dose-response relationship (*i.e.*, increasing or decreasing with dose, with or without a plateau) will result in a linear relationship between the response variable and treatment. As a result, monotonic dose-response relationships were evaluated using sequential linear trend tests based on ordinal (*i.e.*, equal) spacing of dose levels. Here, and additional term representing the interaction of the linear trend in treatment by time (LinTrt*Time) is evaluated (the LinTrt*Time interaction is a subset of the TRT*TIME interaction). This interaction evaluated the degree to which the linear trend in treatment is consistent over time. If this interaction, LinTrt*Time, was significant (indicating that the dose response relationship changes over time), trend tests on treatment means were performed for each time interval of 0-10, 11-20, 21-30, 31-40, 41-50, and 51-60 minutes separately. If LinTrt*Time was not significant, the trend test was conducted across the pooled time intervals of the entire 1 hour session only. Effects were considered statistically significant if the p-value for that effect was less than 0.05 (*i.e.*, 5%)

- Nonmonotonic dose responses (*e.g.*, hump shaped, U-shaped, or other responses to dose) were evaluated whenever no significant linear trends were detected but a significant treatment effect was detected. Such cases do not fit the expectation of a classical dose-response relationship, and were considered only when the p-value for the TRT effect, or the TRT*TIME effect, was lower than 0.01 (*i.e.*, 1%). Within the framework of the RMANOVA, pairwise comparisons were made for each individual test substance-treated group with the control group. If TRT*TIME was significant, the comparisons were conducted for each time interval of 10 minutes. If only TRT was significant, the comparisons were conducted by pooling motor activity counts from the entire 1 hour session. These nonmonotonic dose-response comparisons were conducted at the 0.01 significance level.

METHODS:

Observations

All animals were observed twice daily, once in the morning and once in the afternoon, for mortality and moribundity.

Clinical examinations were performed once daily on all animals. The absence or presence of findings was recorded for all animals. On the days that the functional observational battery was conducted, no additional clinical findings were recorded.

Body weight

Individual body weights were recorded weekly, beginning 1 week prior to test substance administration (study day -7). Mean body weights and body weight changes were calculated for the corresponding intervals. In addition, cumulative mean body weight change is presented for study days 0-14.

FOB Assessments

FOB findings were recorded for all animals prior to the initiation of dose administration (study day -6), at the time of peak effect (approximately 2 hours post-dosing) on study day 0, and on study days 7 and 14. The time of peak effect was considered to be 2 hours post-dosing based on previous pharmacokinetic data which indicated a T_{max} of 2 hours following dose administration for male and female rats administered 1000 mg/kg Valifenalate Technical (See annex conf. 22). The FOB used at WIL Research is based on previously developed protocols (Gad, 1982; Haggerty, 1989; Irwin, 1968; Moser *et al*., 1988; Moser *et al*., 1991; and O'Donoghue, 1989). Testing was performed by the same biologists, to the extent possible, without knowledge of the animal's group assignment. The FOB was performed in a sound-attenuated room equipped with a white-noise generator set to operate at 70 ± 10 dB. All animals were observed for the following parameters as described below:

Home Cage Observations

Posture Biting

Convulsions/tremors	Palpebral (eyelid) closure
Feces consistency	
Handling Observations	
Ease of removal from cage	Ease of handling animal in hand
Lacrimation/chromodacryorrhea	Salivation
Piloerection	Fur appearance
Palpebral closure	Respiratory rate/character
Eye prominence	Mucous membranes/eye/skin color
Red/crusty deposits	Muscle tone
Open Field Observations	
Mobility	Gait
Rearing	Arousal
Convulsions/tremors	Urination/defecation
Grooming	Gait score
Bizarre/stereotypic behavior	Backing
Time to first step (seconds)	

Note: Open field observations were evaluated over a 2-minute observation period.

Sensory Observations	
Approach response	Touch response
Startle response	Tail pinch response
Pupil response	Eyeblink response
Forelimb extension	Hindlimb extension
Air righting reflex	Olfactory orientation
Neuromuscular Observations	
Hindlimb extensor strength	Grip strength-hind and forelimb
Hindlimb foot splay	Rotarod performance
Physiological Observations	
Catalepsy	Body weight
Body temperature	

Motor Activity

Motor activity was assessed for all animals prior to the initiation of dose administration (study day -6), at the time of peak effect (approximately 2 hours post-dosing) on study day 0, and on study days 7 and 14. Motor activity, recorded after completion of the FOB, was measured automatically using a personal computer-controlled system that utilizes a series of infrared photobeams surrounding an amber plastic, rectangular cage to quantify each animal's motor activity. Four-sided black plastic enclosures were used to surround the transparent plastic boxes and decrease the potential for distraction from extraneous environmental stimuli or activity by technicians or adjacent animals. The black enclosures rested on top of the photobeam frame and did not interfere with the path of the beams. The motor activity assessment was performed in a sound-attenuated room equipped with a white-noise generator set to operate at 70 ± 10 dB. The testing of treatment groups was conducted according to replicate sequence. Each animal was tested separately. Data

were collected in 5-minute epochs (print intervals), and the test session duration was 60minutes. These data were compiled as six 10-minute subintervals for tabulation.

Data for ambulatory and total motor activity were tabulated. Total motor activity was defined as a combination of fine motor skills (*i.e.*, grooming, interruption of 1 photobeam) and ambulatory motor activity (interruption of 2 or more consecutive photobeams).

Sacrifice and pathology

On study day 15, all animals were anesthetized by an intraperitoneal injection of sodium pentobarbital and then perfused *in situ* with a 4.0% paraformaldehyde/0.1M phosphate buffered solution. The central and peripheral nervous system tissues were dissected and preserved. Fixed brain weight and brain dimensions (length [excluding olfactory bulbs] and width) were recorded. Any observable gross changes and abnormal coloration or lesions of the brain and spinal cord were recorded.

The following nerve tissues were collected from all animals perfused *in situ*:

Brain - olfactory bulbs, cerebral cortex (2 levels), hippocampus/dentate gyrus, basal ganglia, thalamus, hypothalamus, midbrain, cerebellum, pons, and medulla oblongata
Spinal cord - at cervical swellings C_3-C_7 and at lumbar swellings T_{13}-L_4
Trigeminal ganglia/nerves[a]
Lumbar dorsal root ganglia at T_{13}-L_4[b]
Lumbar dorsal root fibers at T_{13}-L_4[b]
Lumbar ventral root fibers at T_{13}-L_4[b]
Cervical dorsal root ganglia at C_3-C_7[b]
Cervical dorsal root fibers at C_3-C_7[b]
Cervical ventral root fibers at C_3-C_7[b]
Cervical spinal nerve
Lumbar spinal nerve
Sciatic nerves (mid-thigh region) (2)[c]
Sciatic nerves (at sciatic notch) (2)[c]
Sural nerves (2)[c]
Tibial nerves (2)[c]
Peroneal nerves (2)[c]
Optic nerves[a]
Eyes[a]
Skeletal muscle (gastrocnemius)
Other sites (if deemed necessary)
[a] - Both processed and evaluated microscopically.
[b] - Four to 6 tissues were collected at necropsy; 2 were evaluated microscopically.
[c] - One processed for microscopic examination.

(2) - Two sections (1 transverse and 1 longitudinal) of the tissue were evaluated from the right hind leg. The tissues from the left hind leg were collected and preserved for possible future evaluation.

The tissues listed above from 5 randomly selected animals/sex in the control and 2000 mg/kg groups were prepared for a qualitative histopathological examination by embedding in paraffin (central nervous system tissues) or plastic (peripheral nervous system tissues), sectioning, and staining with hematoxylin and eosin. In addition, potential target tissues (cervical spinal nerve, cervical dorsal root fibers, cervical ventral root fibers, lumbar spinal nerve, lumbar dorsal root fibers, lumber ventral root fibers, sciatic nerve, sural nerve, tibial nerve, and peroneal nerve) were examined from all animals in all groups.

RESULTS AND DISCUSSION

MORTALITY:

All animals survived to the scheduled euthanasia (study day 15).

CLINICAL SIGNS:

There were no test substance-related clinical findings noted during the daily clinical examinations.

BODY WEIGHT AND BODY WEIGHT GAIN:

Body weights were unaffected by test substance administration. There were no statistically significant differences when the control and test substance-treated groups were compared.

FOB ASSESSMENTS:

Home cage parameters were unaffected by test substance administration. There were no statistically significant differences for the test substance-treated males and females when compared to the control group at the time of peak effect on study day 0, and on study days 7 and 14, with the following exception. On study day 7, a significantly ($p<0.05$) higher number of females (9 out of 10) in the 1000 mg/kg group were noted as sitting or standing normally. This difference noted for the females in the 1000 mg/kg group did not occur in a dose-related manner. Furthermore, animals sitting or standing normally are considered to have a normal posture and this is a common home cage observation and was not considered test substance-related.

Handling parameters were unaffected by test substance administration.

Open field parameters were unaffected by test substance administration.

Sensory parameters were unaffected by test substance administration.

Neuromuscular parameters were unaffected by test substance administration.

Physiological parameters were unaffected by test substance administration.

MOTOR ACTIVITY:

Within-session repeated measures analyses of variance were conducted across the subintervals of each test session for total and ambulatory counts and for the overall interval means (representing the entire 60-minute session activity) during each test session. Motor activity patterns (mean ambulatory and total motor activity counts) were unaffected by test substance administration. There were no statistically significant differences between the control and test substance treated groups when values obtained from the 6 subintervals (0-10 minutes, 11-20 minutes, 21-30 minutes, 31-40 minutes, 41-50 minutes, and 51-60 minutes) and the overall 60-minute test session values were evaluated at the time of peak effect on study day 0 and on study days 7 and 14. No remarkable shifts in the pattern of habituation occurred in any of the test substance-treated groups when the animals were evaluated on study days 0, 7, and 14.

PATHOLOGY:

There were no macroscopic changes observed for males and females at any dosage level.

Brain weights and measurements were unaffected by administration of Valifenalate Technical at 500, 1000, and 2000 mg/kg. There were no statistically significant differences between the control and test substance-treated groups.

There were no test substance-related histologic changes.

All histologic changes were considered to be incidental findings or related to some aspect of experimental manipulation other than administration of the test substance. There was no test substance-related alteration in the prevalence, severity, or histologic character of those incidental tissue alterations, with the exception of minimal to mild axonal degeneration in multiple nerves without any definitive pattern in nerve type or gender affected, as detailed below.

The initial subset of 5 rats/sex/group, histologic changes of note were limited to a slight increase in the incidence of axonal degeneration in multiple nerves, as presented in Table 3.11.1.1-02.
Minimal to mild axonal degeneration was observed sporadically in control and test substance-treated group animals in multiple nerves. The axonal degeneration was characterized by small numbers of “digestion chambers” consisting of axonal swelling and fragmentation and myelin vacuolation and disruption, often

arranged in a linear array affecting a single nerve fiber. While axonal degeneration can be an incidental finding consistent with spontaneous background findings in rats (Eisenbrandt *et al.*, 1990), the incidence of this finding was slightly higher in the 2000 mg/kg group animals compared to the control group animals. This was of particular note in the lumbar spinal nerve (females), lumbar dorsal root fibers, sciatic nerves, and peroneal nerve (females). In addition, when all nerve types were considered together, there was a seeming dose-related increase in the incidence of axonal degeneration in any nerve (males: 3, 5, 8, and 12 nerves had axonal degeneration in the 0, 500, 1000, and 2000 mg/kg groups, respectively, out of the above 50 nerves examined in each dose group; females: 3, 3, 4, and 15 nerve sections had axonal degeneration in the 0, 500, 1000, and 2000 mg/kg groups, respectively, out of the above 50 nerves examined in each dose group). The number of animals in each dose group with axonal degeneration of one or more nerves showed a potential dose-related response in the males only (males: 2, 3, 3, and 5 animals affected; females: 2, 2, 3, 4 animals affected). The described changes of uncertain relationship to the test substance warranted further examination.

Table 3.11.1.1-02 Incidence of Selected Histopathologic Findings, Study Day 15 Necropsy, Initial Subset of Animals

	Males				Females			
Dose (mg/kg):	**0**	**500**	**1000**	**2000**	**0**	**500**	**1000**	**2000**
Cervical Spinal Nerve [a]	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
Degeneration, Axonal	0	0	0	0	0	0	0	1
Minimal	-	-	-	-	-	-	-	1
Cervical Dorsal Root Fibers [a]	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
Degeneration, Axonal	0	0	0	0	0	0	0	1
Mild	-	-	-	-	-	-	-	1
Cervical Ventral Root Fibers [a]	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
Degeneration, Axonal	0	0	0	0	0	0	0	0
Minimal	-	-	-	-	-	-	-	-
Lumbar Spinal Nerve [a]	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
Degeneration, Axonal	0	0	0	1	0	0	0	3
Minimal	-	-	-	1	-	-	-	3
Lumbar Dorsal Root Fibers [a]	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
Degeneration, Axonal	0	0	1	3	0	2	0	3
Minimal	-	-	1	3	-	2	-	1
Mild	-	-	0	0	-	0	-	2
Lumbar Ventral Root Fibers [a]	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
Degeneration, Axonal	0	3	1	1	1	0	1	1
Minimal	-	3	1	1	1	-	1	1
Sciatic Nerve [a]	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
Degeneration, Axonal	2	1	3	4	2	1	2	4
Minimal	2	1	3	4	2	1	2	4
Sural Nerve [a]	**5**	**5**	**5**	**5**	**5**	**5**	**4**	**5**
Degeneration, Axonal	0	0	0	1	0	0	1	0
Minimal	-	-	-	1	-	-	1	-

	Males				Females			
Dose (mg/kg):	**0**	**500**	**1000**	**2000**	**0**	**500**	**1000**	**2000**
Tibial Nerve [a]	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
Degeneration, Axonal	1	0	2	1	0	0	0	0
Minimal	1	-	2	1	-	-	-	-
Peroneal Nerve [a]	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
Degeneration, Axonal	0	1	1	1	0	0	0	2
Minimal	-	1	1	1	-	-	-	2

[a] - Number of tissues examined from each group.

Following microscopic examination of the nerves from the remaining 5 perfused rats/sex/group and combination of the findings from all nerves from all perfused animals (10 rats/sex/group), there was a slight increase in the incidence of axonal degeneration in multiple nerves, as presented below.

Table 3.11.1.1-03 Incidence of Selected Histopathologic Findings, Study Day 15 Necropsy, All Perfused Animals

	Males				Females			
Dose (mg/kg):	**0**	**500**	**1000**	**2000**	**0**	**500**	**1000**	**2000**
Cervical Spinal Nerve [a]	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**
Degeneration, Axonal	0	0	0	0	0	0	1	1
Minimal	-	-	-	-	-	-	1	1
Cervical Dorsal Root Fibers [a]	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**
Degeneration, Axonal	0	0	0	0	0	0	1	2
Minimal	-	-	-	-	-	-	1	1
Mild	-	-	-	-	-	-	0	1
Cervical Ventral Root Fibers [a]	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**
Degeneration, Axonal	0	0	0	0	0	0	0	0
Minimal	-	-	-	-	-	-	-	-
Lumbar Spinal Nerve [a]	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**
Degeneration, Axonal	1	0	0	1	0	1	1	3
Minimal	1	-	-	1	-	1	0	3
Mild	0	-	-	0	-	0	1	0
Lumbar Dorsal Root Fibers [a]	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**
Degeneration, Axonal	1	1	2	5	1	5	1	4
Minimal	1	1	2	5	1	3	0	2
Mild	0	0	0	0	0	2	1	2
Lumbar Ventral Root Fibers [a]	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**
Degeneration, Axonal	0	4	1	3	1	0	2	1
Minimal	-	4	1	3	1	-	2	1
Sciatic Nerve [a]	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**
Degeneration, Axonal	4	5	3	8	4	4	4	6
Minimal	4	5	3	8	4	3	4	5
Mild	0	0	0	0	0	1	0	1

	Males				Females			
Dose (mg/kg):	**0**	**500**	**1000**	**2000**	**0**	**500**	**1000**	**2000**
Sural Nerve [a]	**10**	**10**	**10**	**10**	**10**	**10**	**9**	**10**
Degeneration, Axonal	0	0	1	1	0	0	1	1
Minimal	-	-	1	1	-	-	1	1
Tibial Nerve [a]	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**
Degeneration, Axonal	2	1	3	1	0	1	0	0
Minimal	2	1	3	1	-	1	-	-
Peroneal Nerve [a]	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**
Degeneration, Axonal	1	5	2	2	1	1	1	4
Minimal	1	5	2	2	1	1	1	4

[a] - Number of tissues examined from each group.

Minimal to mild axonal degeneration was observed sporadically in the remaining control and test substance-treated group animals in multiple nerves, as was described in the initial subset of examined animals. When histologic findings from the nerves of all perfused animals (original subset of 5 rats/sex/group plus remaining 5 rats/sex/group for a total of 10 rats/sex/group) were combined, the incidence of axonal degeneration was slightly higher in the 2000 mg/kg group animals compared to the control group animals in this study. This was of particular note in the lumbar spinal nerve (females), lumbar dorsal root fibers (males), and sciatic nerves. In addition, when all nerve types were considered together, there was a seeming increase in incidence of axonal degeneration in any nerve in the 2000 mg/kg group animals (males: 9, 16, 12, and 21 nerves had axonal degeneration in the 0, 500, 1000, and 2000 mg/kg groups, respectively, out of the above 100 nerves examined in each dose group; females: 7, 12, 12, and 22 nerve sections had axonal degeneration in the 0, 500, 1000, and 2000 mg/kg groups, respectively, out of the above 100 nerves examined in each dose group). However, the number of animals in each dose group with axonal degeneration of one or more nerves did not show a clear dose-related response (males: 5, 8, 5, and 9 animals affected; females: 4, 6, 5, 7 animals affected).

The relationship of the axonal degeneration was considered to be incidental and of unlikely relationship to the administration of the test substance for the following reasons. The axonal degeneration was of limited severity (minimal to mild) and was within a severity range consistent with the spontaneous background finding of axonal degeneration in rats (Eisenbrandt *et al.*, 1990). Multiple nerves were affected without a consistent pattern between males and females or of nerve type affected. Axonal degeneration was noted in both control and test substance-treated groups without a remarkable difference in the number of animals affected (having one or more nerves with axonal degeneration) between groups. In addition, there was no correlation of these findings to clinical or functional neurological assessments performed in this study.

CONCLUSION:

Based on the results of the FOB and motor activity evaluations, none of the functional domains described by Moser (1991) were affected by a single dose of Valifenalate Technical at 500, 1000, or 2000 mg/kg. Histologic changes of note were limited to a seemingly slight increase in the incidence of axonal degeneration in multiple nerves when considered in combination [with particular emphasis on the lumbar spinal nerve (females), lumbar dorsal root fibers (males), and sciatic nerve] in the 2000 mg/kg group animals; however, the number of animals in each dose group with axonal degeneration of 1 or more nerves did not show a clear dose-response. Based on the results of this study following a single oral dose of Valifenalate Technical to rats, the no-observed-adverse-effect level (NOAEL) was 2000 mg/kg.

3.11.2 Human data

No human data available on specific target organ toxicity – single exposure

3.11.3 Other data

No other data available on specific target organ toxicity – single exposure

3.12 Specific target organ toxicity – repeated exposure

3.12.1 Animal data

3.12.1.1 Oral 28-day study on rat – IR5885

Reference Number:	KII- Section 3, Volume 2, Annex IIA, point 5.3.1/02
Report:	See Annex conf. 48
Guidelines:	OECD 407
Deviation:	As a preliminary to a 90-d study, no specific neurobehavioural observations were carried out; organ weight and histopathology only on selected organs/tissues
GLP:	This study was in general performed in accordance with GLP, but the data and report were not fully audited by the Quality Assurance Unit

Executive summary:

In a rat range-finding study, doses of 0, 120, 600, 3000 and 15000 ppm (mean achieved doses approximately 0, 13, 63, 311 and 1518 mg/kg/day for males and 0, 13, 64, 314 and 1537 mg/kg/day for females), were administered in the feed for four weeks to groups of Han Wistar rats (5 animals of each sex per dose group).

There were no mortalities, no clinical signs or macroscopic finding at necropsy, to be considered related to treatment with IR5885. Overall reduction in body weight gain was observed at the high doses as well as abnormalities following blood chemistry and haematological analyses. Slight low thymus weights were found at the two highest doses in both sexes and an increased lymphocytolysis in the thymus was observed for animals receiving 15000 ppm and for males receiving 600 or 3000 ppm. It is concluded that oral administration, through the diet, of IR5885 to Han Wistar rats for four weeks up to 15000 ppm was well tolerated. The NOAEL was considered to be 3000 ppm (equivalent to 311 mg/kg/day for males and 314 mg/kg/day for females).

MATERIALS AND METHODS

MATERIALS:

Test Item:	IR5885
Description:	White powder
Lot/Batch #:	FCF/T/180-00 (ex ZI 068)
Purity:	98.9%
CAS #:	283159-90-0
Stability of test item:	Stable in the dark at room temperature and also for at least 14 days at 54 °C and was distributed homogeneously in the feed. Batches of test diets were prepared weekly and maintained in sealed metal container.
Vehicle and/or positive control:	None

Test animals –
Species: Rat
Strain: Han Wistar
Age: 26 - 30 days
Weight at dosing: 94-106 g males; 87-99 g females
Source: Harlan UK, Limited, Beicester, Oxfordshire, England (UK)
Acclimation period: 13 days
Diet: Rat and mouse No. 1 diet (Special Diets Service, Ltd) *ad libitum*
Water: Tap water from the municipal water main system, *ad libitum*
Housing: 5 animals/sex in cages made of stainless steel body with a stainless steel mesh lid and floor.

Environmental conditions -
Temperature: 19 – 23 °C
Humidity: 40 – 70 %
Air changes: At least 15 per hour
Photoperiod: 12-hour light : 12-hour dark cycle

STUDY DESIGN AND METHODS

In life dates: May 24 – July 5, 2001

Animal assignment and treatment

5 animals of each sex per dose group were used. Animals were non-selectively allocated to cages and treatment groups. IR5885 was administered in the diet at the following doses – 0, 120, 600, 3000 and 15000 ppm (Table 3.12.1.1-01) to all males and females, for 28 and 29 consecutive days, respectively. A negative control group received untreated diet.

Table 3.12.1.1-01 Study design

Test group	Concentration in diet (ppm)	Dose per animal (study averages)		Animals assigned	
		Male (mg/kg bw/day)	Female (mg/kg bw/day)	male	female
1	0	0	0	5	5
2	120	13	13	5	5
3	600	63	64	5	5
4	3000	311	314	5	5
5	15000	1518	1537	5	5

Diet preparation and analysis

The test item (approximately 1045 g) was incorporated into the diet to provide the required concentrations by initial preparation of a premix at a concentration of 30000 ppm. This premix was then diluted with further quantities of plain diet to prepare the two highest concentrations test mixes (15000 and 3000 ppm) and a second premix (3000 ppm). This second premix was used to prepare the two lowest concentrations test mixes (120 and 600 ppm). Batches of test diets were prepared weekly and maintained in sealed metal containers. The unused residue at the end of each week was discarded.
The Sponsor provided the test material IR5885 and the analytical standard. The homogeneity and the stability of the test item in the rodent diet formulation were confirmed at nominal concentrations of 50 and 20000 ppm during an earlier study. The stability was confirmed during storage in the freezer for 21 days and at room temperature for 22 days following preparation. At a specified time (Week 1) during treatment, representative samples of the test diets were taken from the mixer and sub-sampled in duplicate before analysis.

Statistics

For organ weight and body weight changes, homogeneity of variance was tested using Bartlett's test. Whenever this was found to be statistically significant, Behrens-Fisher test was used to perform pairwise comparisons, otherwise a Dunnett's test was used.
Inter-group differences in macroscopic pathology and histopathology were assessed using Fisher's Exact test.
In clinical pathology data, treatment groups were compared using a Mantel's test for trend in proportions and also pairwise Fisher Exact tests for each dose group against the control.

Observations

Animals were inspected at least twice daily for evidence of reaction to treatment or ill health. In addition, a more detailed weekly physical examination was performed on each animal for signs of toxicity.

Body weight and food consumption

Each animal was weighed during the acclimatisation period (Day –7), on the first day of treatment (Week 0), at weekly intervals throughout the treatment period and before necropsy.
Food consumption was measured weekly.

Haematology & clinical chemistry

After 4 weeks of treatment (at termination), blood samples from all animals were withdrawn from the retro-orbital sinus, with the animals held under Isofluorane anaesthesia, and collected into EDTA anticoagulant. All samples were examined for the following parameters: haematocrit (Hct), haemoglobin concentration (Hb), erythrocyte count (RBC), total and differential leucocyte count (WBC), platelet count (Plt), mean cell haemoglobin concentration (MCHC), mean cell haemoglobin (MCH), mean cell volume (MCV). Additional blood samples were taken into citrate anticoagulant and examined in respect of prothrombin time (PT) and activated partial thromboplastin time (APTT).
At the same time as for peripheral haematology, further blood samples were taken and collected into lithium heparin as anticoagulant. After separation the plasma was analysed for: alkaline phosphatase (AP), alanine amino-transferase (ALT), aspartate amino-transferase (AST), glucose concentration (Gluc), total bilirubin concentration (Bili.Total), total cholesterol concentration (Chol.Total), total triglyceride concentration (Trig), creatinine concentration (Creat), urea concentration, total protein concentration (Total Prot), albumin (Alb), albumin/globulin ratio (A/G Ratio), sodium (Na), potassium (K) and chloride (Cl), calcium concentration (Ca Total) and inorganic phosphorus (Phos).

Sacrifice and pathology

At study termination, all animals were sacrificed by carbon dioxide inhalation. All animals were subjected to a detailed necropsy and the following organs were weighed: adrenals, brain, epididymides, heart, kidneys, liver, lungs (included bronchi), ovaries, spleen, testes, thymus, thyroid with parathyroids, uterus with cervix.
Samples of the following tissues were preserved in 10% neutral buffered formalin and subsequently processed for histopathological examination: brain, kidneys, liver, Lymph nodes (mandibular and mesenteric), spinal cord, spleen and thymus.
Microscopic examination was performed as follows: i) The brain, kidneys, liver and spinal cord were examined for all animals of group 1 and 5. ii) The spleen, mandibular and mesenteric lymph nodes were examined for all animals of groups 1, 4 and 5. iii) The thymus was examined for all animals. iv) Tissues reported at macroscopic examination as being abnormal were examined for all animals.

RESULTS AND DISCUSSION

Clinical signs of toxicity and mortality

The appearance and behaviour of the animals were unaffected by the treatment.
There were no deaths.

Body weight and body weight gain

Overall bodyweight gain for males receiving 15000 ppm was low when compared with that of the Controls (75% of the Control value, p<0.05).

Food consumption and compound intake

Table 3.12.1.1-02 Achieved intakes

Test group	Concentration in diet (ppm)	Dose per animal (study averages)	
		Male (mg/kg bw/day)	Female (mg/kg bw/day)
1	0	0	0
2	120	13	13
3	600	63	64
4	3000	311	314
5	15000	1518	1537

Overall food conversion efficiency for males receiving 15000 ppm was low when compared with that of the Controls. Conversely, food conversion efficiency for treated females was higher than that of the Controls.
Mean concentrations of IR5885 in diet formulation analysed during toxicity study where between 4.7% and 9.3% above nominal concentrations, confirming the accuracy of formulation. The precision of duplicate analysis was <3%, the values for the four procedural recoveries analysed concurrently with the test diets were within 94.4% and 97.4% confirming the accuracy of the method.

Blood analysis

Haematological findings
At the end of treatment period, slight low packed cell volume (haematocrit) and haemoglobin concentration for males receiving 600, 3000 or 15000 ppm and for females receiving 15000 ppm was evidenced, if compared with the Controls. The changes were, however, minimal and without dosage-relationship in the males and therefore considered of no toxicological significance. Lymphocyte counts were slightly low for males and females receiving 3000 or 15000 ppm (Table 3.12.1.1-03). This produced a concomitant reduction of total white cell count in these animals.

Clinical chemistry findings
Blood chemistry examination revealed, compared with Controls, high plasma amino-transferase activity, compared with the Controls, for females receiving 15000 ppm. Calcium concentrations were slightly low for all treated animals. Phosphorus concentrations were also slightly low for females receiving at least 600 ppm. Total plasma protein concentrations were slightly low for males receiving 600 ppm or above and for all treated female groups. Albumin/globulin ratios were slightly high for females receiving 600 ppm or above (Table 3.12.1.1-03).

Sacrifice and pathology

Organ weight
Analysis of organ weights of rats killed after four weeks of treatment revealed slightly low absolute and bodyweight-relative thymus weights for males and females which received 3000 or 15000 ppm.
Gross and histopathology
There were no macroscopic findings at necropsy which were considered to be related to treatment with the test item. Treatment related changes were confined to the thymus where there was lymphocytolysis in males at 600 ppm or more and a slight lymphocytolysis in females at 15000 ppm (Table 3.12.1.1-03).

Table 3.12.1.1-03 Selected haematology and pathology findings among rats (at termination)

	Males					Females				
Feeding Level (ppm)	**0**	**120**	**600**	**3000**	**15000**	**0**	**120**	**600**	**3000**	**15000**
Number in group	5	5	5	5	5	5	5	5	5	5
HAEMATOLOGY										
Hct (L/L)	0.474	0.462	0.452*	0.448*	0.451*	0.442	0.432	0.424	0.429	0.423*
Hb (g/dL)	16.3	16.1	15.7*	15.2#	15.5#	15.4	15.2	14.8	15.2	14.8*
Lymphocytes ($x10^9$/L)	7.12	8.96	7.01	6.44	5.19*	6.15	6.10	5.92	4.01#	4.03#
APTT (sec)	16.9	17.9	16.8	16.9	20.8*	19.3	17.3*	16.9*	17.9*	16.4#
BLOOD CHEMISTRY										
AST (U/L)	74	65	79	78	79	59	69	63	64	73#
Ca (mmol/L)	2.84	2.76#	2.72#	2.74#	2.75#	2.89	2.73#	2.76#	2.74#	2.75#
Phos (mmol/L)	3.02	3.08	2.97	2.87	2.99	2.17	2.09	1.85*	1.75#	1.72#
Total Protein (g/L)	63	63	61*	61*	61*	70	63*	66*	64*	65*
Alb (g/L)	1.36	1.33	1.47	1.36	1.39	1.35	1.45	1.47*	1.52*	1.44*
HISTOPATHOLOGY										
Lymphocytolysis	0	0	3*	4*	2	2	1	3	3	4
Slight	0	0	3	4	2	2	1	3	3	4
Moderate	0	0	0	0	0	0	0	0	0	0

* p<0.05 with respect to the control # p<0.01 with respect to the control

CONCLUSIONS

It is concluded that oral administration, via the diet, of IR5885 to Han Wistar rats for four weeks at concentrations up to 15000 ppm was well tolerated, producing non-specific toxicity at 15000 only. The NOAEL in this study was considered to be 3000 ppm (equivalent to 311 mg/kg/day for males and 314 mg/kg bw/day for females). Concentrations up to 1000 mg/kg bw/day are considered to be suitable for the subsequent 13-week toxicity study.

3.12.1.2 Oral 90-day study on rats – IR5885

Reference Number: KII- Section 3, Volume 3, Annex IIA, point 5.3.2/02

Report: See annex conf. 49

Guidelines: OECD 408

Deviation: None

GLP: Yes (Certified laboratory)

Executive summary:

In a 13-week toxicity study, groups of 10 male and 10 female Han Wistar rats received IR5885 in their diet (7 days/week) at doses designed to achieve 0, 7, 150 or 1000 mg/kg bw/day. A further 5 male and 5 female rats were assigned to the Control and high dose groups for a 4-week recovery period following the 13-week treatment period.

There were no mortalities and no effect of treatment on appearance, nor was there any evidence of neurotoxicity during arena observations, sensory reactivity, grip strength or motor activity investigations.

Bodyweight gain, food consumption and food conversion efficiency were unaffected by treatment. There were no opthalmic findings related to treatment.
During Week 13 of treatment, several haematological parameters resulted to be slightly different from the respective values in Control for animals receiving 150 or 1000 mg/kg bw/day. All these changes were minimal and close to the mean of background data; furthermore none of them was apparent at the end of the recovery period. Blood chemistry investigations during Week 13 revealed some changes in females receiving the highest dose, however, all of these changes showed recovery at the end of 4-week recovery period. Urinalysis revealed slightly altered urine volume, pH and specific gravity for animals given 1000 mg/kg bw/day. Liver weights were slightly high after 13 weeks of treatment for animal receiving 1000 mg/kg bw/day but they had returned to normal at the end of the recovery period. Macroscopic examination of animals killed after 13 weeks of treatment revealed distended caecum for seven males and one female receiving 1000 mg/kg bw/day. This was not evident at the end of the recovery period.
It is concluded that oral administration, through the diet, of IR5885 to Han Wistar rats for 13 weeks at dosages of 7, 150 and 1000 mg/kg bw/day was well tolerated. Treatment produces changes in caecum and adaptive changes in the liver and the kidney. All findings that were attributable to treatment showed recovery four weeks after cessation of treatment. The NOAEL was considered to be 150 mg/kg bw/day.

MATERIALS AND METHODS

MATERIALS:

Test Item:	IR5885
Description:	White powder
Lot/Batch #:	FCF/T/180-00 (ex ZI 068)
Purity:	98.9%
CAS #:	283159-90-0
Stability of test item:	Stable in the dark at room temperature and also for at least 14 days at 54 °C. Distributed homogeneously in the feed. Batches of test diets were prepared weekly or at two-week intervals and maintained in sealed metal container.
Vehicle and/or positive control:	None
Test animals –	
Species:	Rat
Strain:	Han Wistar
Age:	26 - 30 days at arrival. 39 - 43 days at dosing
Weight at dosing:	130 – 164 g males; 103 – 128 g females
Source:	Harlan UK Limited, Beicester, Oxfordshire, England
Acclimation period:	13 days
Diet:	Rat and mouse No. 1 Maintenance diet (Special Diets Service Ltd, Witham, Essex, England) *ad libitum*
Water:	Tap water from the municipal water main system, *ad libitum*
Housing:	5 animals/sex in cages with a stainless steel body with a stainless mesh lid and floor
Environmental conditions -	
Temperature:	19 – 23 °C
Humidity:	40 – 70 %
Air changes:	At least 15 per hour
Photoperiod:	12-hour light : 12-hour dark cycle

STUDY DESIGN AND METHODS:

In life dates:	August 9, 2001 – December 19, 2001

Animal assignment and treatment

10 animals of each sex per dose group were used. A further 5 male and 5 female rats were assigned to the Control and high concentration groups for a 4-week recovery period following the 13-week treatment period. Animals were non-selectively allocated to cages and treatment groups. IR5885 was continuously administered via the diet throughout the 13-week treatment period at the following fixed doses – 0, 7, 150 and 1000 mg/kg bw/day (Table 3.12.1.2-01) to all males and females. A negative control group received untreated diet.

Table 3.12.1.2-01 Study design

Test group	Dosage (mg/kg bw/day)	Animals assigned	
		male	female
1	0	15	15
2	7	10	10
3	150	10	10
4	1000	15	15

Diet preparation and analysis

The test item (approximately 1045 g) was incorporated into the diet to provide the required concentrations by initial preparation of a premix. This premix was then diluted with further quantities of plain diet to prepare the intermediate and the high concentrations test mixes and a second premix. This second premix was used to prepare the lowest concentration test mixes. Batches of test diets were prepared weekly or at two-week intervals and maintained in sealed metal containers. The unused residue at the end of each week was discarded.

The Sponsor provided the test material IR5885 and the analytical standard. The homogeneity and the stability of the test item in the rodent diet formulation were confirmed at nominal concentrations of 50 and 20000 ppm during an associated analytical study performed at Huntingdon laboratories (Huntingdon Life Science Report No. IGA012/013038). The stability was confirmed during storage in the freezer for 21 days and at room temperature for 22 days following preparation. At specified intervals (Weeks 1, 6 and 12) during treatment, representative samples of the test diets were taken from the mixer and sub-sampled in duplicate before analysis (HRC/FCH/M20/01 issue 01/240501).

Table 3.12.1.2-02 Homogeneity and stability of test material

Nominal Inclusion (ppm)	Analysed concentration (ppm)	% of nominal	Mean of mix (T+M+B)	CV (%)
50	T=54.4	109		
	M=53.7	107	54.1	2.18
	B=54.3	109		
	S_{RT}=53.3	107		
	S_{-20}=51.9	104		
20000	T=21400	107		
	M=22150	111	21400	4.06
	B=20500	103		
	S_{RT}=20000	100		
	S_{-20}=21400	107		

T= top; M= middle; B= bottom;
S_{RT}= 22-d stability at room temperature; S_{-20}= 21-d stability at –20 °C

The homogeneity of IR5885 in rodent diet formulation was confirmed at nominal concentrations of 50 and 20000 ppm. Each formulation achieved accuracy within 8.5% of the nominal concentration and a precision, measured by the coefficient of variation, of <4.5%. The stability was confirmed, at above-mentioned nominal concentrations, during a 21-day frozen storage period and a storage period of 22 days at room temperature following fresh preparation (Table 5.3.2-06).

Statistics

For organ weight and body weight changes, homogeneity of variance was tested using Bartlett's test. Whenever this was found to be statistically significant, Behrens-Fisher test was used to perform pairwise comparisons, otherwise Dunnett's test was used.
Inter-group differences in macroscopic pathology and histopathology were assessed using Fisher's Exact test.
In clinical pathology data, treatment groups were compared using Mantel's test for trend in proportions and also pairwise Fisher Exact tests for each dose group against the control.

Observations

Animals were inspected at least twice daily for evidence of reaction to treatment or ill-health. In addition, blind detailed weekly physical examinations and arena observations were performed on each animal for signs of toxicity.

Body weight, food consumption and achieved dosage

Each animal was weighed during the acclimatisation period (Day –7), on the first day of treatment (Week 0), at weekly intervals throughout the treatment and recovery periods and before necropsy.
Food consumption was measured at weekly intervals. The mean weekly consumption per animal was calculated from the data recorded for each cage. Group mean conversion efficiencies were calculated for each week of treatment. The group mean achieved dosage for each sex, expressed in mg/kg bw/day, was calculated for each week from the nominal dietary test material concentration, food consumption and body weight data.

Sensory reactivity, grip strength and motor activity

During Week 12 of treatment, assessment of sensory reactivity, grip strength and motor activity were performed on each male and female, including the five male and female recovery animals. These assessments were performed at a similar time of day. As no-treatment related changes were observed these investigations were not performed during the recovery phase. Each rat was subjected to the procedures detailed below by an observer who was unaware of the treatment applied to the animal.

Approach response
touch response
auditory startle reflex
tail pinch response
grip strength
motor activity.

Ophthalmic examination

During Week 13 of treatment, both eyes of all surviving animals from group 1 and 4 (Main study and Recovery) were examined by means of an indirect ophthalmoscope, after the instillation of 0.5% tropicamide. The structures examined include the following: adnexa – conjunctiva – cornea and sclera – anterior chamber and iris – lens and vitreous – ocular fundus. As no treatment-related changes were observed, the examination was not performed during the recovery phase or for animals of group 2 and 3.

Haematology & clinical chemistry

During Week 13 of treatment, after overnight starvation, blood samples from all Main study animals were withdrawn from the retro-orbital sinus, with the animals held under Isofluorane anaesthesia, and collected into EDTA anticoagulant. All samples were examined for the following parameters: haematocrit (Hct), haemoglobin concentration (Hb), erythrocyte count (RBC), total and differential leucocyte count (WBC), platelet count (Plt), mean cell haemoglobin concentration (MCHC), mean cell haemoglobin (MCH), mean cell volume (MCV). Additional blood sample was taken into citrate anticoagulant and examined in respect of prothrombin time (PT) and activated partial thromboplastin time (APTT). Further blood samples were similarly taken during Week 4 of the recovery period from all recovery group animals. Samples were

examined for the following parameters which suggested an effect of treatment in Week 13: Hct (males only), Hb (males only), RBC (males only), WBC (males and females), Plt (males only), PT (males only). Bone marrow samples were obtained at necropsy from the femur of all animals killed on completion of the treatment and recovery periods. Smears from all animals of groups 1 and 4 sacrificed in Week 13 were examined computing the myeloid : erythroid ratio and also assessing the cellularity and composition of the marrow. At the same time as for peripheral haematology, further blood samples were taken and collected into lithium heparin as anticoagulant. After separation the plasma was analysed for: alkaline phosphates (ALP), alanine amino-transferase (ALT), aspartate amino-transferase (AST), gamma glutamyl transpeptidase (gGT), glucose concentration (Gluc), total bilirubin concentration (Bili.), total cholesterol concentration (Chol.), total triglyceride concentration (Trig), creatinine concentration (Creat), urea concentration, total protein concentration (Total Prot), albumin (Alb), albumin/globulin ratio (A/G Ratio), sodium (Na), potassium (K) and chloride (Cl), calcium concentration (Ca Total) and inorganic phosphorus (Phos).

Urinalysis

During Week 13 of treatment overnight urine samples were collected from all Main study animals and examined in respect of: appearance (App), volume (Vol), pH, specific gravity (SG), protein (Prot), glucose (Gluc), ketones (Keto), bilirubin, blood. The sediment from centrifugation was examined microscopically for epithelial cells (Epi), polymorphonuclear leukocytes (Leuc), erythrocytes (RBC), crystals (Cryst), casts, spermatozoa and precursors (Sperm) or other abnormalities (Abn). During Week 4 of the recovery period overnight urine samples were similarly collected from all recovery animals. Samples were examined in respect of the following parameters, which were considered to have shown an effect of treatment in Week 13: Vol, pH and SG.

Sacrifice and pathology

At study termination, all animals were sacrificed by carbon dioxide inhalation. All animals were subjected to a detailed necropsy and the following organs were weighed: adrenals, brain, epididymides, heart, kidneys, liver, lungs (included bronchi), ovaries, spleen, testes, thymus, thyroid with parathyroid, uterus with cervix.
Samples of appropriate tissues were preserved and subsequently processed for histopathological examination.
Microscopic examination was performed as follows: i) The adrenals, brain, femur, heart, kidneys, liver, lungs, spinal cord, sternum, stomach, thyroid and uterus were examined for all animals of group 1 and 4 sacrificed on completion of the 13 weeks of treatment. ii) The kidney, liver and lungs were examined for all animals of groups 2 and 3 killed on completion of the 13 weeks of treatment and the 4 weeks of recovery. iii) Tissues reported at macroscopic examination as being abnormal were examined for all animals. Findings were either reported as “Present” or assigned a severity grade. In the latter case one of the following five grades was used: minimal, slight, moderate, marked or severe.

RESULTS AND DISCUSSION

Clinical signs and arena observations, mortality

The appearance and behaviour of the animals were unaffected by treatment. Arena observations were also unaffected by treatment.
There were no deaths.

Body weight and body weight gain, food consumption and food conversion efficiency

Body weight gain was unaffected by treatment.

Table 3.12.1.2-03 Achieved intakes

Test group	dosage (mg/kg bw/day)	Dose per animal (averages over the 13 Weeks)	
		Male (mg/kg bw/day)	Female (mg/kg bw/day)
1	0	0	0
2	7	7.0	7.0

3	150	151.0	150.0
4	1000	1013	1020

Food consumption and food conversion efficiency were considered unaffected by treatment.
The mean concentrations of IR5885 in test diet formulations analysed during the toxicity study were between 12.4% above and 5% below nominal concentrations, which, although slightly above the normal limits (+10/-15%), were not considered to have an adverse effect on the toxicological interpretation of the study. The precision of the duplicate analyses was <5%. The values for the procedural recoveries were within 94.1% and 100.7%, confirming the accuracy of the method.

Sensory reactivity, grip strength and motor activity

Sensory reactivity and grip strength were unaffected by treatment. Motor activity scores for males and females showed considerable inter-group variation but the differences were not considered to be associated with treatment.

Ophthalmoscopy

There were no ophthalmic findings related to treatment.

Blood analysis

Haematological findings (peripheral blood and bone marrow)

Haematology investigations during Week 13 of treatment revealed, when compared with the Controls, slight low haematocrit, haemoglobin concentrations and red blood cell counts for males receiving 150 or 1000 mg/kg bw/day though the differences were not always statistically significant. Lymphocytes and monocyte counts for males receiving 150 or 1000 mg/kg bw/day were slightly low when compared with the Controls. Neutrophil counts were also slightly low for females receiving 1000 mg/kg bw/day. These differences, however, did not show any dose-relationship in males and are unlikely to be of toxicological significance. Slight high platelet count (no dose-related) and slightly shorter prothrombin times were apparent for males given 150 or 1000 mg/kg bw/day. All the changes were resolved by the end of the recovery period.
Examination of bone marrow smear revealed an apparently high total myeloid count for males given 1000 mg/kg bw/day (associated with the reduction of leukocytes, kariocytes or broken down unidentifiable cells). Since the differences with the control were small and females were unaffected, this finding was not considered toxicologically significant.

Clinical chemistry findings

Blood chemistry examination during week 13 of treatment revealed, when compared with the Controls, low plasma triglyceride and high chloride concentrations for males receiving 150 or 1000 mg/kg bw/day and slightly higher calcium concentrations for females receiving 1000 mg/kg bw/day. After the 4-week recovery period triglyceride concentrations were still slightly lower than those of the Controls, although there was partial recovery and the difference did not attain statistical significance. Other differences showed a total recovery.

Urinalysis

Urinalysis investigations revealed slightly high volume and pH and slightly low specific gravity for males and females receiving 1000 mg/kg bw/day. Slightly high pH was also observed for males and females receiving 150 mg/kg bw/day and for males receiving 7 mg/kg bw/day. These changes had resolved by the end of the recovery period.

Sacrifice and pathology

Organ weight

Analysis of organ weights of rats killed after 13 weeks of treatment revealed high absolute and bodyweight-relative liver weights in males and females given 1000 mg/kg bw/day. After 4 weeks of recovery liver weights for previous treated animals was similar to those of Controls. A few differences attained statistical significance after the 4-week recovery phase (low absolute and bodyweight-relative thymus and high bodyweight-relative kidney weights in males), but since these differences were not evident for animals killed after 13 weeks of treatment, they were considered to have arisen by chance and are not of any toxicological significance.

Gross and histopathology

Distended caecum for seven males and one female given 1000 mg/kg bw/day was observed at macroscopic pathology examination. No other findings attributable to treatment were found. There were no macroscopic changes related to previous treatment with IR5885 for animals killed after the 4-week recovery phase.

Table 3.12.1.2-04 Selected haematology, clinical chemistry, urinalysis and pathology findings among rats

	Males						**Females**					
dosage mg/kg bw/day	0	7	150	1000	0 R	1000 R	0	7	150	1000	0 R	1000 R
Number in group	10	10	10	10	5	5	10	10	10	10	5	5
HAEMATOLOGY (peripheral blood and bone marrow)												
Hct (L/L)	0.446	0.443	0.436	0.425#	0.443	0.439	0.414	0.407	0.405	0.411	n.a	n.a.
Hb (g/dL)	15.8	15.6	15.3*	15.1#	15.8	15.9	14.6	14.4	14.3	14.5	n.a.	n.a.
RBC ($x10^{12}$/L)	8.39	8.43	8.29	8.19	8.36	8.50	7.53	7.44	7.29	7.45	n.a.	n.a.
Lymphocytes ($x10^9$/L)	6.91	5.91	4.99*	5.96*	6.04	6.43	3.38	3.15	2.86	3.18	4.22	4.44
Monocytes ($x10^9$/L)	0.25	0.21	0.19*	0.18*	0.23	0.24	0.12	0.12	0.09	0.10	0.17	0.14
Neutrophils ($x10^9$/L)	1.13	0.94	1.14	1.12	1.33	1.22	0.68	0.62	0.60	0.47*	1.05	0.85
Plt ($x10^9$/L)	833	786	928#	895#	893	836	876	908	915	890	n.a.	n.a.
PT (sec)	15.7	15.0	14.5#	14.2#	15.1	15.6	14.2	14.0	14.3	14	n.a.	n.a.
Total myelocytes(%)	n.a	n.a	n.a	n.a	50.7	55.4*	n.a	n.a	n.a	n.a	41.9	43.8
BLOOD CHEMISTRY												
Trig (mmol/L)	1.36	1.20	0.90#	0.87#	1.63	1.11	0.52	0.58	0.49	0.49	n.a.	n.a.
Cl (mmol/L)	106	106	107*	108#	104	103	107	107	108	108	n.a.	n.a.
Ca (mmol/L)	2.93	2.91	2.87	2.98	n.a.	n.a.	2.93	2.96	2.93	3.02*	2.94	2.97
URINALYSIS												
Vol (mL)	3.5	2.5	4.8	5.6#	4.8	3.6	1.9	1.4	2.3	3.2	2.5	2.9
pH	6.9	7.4*	7.3*	7.3*	7.0	7.1	5.9	5.8	6.4*	6.4#	5.6	5.7
SG (g/L)	1038	1041	1035	1034	1036	1035	1050	1052	1045	1039*	1051	1044
ORGAN WEIGHTS												
Liver-absolute (g)	11.96	10.89	12.29	14.20	14.12	13.71	7.66	7	7.46	8.53	7.42	7.55
Liver-relative (%)	3.336	3.201	3.380	3.846#	3.627	3.553	3.546	3.534	3.632	4.005#	3.496	3.704
Thymus-absolute (g)	0.320	0.329	0.355	0.344	0.361	0.285*	0.312	0.269	0.304	0.298	0.265	0.247
Thymus-relative (g)	0.090	0.098	0.098	0.095	0.093	0.704*	0.144	0.137	0.148	0.141	0.125	0.121
Kidney-relative (g)	0.550	0.549	0.540	0.585	0.505	0.567 *	0.601	0.657#	0.637	0.601	0.600	0.626
MACROSCOPIC PATHOLOGY (incidence)												
Distended caecum	0	0	0	7#	0	0	0	0	0	1	0	0

* p<0.05 with respect to the control
# p<0.01 with respect to the control
n.a. not analysed

CONCLUSIONS

It is concluded that oral administration, via the diet, of IR5885 to Han Wistar rats for 13 weeks at dosages of 7, 150 and 1000 mg/kg bw/day was well-tolerated with no overt signs of toxicity. Treatment produced changes in the caecum and adaptive changes in the liver and kidneys. All findings that were attributable to treatment showed recovery four weeks after cessation of treatment. The NOAEL in this study was considered to be 150 mg/kg bw/day.

3.12.1.3 Combined long-term and carcinogenicity study – IR5885

Reference Number:	KII- Section 3, Volume 5-6-7, Annex IIA, point 5.5.1/01
Report:	See annex conf. 51
Guidelines:	OECD 453 – US OPPTS 870.4300
Deviation:	None
GLP:	Yes (Certified laboratory)

Executive summary:

The carcinogenic and toxic potential of the fungicide IR5885, given by dietary administration to Han Wistar rats over a period of 104 and 52 weeks, respectively, was assessed in this study. Three groups of 50 male and 50 female rats received the test item at concentrations designed to achieve dosages of 15, 150 or 1000 mg/kg bw/day for 104 weeks. Together with a similarly constituted group that received the vehicle (untreated diet), these animals comprised the Carcinogenicity phase of the study. A further 20 male and 20 female rats assigned to each group were sacrificed after completion of 52 weeks of treatment and comprised the Toxicity phase of the study.
During the study, clinical condition, detailed physical and arena observations, sensory reactivity, grip strength, motor activity, bodyweight, food consumption, water consumption, ophthalmic examination, haematology, blood chemistry, urinalysis, organ weight, macropathology and histopathology investigations were undertaken.
Mortality was unaffected by treatment.
Slight increased incidence of yellow staining in the perigenital area was observed in females receiving 1000 mg/kg bw/day. Brown urine staining on the cage paper was observed in males and females at this dosage, up to Week 23
The group distribution, multiplicity and mean onset time of palpable swellings were unaffected by treatment.
There was no evidence of neurotoxicity from arena observations or assessment of sensory reactivity and grip strength. Animals receiving 1000 and (to a lesser extent) 150 mg/kg bw/day showed slightly increased motor activity scores during Week 50.
Overall body weight gain was slightly low in males receiving 150 or 1000 mg/kg bw/day assigned to the Carcinogenicity phase and food consumption was slightly low during the first two weeks of treatment in males receiving the two highest doses.
There were no treatment-related ophthalmic findings.
Hematological investigations indicated persistently low hemoglobin concentration in males receiving 1000 mg/kg bw/day. In Week 13 and 26 this finding was associated with reduced erythrocytes counts and mean cell hemoglobin concentration. Hematocrit, hemoglobin concentration and erythrocyte counts were slightly low during Weeks 13, 26 and 52, respectively, also in females receiving 1000 mg/kg bw/day.

Examination of tail vein smears taken from Carcinogenicity phase animals did not reveal any treatment-related change.
There were no toxicological changes in the blood plasma.
Urinalysis investigations revealed some alterations in pH, ketones (in males) and urine volume (in females) treated at the two highest concentrations, which resulted to be slightly higher than respective control values. Specific gravity and protein concentration, in general, resulted to be slightly lower in females at 150 and 1000 mg/kg bw/day during Week 25 whereas a slight increase in males treated at 1000 mg/kg bw/day during weeks 25 and 51 was observed. The appearance and composition of urine during weeks 77 and 103 were unaffected by treatment.
Slight high absolute and bodyweight-relative liver weight was registered in both Carcinogenicity and Toxicity animals treated at 1000 mg/kg bw/day. Bodyweight-relative higher kidney weights were also observed in Toxicity phase males treated at 1000 mg/kg bw/day.
Macroscopic examinations after 52 and 104 weeks of treatment did not reveal any findings related to treatment.
There were no neoplastic changes due to treatment with IR5885.
Histopathology examinations performed in animals killed after 52 weeks of treatment indicated an increased incidence of pelvic/papillary epithelial hyperplasia in the kidney of females at 1000 mg/kg bw/day and follicular cell hypertrophy in the thyroids of males receiving 1000 mg/kg bw/day. This latter change was not present in Carcinogenicyty phase animals.
It is concluded that oral administration of IR5885, a fungicide, via the diet to Han Wistar rats for 104 weeks at concentrations designed to achieve dosages of up to 1000 mg/kg bw/day was generally well tolerated, with no evidence of significant toxicity or any increase in tumour incidence. The thyroid and kidney were identified as target organs. Based on the above-reported findings, the NOAEL in this study was considered to be 150 mg/kg bw/day in males and 1000 mg/kg bw/day in females.

MATERIALS AND METHODS

MATERIALS:

Test Item:	IR5885
Description:	White powder
Lot/Batch #:	T 025/02 (Total amount of test item was deliver in two times)
Purity:	99.56% (weeks 1-103) – 99.63% (weeks 104-106)
CAS #:	283159-90-0
Stability of test item:	Stable in the dark at room temperature
Vehicle:	None
Test animals –	
Species:	Rat
Strain:	Han Wistar
Age:	26-30 days at arrival; 37-41 at beginning of dosing
Weight:	109-150 g males; 90-122 g females
Source:	Harlan UK Ltd, Bicester, Oxfordshire, England.
Acclimation period:	11 days
Diet:	Rat and mouse No.1 Maintenance Diet, *ad libitum*
Water:	Tap water from the municipal water main system, *ad libitum*
Housing:	Five per sex/cage. Cages were made of stainless steel body with a stainless steel mesh lid and floor.
Environmental conditions -	
Temperature:	21 °C ± 2
Humidity:	55% ± 15
Air changes:	Periodically (frequency not specified)
Photoperiod:	12-hour light : 12-hour dark cycle (7 a.m. – 7 p.m.)

STUDY DESIGN AND METHODS:

In life dates: February 28, 2002 to March 18, 2004

Animal assignment and treatment

As shown in table 3.12.1.3-01, IR5885 was administered over a period of 52 consecutive weeks to animals assigned to the Toxicity phase and 104 consecutive weeks to animals assigned to the Carcinogenicity phase. On completion of the respective phases the animals were sacrificed. The animal numbering system of the Toxicity phase was such that it was not easy to identify a treatment group from the animal number.

Table 3.12.1.3-01 Study design

Test group	Toxicity phase (52 weeks)		Carcinogenicity phase (104 weeks)		Dosage (mg/kg bw/day)
	Males	Females	Males	Females	
1	20	20	50	50	0
2	20	20	50	50	15
3	20	20	50	50	150
4	20	20	50	50	1000

Test item formulate preparation and analysis

IR5885 was incorporated into the diet to provide the required concentration by dilution of an appropriate premix. All formulated diets were prepared freshly each week, with diets for males and females being prepared separately. The concentrations for each group were adjusted at the same frequency as food and bodyweight recordings were made in order to achieve the required dosage in terms of mg/kg bw/day.
Two primary premixes were prepared in order to cover the preparation of the high and low concentration diets. For each premix the required amount of test item was gradually mixed with half the required final weight of diet, which was finely sieved and finally mixed in a coffee grinder. The resultant mix was diluted with coarse diet to achieve the final weight and blended in a Turbula mixer for 100 revolutions. All formulations were stored at room temperature. The unused residue at the end of each week was discarded for all groups.
The suitability of the mixing procedure and the homogeneity and stability of the test material in the diet matrix following room temperature storage for 22 days and frozen storage (-20 °C) for 21 days, were demonstrated at concentrations of 50 and 20000 ppm (Report No. IGA 012/013038). Further homogeneity and stability assessments were performed on the current study at a dietary concentration of 30000 ppm to cover the range of concentrations expected to be used in this study. The homogeneity was assessed as analysed concentration at the top, middle and bottom of the freshly prepared diet formulation. The stability was assessed on samples taken from the homogeneity mix and was reported as the concentration of samples taken at intervals during ambient temperature storage up to 22 days. Each homogeneity and stability sample taken on day 8 and 22 was analysed in duplicate. The precision of the duplicate analyses of each sample and of the homogeneity samples was expected to be within 5%. The accuracy of each formulation was expected to be within –15% and +10% of nominal concentrations.

Statistics

All statistical analyses were carried out separately for males and females and considering the individual animal as the basic experimental unit. For categorical data, including pathological findings, the proportion of animals was analysed using Fisher Exact test for each treated group versus control.
For continuous data, Bartlett's test was first applied to test the homogeneity of variance among the groups. Using tests dependent on the outcome of Bartlett's test, treated groups were then compared to the control group, incorporating adjustment for multiple comparisons where necessary. For bodyweight gains and organ weights, whenever Bartlett's test was found to be statistically significant, a Behrens Fisher test was used to perform pairwise comparisons, otherwise a Dunnett's test was used.

Significant differences between control and treated groups were expressed at the 5% ($p<0.05$), 1% ($p<0.01$) or 0.1% ($p<0.01$) level. The following statistical ciphers were used throughout the report:
a – $p<0.05$; b – $p<0.01$ – using categorical or parametric tests
A – $p<0.05$; B – $p<0.01$ – using non-parametric tests
Inter-group differences in mortality and tumour incidence were performed using the approach of Peto *et al*. (British J. Cancer 29 (1974) pp. 101-105).

METHODS:

Mortality and clinical observations

Animals were inspected visually at least twice a day for evidence of ill-health or reaction to treatment. Cages were inspected daily for evidence of any abnormality, such as loose faeces. Before treatment commenced and weekly thereafter a detailed physical examination, which included palpation, was performed on all animals to monitor general health.
Debilitated animals were observed carefully and, where necessary, isolated to prevent cannibalism. Where possible, blood smears were taken *ante mortem* from the Carcinogenicity phase animals.

Detailed physical examination

For Toxicity phase animals, arena observations were performed each week in addition to the physical examination, at approximately the same time of a day by an observer unaware of the experimental group to which the animal belong. Particular attention was paid to possible signs of neurotoxicity such as convulsion, tremor and abnormalities of gait or behaviour.

Sensory reactivity and grip strength

During Week 50 of treatment sensory reactivity and grip strength assessment was performed on the 10 males and 10 females with the lowest animal numbers in each group of the Toxicity phase by an observer unaware of the experimental group to which the animal belonged. The following measurements, reflexes and responses were recorded: approach response – grip strength - auditory startle reflex – tail pinch response – touch response.

Motor activity

During Week 50 of treatment the motor activity of the 10 males and 10 females with the lowest animal numbers in each group of the toxicity phase was measured using an automated equipment. Activity measurements were recorded for ten six-minute periods (a total of one hour).

Bodyweight

The weight of each rat was recorded on the day that treatment commenced (Week 0), at weekly intervals for the first 16 weeks, and once every four weeks thereafter (in coincidence with the end of a food consumption measurement period) and before necropsy.

Food consumption

The weight of food supplied to each cage, that remaining and an estimate of any spilled was recorded weekly for the first 16 weeks and every four week throughout the remaining treatment period. From these records the mean weekly consumption per animal (g/rat/week) was calculated for each cage.

Water consumption

During Week 24 of treatment, water consumption was recorded by weight (over a 3-day period) for each cage of animals, using water bottles fitted with sipper tubes.

Ophthalmic examination

Before treatment commenced, the eyes of all animals allocated to the study (including spare animals) were examined by means of a binocular indirect ophthalmoscope. Rejected animals were replaced with animals with no adverse ocular abnormality. During Week 51 of treatment the eyes of all animals of groups 1 and 4 assigned to the Toxicity phase were similarly examined.

Hematology, peripheral blood

During Weeks 13, 26 and 52 of treatment, blood samples were obtained from all surviving Toxicity phase animals after overnight starvation. Similarly, during Weeks 78 and 104 of treatment, blood samples were obtained from the 10 males and 10 females from each group with the highest animal numbers assigned to the Carcinogenicity phase. Animals were held under light general anaesthesia induced by isofluorane and blood samples were withdrawn from the retro-orbital sinus. Blood samples were collected into EDTA as anticoagulant and examined for the following characteristics: haematocrit (Hct), haemoglobin concentration (Hb), erythrocyte count (RBC), total and differential leucocyte count (WBC), platelet count (Plt), mean cell haemoglobin concentration (MCHC), mean cell haemoglobin (MCH), mean cell volume (MCV). Additional blood sample was taken into citrate anticoagulant and examined in respect of prothrombin time (PT) and activated partial thromboplastin time (APTT).

Hematology, blood smears

During Week 52, 78 and 104 of treatment, blood smear preparations were prepared from tail vein samples obtained without the use of anaesthesia from all surviving animals assigned to the Carcinogenicity phase that were not used for routine blood sampling. The smears of groups 1 and 4 were examined for: differential leukocyte count, abnormal morphology and unusual cell types, including normoblasts.

Blood chemistry

During weeks 26 and 52 of treatment, further blood samples were collected from the 10 males and 10 females from each group with the highest animal numbers assigned to the Toxicity phase. During week 78 and 104 of treatment blood samples were collected from the 10 males and 10 females from each group with the highest animal numbers assigned to the Carcinogenicity phase. Samples were taken after overnight starvation from the retro-orbital sinus under light anaesthesia induced by isofluorane and were collected into tubes containing lithium heparin as anticoagulant. After separation the plasma was analysed for: alkaline phosphatase (ALP), alanine amino-transferase (ALT), aspartate amino-transferase (AST), gamma glutamyl transpeptidase (gGT), creatine phosphokinase (CPK), glucose concentration (Gluc), total bilirubin concentration (Bili.), total cholesterol concentration (Chol.), creatinine concentration (Creat), urea concentration, total protein concentration (Total Prot), albumin (Alb), albumin/globulin ratio (A/G Ratio), sodium (Na), potassium (K) and chloride (Cl), calcium concentration (Ca Total) and inorganic phosphorus (Phos).

Urinalysis

During Week 12, 25 and 51 of treatment overnight urine samples were collected from the 10 males and 10 females from each group with the highest animal numbers assigned to the Toxicity phase. During Weeks 77 and 103 of treatment, samples were collected from the 10 males and 10 females from each group with the highest animal numbers assigned to the carcinogenicity phase. The individual samples were examined for the following characteristics: appearance (App), volume (Vol), pH, specific gravity (SG), sodium (Na), potassium (K) and chloride (Cl), glucose (Gluc), ketones (Keto), bile pigments (Bili), blood. The sediment from centrifugation was examined microscopically for epithelial cells (Epi), leukocytes (Leuc), erythrocytes (RBC), crystals (Cryst), casts, spermatozoa and precursors (Sperm) or other abnormalities (Abn).

Necropsy and histology

Animals killed during the study and those surviving until the end of the scheduled Toxicity or Carcinogenicity phase were killed by carbon dioxide asphyxiation.
All animals were subjected to a detailed necrospy. After a review of the history of each animal, a full macroscopic examination of the tissues was performed. The requisite organs were weighed.

Organ weights – The following organs, taken from each animal killed after 52 or 104 weeks of treatment, were weighed: adrenals, brain, epididymides, heart, kidneys, liver, ovaries, spleen, testes, thymus, thyroid with parathyroids, uterus with cervix.

Histology – For those animals specified in the Pathology section, the relevant tissues were subjected to histological processing. Tissues and samples were dehydrated, embedded in paraffin wax, sectioned at approximately four to five micron thickness and stained with haematoxylin and eosin. Those tissues subject to histological processing included the following regions: adrenals (cortex and medulla) – brain (cerebellum, cerebrum and midbrain) – femur with joint (bone marrow, epiphysal plate and longitudinal section including articular surface) – heart (auricular and ventricular regions) – kidneys (cortex, medulla and papilla regions) – liver (section from all main lobes) – lungs (section from two major lobes, to include bronchi) – spinal cords (transverse and longitudinal section at the cervical, lumbar and thoracic levels) – sternum (included bone marrow) – stomach (included keratinised, glandular and antrum in sections) – thyroid (included parathyroids in section, where possible) – uterus (uterus section separate from cervix section).

Pathology

All tissues preserved for examination were examined for all Toxicity and Carcinogenicity phase animals of Groups 1 and 4 sacrificed on completion of the scheduled 52 or 104 weeks treatment period and for all animals killed or dying during these periods. The kidneys, liver and lungs were examined for all Toxicity and Carcinogenicity phase animals and the thyroid (with parathyroid) was examined for all Toxicity phase males of groups 2 and 3 sacrificed on completion of the scheduled 52-week treatment period. Tissues reported at macroscopic examination as being grossly abnormal were examined for all animals.

RESULTS AND DISCUSSION

Formulation chemistry:

The test material was found to be homogeneous and stable in the diet matrix at a concentration of 30000 ppm for up to 22 days at room temperature.

The mean concentrations of IR5885 in test formulations analysed during the study were generally within ± 9% of nominal concentrations. Three deviations from acceptable limits (-15% / +10%) occurred in Week 65 when the values for Group 2F and Group 3M and 3F were 10.2, 11.0 and 11.2% above the nominal concentration, respectively. The small isolated variations were not considered to indicate any inherent problems in the diet preparation procedure. Consequently, these results confirmed the accuracy of formulation.

Mortality

A total of six animals assigned to the Toxicity phase died or were killed during the 52-week treatment period. There were no treatment-related findings observed at necropsy or at histopathology examination. These deaths were considered incidental to treatment.

A total of 44 males and 55 females assigned to the Carcinogenicity phase died or were killed during the 104-week treatment period. The group distribution of deaths was unaffected by treatment.

Table 3.12.1.3-02 Premature deaths

Group/sex	1M	2M	3M	4M	1F	2F	3F	4F
Dosage (mg/kg bw/day)	**0**	**15**	**150**	**1000**	**0**	**15**	**150**	**1000**
Toxicity phase								
Group size	**20**	**20**	**20**	**20**	**20**	**20**	**20**	**20**
Total number of death	1	1	0	0	0	0	1	3
% Survival	95	95	100	100	100	100	95	85
Carcinogenicity phase								
Group size	**50**	**50**	**50**	**50**	**50**	**50**	**50**	**50**
Total number of death	17	7	9	11	13	16	11	15
% Survival	66	86	82	78	74	68	78	70

Detailed physical and arena observations

A slightly increased incidence of yellow staining in the perigenital area was observed in females receiving 1000 mg/kg bw/day.
Brown staining on the cage paper was observed between Weeks 4 and 23 in males receiving 1000 mg/kg bw/day. The staining was identified as being dark between Week 4 and 8. From Week 9 it was described as light brown. Females receiving 1000 mg/kg bw/day had dark brown staining of the cage tray paper in Weeks 4 and 5 only. There were occasional observations of light brown staining on the cage tray paper of low and intermediate dosage animals between Weeks 13 and 23.
Arena observations were unaffected by treatment.

- **Palpable swelling**

Palpable swelling was unaffected by treatment.

- **Motor Activity – Sensory reactivity - Grip strength**

Animals receiving 1000 mg/kg bw/day and, to a lesser extent, those receiving 150 mg/kg bw/day, showed slightly increased motor activity scores during Week 50, when compared to the Controls. Sensory reactivity and grip strength were unaffected by treatment.

Bodyweight

The overall bodyweight gain (Weeks 0 to 104) was low for Carcinogenicty phase males receiving 150 or 1000 mg/kg bw/day (92 and 91% of Controls, respectively), though only the difference at the highest dosage attained statistical significance ($p<0.05$). Female bodyweights were unaffected by the treatment. There was no clear effect of treatment on the Toxicity phase animals.

Food consumption and food conversion efficiency

Food consumption was slightly low, when compared to the control values, during the first two weeks of treatment in males receiving 1000 mg/kg bw/day. However, overall food consumption values (Weeks 1 to 104) were similar for Control and treated animals.
Food conversion efficiency during the first 16 weeks of treatment was unaffected by the treatment.

Achieved dosages and water consumption

The overall achieved dosages were within ±5% of the nominal dosage and were therefore considered satisfactory. The weekly values were generally within ±10% of the target dosages and were also considered satisfactory. Occasional excursions outside this range occurred but in view of their isolated nature they were

not considered significant. Water consumption investigation, during Week 24 did not reveal any effect of treatment.

Ophthalmic examination

There were no ophthalmic findings related to treatment.

Hematology

Hematological investigations indicated persistently low hemoglobin concentration in males receiving 1000 mg/kg bw/day. In Week 13 and 26 this finding was associated with reduced erythrocytes counts and mean cell hemoglobin concentration. Hematocrit, hemoglobin concentration and erythrocyte counts were slightly low during Weeks 13, 26 and 52, respectively, also in females receiving 1000 mg/kg bw/day, although these differences were less consistent than in males. Other parameters were found to be slightly different from their respective control values. However, due to their isolated occurrence and/or persisting in time as well as due to a lack of any-dose relationship, these findings, although sometimes statistically significant were considered not to be related to the treatment.

Hematology, blood smears

Examination of tail vein smears prepared in Week 52, 78 or 104 of treatment from all Carcinogenicity phase animals did not reveal any changes considered to be related to treatment.

Blood chemistry

There were no toxicological significant changes in the biochemical composition of the blood plasma. Reduction in plasma alkaline phosphatase as well as plasma glucose concentration in females or slight increase in plasma cholesterol concentration in males occurred throughout the study but, in view of the small and inconsistent nature of these differences and/or the absence of a clear trend with dosage, they could not be attributed to the treatment.

Urinalysis

Urinalysis revealed, when compared to the Controls, a generally slightly high urine volume, low specific gravity and low protein concentration for females receiving 1000 and (to a lesser extent) 150 mg/kg bw/day during investigations performed in Week 12, 25 and 51. Conversely, specific gravity and protein concentration were slightly high in males receiving 1000 mg/kg bw/day during Week 25 (and also Week 51 for protein concentration, only). A slight increase in ketones in the urine, when compared to the Controls, was observed during Weeks 25 and 51 in males receiving 1000 mg/kg bw/day.
The appearance and composition of urine during Weeks 77 and 103 were unaffected by treatment.

Organ weights

Analysis of organ weights in animals killed after 52 weeks of treatment revealed, when compared to controls, slightly high absolute and bodyweight-relative liver weights in males and females receiving 1000 mg/kg bw/day. Bodyweight-relative kidney weights were also marginally higher than those of controls for males at the highest dosage.
After 104 weeks of treatment, slightly high absolute and bodyweight-relative liver weights, when compared to the Controls, were observed in males and females, which received 1000 mg/kg bw/day. However, these differences were minimal, with only the bodyweight-relative values attaining statistical significance.
All other inter-group differences were minor, lacked dosage-relationship and were therefore attributed to normal biological variation.

Table 3.12.1.3-03 Liver and kidney absolute and bodyweight-relative weights

	Group		1M	2M	3M	4M	1F	2F	3F	4F
	Dosage (mg/kg/day)		**0**	**15**	**150**	**1000**	**0**	**15**	**150**	**1000**
Toxicity Phase (Week 52)	**Liver**									
		Absolute	14.04	14.37	14.50	16.05*	8.54	8.66	9.00	9.53*
		bw-relat.	2.890	3.093	3.095	3.441**	3.196	3.178	3.301	3.587**
	Kidney									
		Absolute	2.28	2.28	2.28	2.35	1.64	1.62	1.63	1.58
		bw-relat.	0.472	0.490	0.490	0.508*	0.617	0.599	0.598	0.595
Carcinogen. Phase (Week 104)	**Liver**									
		Absolute	16.27	15.46	15.55	16.71	11.08	10.90	10.76	11.90
		bw-relat.	2.876	2.805	2.906	3.161**	3.203	3.071	3.083	3.446**
	Kidney									
		Absolute	2.78	2.76	2.70	2.72	1.98	2.03	1.96	2.03
		bw-relat.	0.491	0.490	0.517	0.519	0.577	0.574	0.563	0.592

* p<0.05; ** p<0.01 when compared to control groups

Macroscopic pathology

Macroscopic examination at necrospy in animals killed after 52 and 104 weeks of treatment did not reveal any findings that were considered to be related to treatment with IR5885. All findings were minor, confined to one sex or lacked dosage-relationship and were therefore attributed to normal biological variation.
After 52 weeks of treatment, a high incidence of thin optic nerves was apparent among treated males. For all animals affected the finding was observed in the right eye only and was, therefore, attributed to the retro-orbital sinus blood sampling procedure.

Microscopic pathology

Toxicity phase – treatment related changes were confined to the thyroids where there was an increased incidence of follicular cell hypertrophy in male rats, which received IR5885 at 1000 mg/kg bw/day, as shown in table 3.12.1.3-04

Table 3.12.1.3-04 Group incidence of changes in the thyroids (Toxicity phase)

Group/sex		1M	2M	3M	4M
Dosage (mg/kg bw/day)		**0**	**15**	**150**	**1000**
Follicular cell hypertrophy	**Total**	**3**	**2**	**5**	**11***
	Slight	3	2	5	10
	Moderate	0	0	0	1
Number of animal examined		20	20	20	20

* p < 0.05 when compared to Group 1M

Follicular cell carcinoma was seen in the thyroids of a single male rat at 1000 mg/kg bw/day and follicular cell adenoma was seen in the thyroids of a single male rat at 150 mg/kg bw/day. In the absence of any increase in the incidence of these tumours in the Carcinogenicity phase of this study, they were considered fortuitous.
Carcinogenicity phase :

Neoplastic findings – There were no treatment-related changes. There were no statistically significant differences from Control with respect to tumour incidences in treated groups.

Non-neoplastic findings – There was an increased incidence of pelvic/papillary epithelial hyperplasia in the kidney of females at 1000 mg/kg bw/day (table 3.12.1.3-05).

Table 3.12.1.3-05 Group incidence of changes in the kidney (Carcinogenicity phase)

Group/sex		1F	2F	3F	4F
Dosage (mg/kg bw/day)		**0**	**15**	**150**	**1000**
Pelvic/papillary epithelial hyperplasia	**Total**	**9**	**10**	**5**	**25****
	Slight	7	7	4	17
	Moderate	2	3	1	7
	Marked	0	0	0	1
Number of animal examined		50	50	50	50

** $p<0.01$ when compared to Group 1F

All other changes observed in the Carcinogenicity phase of this study were of the type normally encountered in Wistar rats at the performing laboratories.

CONCLUSIONS

The oral administration of IR5885, a fungicide, via the diet to Han Wistar rats for up to 104 weeks at concentrations designed to achieve dosages of up to 1000 mg/kg bw/day was generally well tolerated, with no evidence of significant toxicity or any increase in tumour incidence. The thyroid and kidney were identified as target organs.

This investigation met all the requirements of a satisfactory carcinogenicity study. Survival, which was not affected by treatment with IR5885, was good, with at least 66% of males and 68% of females surviving in each group up to 104 weeks. The slightly low overall bodyweight gain for males at the two highest dosages suggested slight non-specific toxicity.

The changes observed in this study indicated that the only finding of possible toxicological significance was the slight anemia observed at the highest dosage in males, though as it is considered a secondary change to an effect upon thyroid hormone and therefore probably not relevant to man. In addition, changes in the kidney function were attributed to the excretion of IR5885.

Based on these findings, the NOAEL in this study was considered to be 150 mg/kg bw/day in males and 1000 mg/kg bw/day in females.

3.12.1.4 IR5885 4-Week Dermal Toxicity Study in Rats

Reference Number: KII- **Post submission 02**, Section 3, Volume 2, Annex IIA, point 5.3.7/01

Report: **See annex conf. 23**

Guidelines: OECD 410, EPA OPPTS 870.3200

Deviation: None

GLP: Yes (certified laboratory)

Executive summary:

The dermal toxicity of IR5885 when given by daily topical administration (6 hours exposure) to rats, has been investigated over a period of 4 consecutive weeks. One group of 10 male and 10 female Wistar Hannover rats received the test item by dermal topical application at a dosage of 1000 mg/kg/day for 4 consecutive weeks. A second similarly constituted group received the vehicle alone (sterile water) and acted as a control.
On the basis of the observed results no signs of effects of the test item were seen at the dose level investigated. Therefore, the dose of 1000 mg/kg/day is considered the No Observed Effect Level (NOEL).

MATERIALS AND METHODS

MATERIALS

Test Material **IR5885**
Batch: **T025/02**
Purity: **> 99%**
CAS number: **283159-90-0**

Vehicle and/or positive control: Animal were dosed using a patch of surgical gauze measuring 2.5 x 2.5 cm. A strip of alluminium foil was placed over the treated site and whole assembly held in place by encircling the trunk of the animal with length of adhesive bandage. Control animals received the vehicle alone (sterile water) at the same dose level.

Test animals-
Species: Rat
Strain: Wistar Hannover
Age: 7-8 weeks old
Weight at dosing: 150-177 g
Source: Harlan Italy s.r.l., 33049 San Pietro al Natisone (UD), Italy
Acclimatation period: 18 days
Diet: Commercially available laboratory rodent diet (4 RF 18, Mucedola S.r.l., via G. Galilei, 4, 20019 Settimo Milanese (MI), Italy
Water: Drinking water, obtained from the municipal water, *ad libitum* to each cage via water bottles
Housing: Animals were individually housed in clear polycarbonate cages measuring 42.5 x 26.6 x 18 cm and equipped with a stainless steel mesh lid and floor.

Environmental conditions-
Temperature: 22°C ± 2°C
Humidity: 55% ± 15%
Air changes: 15 to 20 air changes per hour
Photoperiod: artificial light for 12 hours each day

STUDY DESIGN

In life dates: February 23 to March 31, 2005

Animal assignment and treatment

The rats were allocated to the 2 groups by computerised stratified randomisation to give approximately equal initial group mean body weights.

Dosing selection

The dose level of 1000 mg/kg/day (limit test) was selected in consultation with the sponsor based on information from previous studies.

Preparation and treatment of anmals

One day before dosing commenced the fur was removed from the dorsal surfaces of the trunk over an estimated area of 10% of the total body weight.
Animal were dosed with the test item onto the preparated skin site using a patch of surgical gauze measuring 2.5 x 2.5 cm. A strip of alluminium foil was placed over the treated site and whole assembly held in place by encircling the trunk of the animal with length of adhesive bandage.
The amount administered to individual animals was determined by most recently recorded body weight. Prior to administration, each weighted aliquot of the supplied test item was mixed to a paste with 1 mL of sterile water.
Control animals received the vehicle alone (sterile water) at the same dose level (1000 mg/kg/day).
After a period of 6 hours, the tape dressing was removed and the skin site washed.
All animals were dosed once a day, 7 days a week, for a minimum of 4 consecutive weeks. They were dosed up until the day before necropsy.

Statistics

For continuous variables the significance of the differences among groups was assessed by analysis of variance. Differences between each treated group and the control group were assessed by Dunnett's test using a pooled error variance. The homogeneity of the data was verified by Bartlett's test before Dunnett's test. If data were found to be inhomogeneous a Modified test (Cochran and Cox) was applied. The mean value, standerd deviations and statistical analysis were calculated from the actual values in the computer without rounding off.

METHODS

Observations

All observation were recorded for individual animals. Examination of individual animals for signs of reaction to treatment was carried out daily before dosing and approximately 1, 2 and 6 hours after dosing up to day 7 of the study. Since no animals showed any post-dose effects, examinations were reduced to pre-dose and approximately 1 and 6 hours after dosing until the end of treatment.

Body weight

Each animal was weighted on the day of allocation to treatment groups, on the day of preparation (Day-1), on the day that treatment commences, weekly thereafter and just prior to necropsy.

Food consumption

The weight of food consumed by each rat was recorded at weekly intervals following allocation. The group mean daily intake was calculated.

Haematology & clinical chemestry

At the end of 4-week treatment period samples of blood were withdrawn, prior to necropsy, under isofluorane anaesthesia from abdominal vena cava.
Haematology parameters examined were: RBC count, Hgb, HCT, MCH, MCHC, MCV, WBC count and differential, platelet count, reticulocyte count, prothrombin time and abnormalities of the blood film.

Clinical chemestry parameters examined were: alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, gamma-glutamyltransferase, urea, creatinine, glucose, triglycerides, phosphorus, total bilirubin, total cholesterol, total protein, albumin, globulin, A/G ratio, sodium, potassium, calcium and chloride.

Urinalysis

At the end of 4-week treatment period, individual overnight urine samples were collected from all surviving animals under conditions of food and water deprivation.
The parameters examined were: appearance, volume, specific gravity, pH, protein, total reducing substances, glucose, ketones, bilirubin, urobilinogen and blood.
The sediment, obtained fron centrifugation, was examined microscopically for: epithelial cells, poly morphonuclear leucocytes, erythrocytes, crystal, spermatozoa and precursors, other abnormal components.

Sacrifice and pathology

Animals were killed by exanguination under isofluorane anaesthesia at the end of the scheduled test period and subjected to necropsy. From all animals the organs were dissected free of fat and weighed. The ratio of organ weight to body weight were calculated. Samples of all tissues were fixed and preserved in 10% buffered formol saline. After dehydration and embedding in paraffin wax, sections of the tissues required for histopathological examination, were cut at 5 micrometers thickness and stained wiyh haematoxylin and eosin.

RESULTS AND DISCUSSION

Clinical signs of toxicity

Deatailed weekly clinical observations did not show any treatment-related signs. No abnormalities were noted at daily post-dose observations during the study.

Mortality

No mortality occurred during the study.

Body weight

No intergroup differences were observed in bodt weight. The reductions of body weights and terminal body weights, noted in all animals on Day 29 when compared to Day 22, were due to overnight fast preceeding the bleeding procedures for clinical pathology analyses.

Food consumption

No changes were observed in food consumption between treated and control animals. The reductions of food consumption, noted in all animals on Day 29 when compared to Day 22, were due to overnight fast preceeding the bleeding procedures for clinical pathology analyses.

Haematological findings

No treatment-related variations in haematological parameters were observed.

Clinical chemestry findings

No variations of toxicological importance were observed in clinical chemestry parameters.

Organ weight

No changes of toxicological importance were observed in in the organ weights.

Histopathology

The histopathological findings observed in all remaining tissues/organs examined were recognised as those commonly observed in this species and were considered to be spontaneous or incidental origin.

CONCLUSIONS

The dermal toxicity of IR5885 when given by daily topic administration (6 hours exposure) to rats at a dose level of 1000 mg/kg/day, has been investigated over a period of 4 consecutive weeks.
On the basis of the results, no signs of an effect of the test item were seen at the dose level investigated. Therefore, the dose level of 1000 mg/kg/day is considered the No Observed Effect Level (NOEL).
The high value of the No Observed Effect Level shows that there is no problem for the impact on human and animal health**.**

3.12.1.5 Oral 28-day study on mouse – IR5885

Reference Number:	KII- Section 3, Volume 2, Annex IIA, point 5.3.1/01
Report:	See annex conf. 48
Guidelines:	OECD 407
Deviation:	As a preliminary to a 90-day study, no specific neurobehavioural observations were carried out; organ weight and histopathology only on selected organs/tissues
GLP:	This study was in general performed in accordance with GLP, but the data and report were not fully audited by the Quality Assurance Unit

Executive summary:

In a mouse range-finding study, doses of 0, 110, 440, 1750, and 7000 ppm (mean achieved doses approximately 0, 18, 68, 266 and 1105 mg/kg/day for males and 0, 27, 96, 402 and 1536 mg/kg/day for females), were administered in the feed for four weeks to groups of CD-1 mice (6 animals of each sex per dose group).

There were no mortalities, no clinical signs or macroscopic finding at necropsy to be considered related to treatment with IR5885. Overall reduction in body weight gain was observed at the high doses as well as abnormalities following blood chemistry and haematological analyses. Slightly high liver and adrenal weights were found as well as centrilobular hepatocyte hypertrophy. It is concluded that oral administration, through the diet, of IR5885 to CD-1 mice for four weeks up to 7000 ppm was well tolerated. The NOAEL was considered to be 440 ppm (equivalent to 68 mg/kg/day) for males and 7000 ppm (equivalent to 1536 mg/kg/day) for females.

MATERIALS AND METHODS

MATERIALS:

Test Item: IR5885
Description: White powder
Lot/Batch #: FCF/T/180-00 (ex ZI 068)
Purity: 98.9%
CAS #: 283159-90-0
Stability of test item: Stable in the dark at room temperature and also for at least 14 days at 54 °C. Distributed homogeneously in the feed. Batches of test diets were prepared weekly and maintained in sealed metal container.
Vehicle and/or positive control: None
Test animals –
Species: Mouse
Strain: CD-1
Age: 26 - 30 days
Weight at dosing: 26 – 30 g males; 19 – 23 g females
Source: Charles River, Limited, Margate, Kent, England (UK)
Acclimation period: 12 days
Diet: Rat and mouse No. 1 diet (Special Diets Service, Ltd) *ad libitum*
Water: Tap water from the municipal water main system, *ad libitum*
Housing: 2 animals/sex in Type M2 polycarbonate cages with a stainless steel mesh lid
Environmental conditions -
Temperature: 19 – 23 °C
Humidity: 40 – 70 %
Air changes: At least 15 per hour
Photoperiod: 12-hour light : 12-hour dark cycle

STUDY DESIGN AND METHODS

In life dates: May 23 – July 3, 2001

Animal assignment and treatment

6 animals of each sex per dose group were used. Animals were non-selectively allocated to cages and treatment groups. IR5885 was administered in the diet at the following doses – 0, 110, 440, 1750 and 7000 ppm (Table 3.12.1.5-01) to all males and females, for 28 and 29 consecutive days, respectively. A negative control group received untreated diet.

Table 3.12.1.5-01 Study design

Test group	Concentration in the diet (ppm)	Dose per animal (study averages)		Animals assigned	
		Male (mg/kg bw/day)	Female (mg/kg bw/day)	male	female
1	0	0	0	6	6
2	110	18	27	6	6
3	440	68	96	6	6
4	1750	266	402	6	6
5	7000	1105	1536	6	6

Diet preparation and analysis

The test item (approximately 1045 g) was incorporated into the diet to provide the required concentrations by initial preparation of a premix at a concentration of 13000 ppm. This premix was then diluted with further quantities of plain diet to prepare the two highest concentrations test mixes (7000 and 1750 ppm) and a second premix (1300 ppm). This second premix was used to prepare the two lowest concentration test mixes (110 and 440 ppm). Batches of test diets were prepared weekly and maintained in sealed metal containers. The unused residue at the end of each week was discarded.

The Sponsor provided the test material IR5885 and the analytical standard. The homogeneity and the stability of the test item in the rodent diet formulation were confirmed at nominal concentrations of 50 and 20000 ppm during an earlier study. The stability was confirmed during storage in the freezer for 21 days and at room temperature for 22 days following preparation. At a specified time (Week 1) during treatment, representative samples of the test diets were taken from the mixer and sub-sampled in duplicate before analysis.

Statistics

For organ weight and body weight changes, homogeneity of variance was tested using Bartlett's test. Whenever this was found to be statistically significant, Behrens-Fisher test was used to perform pairwise comparisons, otherwise a Dunnett's test was used.
Inter-group differences in macroscopic pathology and histopathology were assessed using Fisher's Exact test.
In clinical pathology data, treatment groups were compared using a Mantel's test for trend in proportions and also pairwise Fisher Exact tests for each dose group against the control.

Observations

Animals were inspected at least twice daily for evidence of reaction to treatment or ill health. In addition, a more detailed weekly physical examination was performed on each animal for signs of toxicity.

Body weight and food consumption

Each animal was weighed during the acclimatisation period (Day –7), on the first day of treatment (Week 0), at weekly intervals throughout the treatment period and before necropsy.
Food consumption was measured weekly.

Haematology & clinical chemistry

After four week of treatment (at termination), blood samples from all animals were withdrawn from the retro-orbital sinus, with the animals held under Isofluorane anaesthesia, and collected into EDTA anticoagulant. All samples were examined for the following parameters: haematocrit (Hct), haemoglobin concentration (Hb), erythrocyte count (RBC), total and differential leucocyte count (WBC), platelet count (Plt), mean cell haemoglobin concentration (MCHC), mean cell haemoglobin (MCH), mean cell volume (MCV). At the same time as for peripheral haematology, further blood samples were taken and collected into lithium heparin as anticoagulant. After separation the plasma was analysed for: alkaline phosphatase (AP), alanine amino-transferase (ALT), aspartate amino-transferase (AST), glucose concentration (Gluc), total bilirubin concentration (Bili.Total), total cholesterol concentration (Chol.Total), total triglyceride concentration (Trig), creatinine concentration (Creat), urea concentration, total protein concentration (Total Prot), albumin (Alb), albumin/globulin ratio (A/G Ratio), sodium (Na), potassium (K) and chloride (Cl), Calcium concentration (Ca Total) and inorganic phosphorus (Phos).

Sacrifice and pathology

At study termination, all animals were sacrificed by carbon dioxide inhalation. All animals were subjected to a detailed necropsy and the following organs were weighed: adrenals, brain, epididymides, heart, kidneys, liver, lungs (included bronchi), ovaries, spleen, testes, thymus, thyroid with parathyroids, uterus with cervix.
Samples of the following tissues were preserved in 10% neutral buffered formalin and subsequently processed for histopathological examination: brain, kidneys, liver, spinal cord and spleen.
Microscopic examination was performed as follows: i) The brain, kidneys, spinal cord and spleen were examined for all animals of group 1 and 5. ii) The liver was examined for all animals. iii) Tissues reported at macroscopic examination as being abnormal were examined for all animals.

RESULTS AND DISCUSSION

Clinical signs of toxicity and Mortality

The appearance and behaviour of the animals were unaffected by treatment.
There were no deaths.

Body weight and body weight gain

Overall bodyweight gain for males receiving 1750 or 7000 ppm was low when compared with that of the Controls (81 and 63% of the Control value, respectively); these differences did not attain statistical significance.
Bodyweight gain for females were highly variable, however, the inter-group differences were not considered to be related to treatment but to unusual bodyweight fluctuations in the Control group.

Food consumption and compound intake

Table 3.12.1.5-02 Achieved intakes

Test group	Concentration in the diet (ppm)	Dose per animal (study averages)	
		Male (mg/kg bw/day)	Female (mg/kg bw/day)
1	0	0	0
2	110	18	27
3	440	68	96
4	1750	266	402
5	7000	1105	1536

There was no clear evidence for any adverse effect of treatment upon food intake.
Mean concentrations of IR5885 in diet formulation analysed during toxicity study where between 1.3% and 8.2% above nominal concentrations, confirming the accuracy of formulation. The precision of duplicate analysis was <4%, the values for the four procedural recoveries analysed concurrently with the test diets were within 93.5% and 97.4% confirming the accuracy of the method.

Blood analysis

Haematological findings

At the end of treatment period, slight low packed cell volume (haematocrit), haemoglobin concentration and red blood cell count for all treated female groups and for males receiving 1750 or 7000 ppm was evidenced, if compared with the Controls (Table 3.12.1.5-03). The changes in females were, however, minimal, without dosage-relationship and individual values were generally within normal background control ranges at the performing laboratories and therefore considered of no toxicological significance.

Clinical chemistry findings

Blood chemistry examination revealed, compared with Controls, high plasma glucose concentrations in males and females receiving 1750 or 7000 ppm. Triglyceride concentrations were low for females receiving 1750 or 7000 ppm and total plasma cholesterol concentrations were slightly high for males receiving 7000 ppm. Plasma potassium concentrations were high for males and females receiving 7000 ppm, sodium and chloride concentrations were slightly low for females receiving 1750 or 7000 ppm and (with the exception of sodium concentrations) in females receiving 440 ppm. Total plasma protein and albumin concentrations were slightly low and albumin/globulin ratios were slightly high for females receiving 1750 or 7000 ppm (Table 3.12.1.5-03).

Sacrifice and pathology

Organ weight

Analysis of organ weights of mice killed after four weeks of treatment revealed slightly high absolute and bodyweight-relative liver weights in males and females receiving 1750 or 7000 ppm and in females receiving 440, 1750 or 7000 ppm. Slightly high absolute and bodyweight-relative adrenal weights were also apparent for males receiving 7000 ppm. Other inter-group differences attained statistical significance but these differences were minor and not attributed to treatment.

Table 3.9.1.2-04 Selected organ weights absolute and body weight-relative

	1M	2M	3M	4M	5M	1F	2F	3F	4F	5f
Dosage (ppm)	**0**	**110**	**440**	**1750**	**7000**	**0**	**110**	**440**	**1750**	**7000**
Liver Absolute	2.033	2.371	2.216	2.350	2.749 **	1.414	1.397	1.523	1.705	2.043*
Liver bw-relat.	4.8638	5.5913*	5.5836*	6.3450**	7.4140**	5.3251	5.7052	5.8798*	6.0769	7.4805**
Adrenal Absolute	0.0041	0.0043	0.0043	0.0042	0.0052	0.0096	0.0089	0.0082	0.0100	0.0100
Adrenal bw relat	0.0098	0.0104	0.0108	0.0113	0.0412*	0.0361	0.0359	0.0318	0.0358	0.0358

* p<0.05; ** p<0.01 when compared to control groups

Gross and histopathology

There were no macroscopic findings at necropsy which were considered to be related to treatment with the test item. Treatment related changes were confined to the liver. Centrilobular hepatocyte hypertrophy was seen in males and females receiving 1750 or 7000 ppm and in males receiving 440 ppm. Focal hepatocyte necrosis seen in the livers of two males which received 7000 ppm and one male which received 110 ppm was not considered to be related to treatment.

Table 3.12.1.5-03 Selected haematology and pathology findings among mice (at termination)

	Males					Females				
Feeding Level (ppm)	**0**	**110**	**440**	**1750**	**7000**	**0**	**110**	**440**	**1750**	**7000**
Number in group	6	6	6	6	6	6	6	6	6	6
HAEMATOLOGY										
Hct (L/L)	0.446	0.446	0.441	0.429	0.403*	0.477	0.450*	0.447#	0.467	0.447#
Hb (g/dL)	14.3	14.3	14.2	13.5*	12.7*	15.3	14.5*	14.0#	14.8#	13.9#
RBC ($x10^{12}$/L)	8.78	8.62	8.77	8.35	7.92#	9.22	8.54#	8.51#	8.86#	8.52#

BLOOD CHEMISTRY										
Gluc (mmol/L)	12.33	12.24	11.45	17.00#	17.13#	8.8	9.29	7.49	11.60*	11.50*
Trig (mmol/L)	1.96	1.91	1.78	1.81	1.86	2.18	2.87	1.94	1.22#	0.63#
Chol Total (mmol/L)	2.85	3.46	3.29	3.16	3.47*	2.33	1.97	2.30	2.31	2.45
K (mmol/L)	4.0	4.3	4.0	4.4	4.6*	3.1	3.1	3.3	3.7	3.7*
Na (mmol/L)	147	147	148	146	147	149	147	147	146*	146*
Cl (mmol/L)	107	108	105	105	108	113	111	110*	109*	110*
Total Protein (g/L)	48	49	48	48	46	50	48	47	48*	45**
Alb (g/L)	1.34	1.33	1.39	1.47	1.39	1.59	1.61	1.58	1.62	1.65
HISTOPATHOLOGY (incidence)										
Hepatocyte hypertrophy, centrilobular	0	0	6#	6#	6#	1	0	1	2	5
Slight	0	0	6	6	4	1	0	1	2	5
Moderate	0	0	0	0	2	0	0	0	0	0

* p<0.05 with respect to the control
# p<0.01 with respect to the control

CONCLUSIONS

It is concluded that oral administration, via the diet, of IR5885 to CD-1 mice for four weeks at concentrations up to 7000 ppm was well tolerated, producing non-specific toxicity at 1750 and 7000 ppm in males. The NOAEL in this study was considered to be 440 ppm in males (equivalent to 68 mg/kg/day) and 7000 ppm in females (equivalent to 1536 mg/kg/day). Concentrations up to 7000 ppm are considered to be suitable for the subsequent 13-week toxicity study.

3.12.1.6 Oral 90-day study on mouse – IR5885

Reference Number: KII- Section 3, Volume 2, Annex IIA, point 5.3.2/01

Report: See annex conf. 50

Guidelines: OECD 408

Deviation: As a preliminary to a carcinogenicity study, limited histopathology was performed

GLP: Yes (Certified laboratory)

Executive summary:

In a 13-week toxicity study, groups of 10 male and 10 female CD-1 mice received IR5885 in their diet (7 days/week) at the concentrations of 0, 110, 900 or 7000 ppm (0, 15.3, 133.7 and 995 mg/kg bw/day for males and 16.7, 147.5 and 1144 mg/kg bw/day for females, respectively).
There were no mortalities and no clinical signs related to treatment with IR5885. Overall reduction in body weight gain was observed for males receiving 7000 ppm and for females receiving 900 ppm but not 7000 ppm. Haematology investigations indicated slightly low haematocrit, haemoglobin concentration, mean cell haemoglobin and mean cell volume in males and females receiving 7000 ppm. After 13 weeks of treatment, liver weights were high in males given 7000 ppm. In these animals an increased centrilobular and periportal hepatocellular vacuolation, due to greater amount of fat than control, was observed.

It is concluded that oral administration, through the diet, of IR5885 to CD-1 mice for 13 weeks up to 7000 ppm was generally well tolerated. The magnitude of reduction of weight gain observed in males given 7000 ppm was too high for a carcinogenicity study and it is therefore considered that the highest dietary

concentration in the subsequent carcinogenicity study should be between 900 and 7000 ppm. The NOAEL was considered to be 900 ppm (equivalent to 133.7 mg/kg bw/day for males and to 147.5 mg/kg bw/day for females).

MATERIALS AND METHODS

MATERIALS:

Test Item: IR5885
Description: White powder
Lot/Batch #: FCF/T/180-00 (ex ZI 068)
Purity: 98.9%
CAS #: 283159-90-0
Stability of test item: Stable in the dark at room temperature and also for at least 14 days at 54 °C. Distributed homogeneously in the feed. Batches of test diets were prepared weekly or at two-week intervals and maintained in sealed metal container.

Vehicle and/or positive control: None

Test animals –
Species: Mouse
Strain: CD-1
Age: 26 - 30 days at arrival. 33 to 37 days at start of dosing
Weight at dosing: 27.3 – 34.0 g males; 22.0 – 26.8 g females
Source: Charles River, Limited, Margate, Kent, England (UK)
Acclimation period: 7 days
Diet: Rat and mouse No. 1 Maintenance diet (Special Diets Service Ltd, Witham, Essex, England) *ad libitum*
Water: Tap water from the municipal water main system, *ad libitum*
Housing: 2 animals/sex in Type M2 polypropilene cages with a stainless steel mesh lid. Wood shavings used as bedding.
Environmental conditions -
Temperature: 19 – 23 °C
Humidity: 40 – 70 %
Air changes: At least 15 per hour
Photoperiod: 12-hour light : 12-hour dark cycle

STUDY DESIGN AND METHODS

In life dates: October 10, 2001 – January 17, 2002

Animal assignment and treatment

10 animals of each sex per dose group were used. Animals were non-selectively allocated to cages and treatment groups. IR5885 was continuously administered via the diet throughout the 13-week treatment period at the following doses – 0, 110, 900 and 7000 ppm (Table 3.12.1.6-01) to all males and females. A negative control group received untreated diet.

Table 3.12.1.6-01 Study design

Test group	Concentration in the diet (ppm)	Dose per animal (study averages)		Animals assigned	
		Male (mg/kg bw/day)	Female (mg/kg bw/day)	male	female
1	0	0	0	10	10
2	110	15.3	16.7	10	10

3	900	133.7	147.5	10	10
4	7000	995.0	1144	10	10

Diet preparation and analysis

The test item (approximately 1045 g) was incorporated into the diet to provide the required concentrations by initial preparation of a premix at a concentration of 16000 ppm. This premix was then diluted with further quantities of plain diet to prepare the two highest concentrations test mixes (900 and 7000 ppm) and a second premix (1600 ppm). This second premix was used to prepare the lowest concentration test mixes (110 ppm). Batches of test diets were prepared weekly or at two-week intervals and maintained in sealed metal containers. The unused residue at the end of each week was discarded.

The Sponsor provided the test material IR5885 and the analytical standard. The homogeneity and the stability of the test item in the rodent diet formulation were confirmed at nominal concentrations of 50 and 20000 ppm during an associated analytical study performed at Huntingdon laboratories (Huntingdon Life Science Report No. IGA012/013038). The stability was confirmed during storage in the freezer for 21 days and at room temperature for 22 days following preparation. At specified intervals (Weeks 1, 6 and 12) during treatment, representative samples of the test diets were taken from the mixer and sub-sampled in duplicate before analysis (HRC/FCH/M20/01 issue 01/240501).

Table 3.12.1.6-02 Homogeneity and stability of test material

Nominal Inclusion (ppm)	Analysed concentration (ppm)	% of nominal	Mean of mix (T+M+B)	CV (%)
50	T=54.4	109		
	M=53.7	107	54.1	2.18
	B=54.3	109		
	S_{RT}=53.3	107		
	S_{-20}=51.9	104		
20000	T=21400	107		
	M=22150	111	21400	4.06
	B=20500	103		
	S_{RT}=20000	100		
	S_{-20}=21400	107		

T= top; M= middle; B= bottom;
S_{RT}= 22-d stability at room temperature; S_{-20}= 21-d stability at –20 °C

The homogeneity of IR5885 in rodent diet formulation was confirmed at nominal concentrations of 50 and 20000 ppm. Each formulation achieved accuracy within 8.5% of the nominal concentration and precision, measured by the coefficient of variation, of <4.5%. The stability was confirmed, at the above-mentioned nominal concentrations, during a 21-day frozen storage period and a storage period of 22 days at room temperature following fresh preparation (Table 3.12.1.6-02).

Statistics

For organ weight and body weight changes, homogeneity of variance was tested using Bartlett's test. Whenever this was found to be statistically significant, Behrens-Fisher test was used to perform pairwise comparisons, otherwise Dunnett's test was used.
Inter-group differences in macroscopic pathology and histopathology were assessed using Fisher's Exact test.
In clinical pathology data, treatment groups were compared using Mantel's test for trend in proportions and also pairwise Fisher Exact tests for each dose group against the control.

Observations

Animals were inspected at least twice daily for evidence of reaction to treatment or ill health. In addition, a more detailed weekly physical examination was performed on each animal for signs of toxicity.

Body weight and food consumption

Each animal was weighed during the acclimatisation period (Day –7), on the first day of treatment (Week 0), at weekly intervals throughout the treatment period and before necropsy.
Food consumption was measured at weekly intervals. The mean weekly consumption per animal was calculated from the data recorded for each cage. Group mean conversion efficiencies were calculated for each week of treatment. The group mean achieved dosage for each sex, expressed in mg/kg bw/day, was calculated for each week from the nominal dietary test material concentration, food consumption and body weight data.

Haematology & clinical chemistry

During Week 13 of treatment, blood samples from all animals were withdrawn from the retro-orbital sinus, with the animals held under Isofluorane anaesthesia, and collected into EDTA anticoagulant. All samples were examined for the following parameters: haematocrit (Hct), haemoglobin concentration (Hb), erythrocyte count (RBC), total and differential leucocyte count (WBC), platelet count (Plt), mean cell haemoglobin concentration (MCHC), mean cell haemoglobin (MCH), mean cell volume (MCV). At the same time as for peripheral haematology, further blood samples were taken and collected into lithium heparin as anticoagulant. After separation the plasma was analysed for: alkaline phosphates (AP), alanine amino-transferase (ALT), apartate amino-transferase (AST), glucose concentration (Gluc), total bilirubin concentration (Bili.Total), total cholesterol concentration (Chol.Total), total triglyceride concentration (Trig), creatinine concentration (Creat), urea concentration, total protein concentration (Total Prot), albumin (Alb), albumin/globulin ratio (A/G Ratio), sodium (Na), potassium (K), chloride (Cl), calcium concentration (Ca Total) and inorganic phosphorus (Phos).

Sacrifice and pathology

At study termination, all animals were sacrificed by carbon dioxide inhalation. All animals were subjected to a detailed necropsy and the following organs were weighed: brain, epididymides, heart, kidneys, liver, lungs (included bronchi), ovaries, spleen, testes, thymus, uterus with cervix.
Samples of the following tissues were preserved in 10% neutral buffered formalin and subsequently processed for histopathological examination: brain, kidneys, liver, spinal cord and spleen.
Microscopic examination was performed as follows: i) The brain, kidneys, spinal cord and spleen were examined for all animals of group 1 and 4. ii) The liver and grossly abnormal tissues were examined for all animals. Findings were either reported as “Present” or assigned a severity grade. In the latter case one of the following five grades was used: minimal, slight, moderate, marked or severe.

RESULTS AND DISCUSSION

Clinical signs of toxicity and mortality

The appearance and behaviour of the animals were unaffected by treatment.
There were no deaths.

Body weight and body weight gain

Overall bodyweight gain for males receiving 7000 ppm was lower when compared with that of the Controls (74% of the Control value, $p<0.05$) and slightly low for males receiving 900 ppm (81% of Control value, p= n.s.).
Bodyweight gain for females given 900 ppm was slightly lower, if compared to the Controls, but in the absence of a similar trend in females receiving 7000 ppm, this difference was considered fortuitous and unrelated to treatment.

Food consumption and food conversion efficiency

Table 3.12.1.6-03 Achieved intakes

Test group	Concentration in the diet (ppm)	Dose per animal (study averages)	
		Male (mg/kg bw/day)	Female (mg/kg bw/day)
1	0	0	0
2	110	15.3	16.7
3	990	133.7	147.5
4	7000	995.0	1144

Food consumption was considered unaffected by treatment.
Overall food intake was low, when compared with the Controls, for all treated female groups. There was, however, no dosage relationship and, consequently, the inter-group differences were attributed to normal biological variation and not to treatment.
Overall food conversion efficiency was slightly low, when compared with the Controls, for males receiving 900 or 7000 ppm.
The mean concentrations of IR5885 in test diet formulations analysed during the toxicity study were between 3% below and 7% above nominal concentrations, which were within the normal limits (+10/-15%), confirming the accuracy of formulation. The precision of the duplicate analyses was <5% and the accuracy of the analytical procedure was confirmed by a mean recovery of 95.7%.

Blood analysis

Haematological findings

During Week 13, slightly low haematocrit, haemoglobin concentration, mean cell haemoglobin and mean cell volume in males and females receiving 7000 ppm were evidenced. A few other inter-group differences attained statistical significance, but these were minor, not dosage-related in severity or were evident in one sex only and were, therefore, considered to have arisen by chance.

Clinical chemistry findings

Blood chemistry examination during Week 13 did not evidence any change in blood chemistry that was considered to be related to treatment.

Sacrifice and pathology

Organ weights

Analysis of organ weights of mice killed after 13 weeks of treatment revealed high absolute and bodyweight-relative liver weights in males given 900 ppm and in both sexes receiving 7000 ppm.

Table 3.12.1.6-04 Liver absolute and bodyweight-relative weights

Group	**1M**	**2M**	**3M**	**4M**	**1F**	**2F**	**3F**	**4F**
Feeding Level (ppm)	**0**	**110**	**900**	**7000**	**0**	**110**	**900**	**7000**
Absolute	2.273	2.191	2.442	3.142**	1.523	1.519	1.530	2.107**
bw-relat.	4.8097	4.6881	5.3887**	7.255**	4.6.99	4.4951	4.7381	6.2411**

** p<0.01 when compared to control groups
M = male, F = female

Gross and histopathology

The liver of three males given 7000 ppm was macroscopically enlarged. Other macroscopic findings after 13 weeks of treatment were of type normally encountered in young CD-1 mice at the performing laboratories.
Findings considered related to treatment were seen in the liver of males given 7000 ppm where there was an increased incidence and severity of centrilobular and periportal hepatocellular vacuolation. This change was not observed in females. Examination of liver sections stained for fat using Oil-Red-O confirmed that the vacuolation in the hepatocytes was a results of fat accumulation. The results (as described in Table 3.12.1.6-04) indicate an increased severity in males given 7000 ppm.

Table 3.12.1.6-05 Selected haematology and pathology findings among mice (during week 13 or at termination)

	Males				**Females**			
Feeding Level (ppm)	**0**	**110**	**900**	**7000**	**0**	**110**	**900**	**7000**
N. in group	10	10	10	10	10	10	10	10
HAEMATOLOGY								
Hct (L/L)	0.416	0.423	0.420	0.398	0.430	0.425	0.420	0.410*
Hb (g/dL)	14.3	14.6	14.3	13.7	14.8	14.6	14.6	14.3
MCH (ρg)	16.1	15.9	15.7	15.3#	16.5	16.4	16.4	16.0
MCV (fL)	47.0	46.0	45.9	44.6#	48.1	47.8	47.0	46.1#
HISTOPATHOLOGY (incidence)								
Hepatocyte vacuolation								
centrilobular	2	2	4	8*	0	0	0	0
Minimal	1	0	3	0	0	0	0	0
Slight	1	1	0	4	0	0	0	0
Moderate	0	1	1	4	0	0	0	0
periportal	0	0	0	3	0	0	0	0
Minimal	0	0	0	1	0	0	0	0
Slight	0	0	0	1	0	0	0	0
Moderate	0	0	0	1	0	0	0	0
Hepatoc. fat	10	10	10	10	0	0	0	0
Unaffected	2	3	1	0	0	0	0	0
Minimal	3	3	4	2	0	0	0	0
Slight	3	2	4	2	0	0	0	0
Moderate	2	2	1	6	0	0	0	0

* $p<0.05$ with respect to the control; # $p<0.01$ with respect to the control

CONCLUSIONS

It is concluded that oral administration, via the diet, of IR5885 to CD-1 mice for 13 weeks at concentrations of 110, 900 and 7000 ppm was generally well tolerated, producing slight changes in erythrocyte parameters and fatty vacuolation in liver of males at 7000 ppm, and non-specific toxicity in males at 900 and 7000 ppm. The magnitude of reduction of weight gain observed for males given 7000 ppm was too high for a carcinogenicity study and it is therefore considered that the highest dietary concentration in the subsequent carcinogenicity study should be between 900 and 7000 ppm. The NOAEL in this study was considered to be 900 ppm (equivalent to 133.7 mg/kg bw/day for males and to 147.5 mg/kg bw/day for females).

3.12.1.7 Oral 28-day study on dog – IR5885

Reference Number: KII- Section 3, Volume 2, Annex IIA, point 5.3.1/03

Report:	See annex conf. 7

Guidelines: OECD 409, OPPTS 870.3150.

Deviation: none

GLP: Yes (certified laboratory)

Executive summary:

In a dog range-finding study, doses of 0, 250, 500 or 1000 mg/kg bw/day of IR5885 were administered through gelatine capsule for 4 consecutive weeks to groups of purebred Beagle dogs (3 animals of each sex per dose group).

There were no mortalities. The only treatment-related changes were restricted to macroscopic and histological changes in the liver (hepatocellular hypertrophy) and enzymatic changes in blood chemistry (alanine aminotransferase, alkaline phosphates and gamma glutamyl-transferase). The NOAEL of the study was considered to be 500 mg/kg bw/day as the changes observed were minimal and/or within historical background ranges.

MATERIALS AND METHODS:

MATERIALS

Test Item:	IR5885
Description:	White powder
Lot/Batch #:	FCF/T/180-00 (ex ZI 068)
Purity:	98.9%
CAS #:	283159-90-0
Stability of test item:	Stable in the dark at room temperature and also for at least 14 days at 54 °C.
Vehicle and/or positive control:	None
Test animals –	
Species:	Dog
Strain:	Beagle, purebred, dewormed and vaccinated against distemper, leptospirosis, contagious hepatitis, rabies and parvovirus
Age:	5 - 6 months
Weight at dosing:	7.4 – 9.6 kg
Source:	Greenhill, 25018 Montichiari (BS) - Italy
Acclimation period:	7 days
Diet:	Standard Kliba 3353 dog maintenance diet (350 ± 1 g) presented approximately one hour after dosing. Any remaining diet withdrawn approximately 3 hours later
Water:	Tap water from the municipal water main system, *ad libitum*
Housing:	Individually during feeding periods and following doses and in groups during the rest part of the day in cages with minimum 2.0 square meters floor space per dog.
Environmental conditions -	
Temperature:	20 ± 3 °C
Humidity:	30 – 70 %
Air changes:	15 - 20 per hour
Photoperiod:	12-hour light : 12-hour dark cycle with at least 8 hour music during the light period

STUDY DESIGN AND METHODS:

In life dates: March 7 – April 25, 2002

Animal assignment and treatment

3 animals of each sex per dose group were used. Animals were computer randomly allocated to cages and treatment groups. IR5885 was administered by gelatine capsule at the following doses – 0 (empty capsules), 250, 500, and 1000 mg/kg/day (Table 3.12.1.7-07) to all males and females, for 28 and 29 consecutive days, respectively.

Table 3.12.1.7-01 Study design

Test group	Dosages (mg/kg bw/day)	Animals assigned	
		males	females
1	0	3	3
2	250	3	3
3	500	3	3
4	1000	3	3

Dose preparation and analysis

The appropriate amount of test item was weighed directly into gelatin capsules. The individual weights of test item required for daily administrations were adjusted weekly based on the most recently recorded body weight of each animal. Control animals received empty gelatine capsule of the same size and number as those given to the highest dosage animals. All capsules were prepared up to 7 days in advance of administration and stored at room temperature, protected from humidity.

Statistics

The following statistical methods were used to analyse body weight, clinical laboratory data and organ weights. Dunnett's test was applied for the comparison of the treated groups and the control groups for each sex. The Steel-test was applied instead of the Dunnett's test when the data cannot be assumed to follow a normal distribution.

Observations

Animals were inspected at least twice daily since the pre-test period for viability. Each animal was examined for any change in behaviour, reaction to treatment or ill health.

Body weight and food consumption

Each animal was weighed weekly from the pre-test period, and before necropsy.
Food consumption was measured daily from commencement of the pre-test period. The daily ration was weighed before and after feeding.

Ophthalmic examination

Each animal was examined during pre-test (20 March, 2002) and Week 4 (22 April, 2002) for abnormalities of the eyes. Examinations were performed using a binocular indirect ophthalmoscope at least 20 minutes after the instillation of 0.5% tropicamide solution. The observation area included the cornea, conjunctiva, sclera, iris, lens and fundus.

Haematology & clinical chemistry

Blood samples were collected during the morning from all animals during pre-test (20 March, 2002) and Week 4 (22 April, 2002). Animals were fastened overnight but allowed access to water, *ad libitum*. Blood samples were collected from the jugular vein. Anticoagulant used during blood collection were K_2-EDTA or sodium citrate 3.8% at 9:1 ratio of blood to anticoagulant for haematology whereas the anticoagulant used for clinical biochemistry was lithium heparin.

Urinalysis

Urine samples were collected during the morning from all animals during pre-test (20 March, 2002) and Week 4 (22 April, 2002). Animals were fastened overnight but allowed access to water, *ad libitum*. Urine was collected into a specimen vial using a catheter.

Sacrifice and pathology

At study termination, all surviving animals were anaesthetised by intravenous injection of sodium pentobarbital and killed by exsanguination. All animals were subjected to a detailed necropsy.
Samples of the following tissues – adrenal glands, brain, heart, kidneys, liver, ovaries, pituitary gland, prostate gland, spleen, testes with epididymides, thymus, thyroid and parathyroids, uterus – were weighed before fixation. Pair organ were weighed separately. Relative organ weights were calculated based on the body and brain weight.
Microscopic examination was performed on all tissues collected at necropsy.

RESULTS AND DISCUSSION

Clinical signs of toxicity and mortality

White particles were noted in faeces of all test item-treated animals. Given that the incidence increased with dose level, this observation is considered likely to be caused by non-absorbed test item and not a toxicological effect. Other signs as watery faeces or vomiting occurred both in treated and control groups at similar incidence during pre-test and dosing periods and are considered not to be related to the treatment.
There were no deaths.

Body weight and body weight gain

Bodyweight gain was comparable across all dose groups and considered to be unaffected by administration of test item.

Food consumption and compound intake

Food consumption during the dosing period was generally comparable with pre-test values and therefore considered to be unaffected by administration of the test item.

Ophthalmoscopy

There were no ophthalmic changes considered to be related to the administration of the test item.

Blood analysis

Haematological findings

Haematology parameters were unaffected by administration of the test item. Differences that occasionally achieved statistical significance did not show a relationship to dosage or to pre-test levels and therefore were considered not to be related to the administration of the test item.

Clinical chemistry findings

Cholesterol and phospholipid levels were reduced compared to control almost in all animals administered 1000 mg/kg bw/day.
Mild increases in alanine aminotransferase activities were noted in animals administered 1000 mg/kg bw/day only, without attaining statistical significance. Dose-dependant increases in alkaline phosphates and gamma glutamyl-transferase were noted across all treated male groups, statistically significant at 1000 mg/kg/day only. In females, alkaline phosphatase activities increased without dose-dependency while gamma glutamyl-transferase increased dose-dependently but without attaining statistical significance.
Total protein levels were reduced across all test item treated groups in a dose-dependent manner, accompanied with reductions in albumin, alpha-2 globulin and albumin-globulin ratios and increases in alpha-1 globulin. Consequently to reduction in albumin, calcium and magnesium levels were also reduced across all test item treated groups, while phosphorus level were increased in males only (Table 3.12.1.7-02).

Urinalysis

Urinary parameters were considered to be unaffected by administration of the test item.

Sacrifice and pathology

Organ weight
Liver weights were increased in all treated groups, dose-dependently in males. No other organ weights were considered to be affected by the administration of the test item.

Table 3.12.1.7-02 Liver absolute and bodyweight-relative weights

Group	**1M**	**2M**	**3M**	**4M**	**1F**	**2F**	**3F**	**4F**
Dosage (mg/kg)	**0**	**250**	**500**	**1000**	**0**	**250**	**500**	**1000**
Absolute	305.2	410.0*	455.4**	507.1**	310.7	368.7	441.3*	411.8
bw-relat.	3.3	4.5*	5.2**	5.3**	3.4	4.1	5.2**	4.8*

* p<0.05; ** p<0.01 when compared to control groups

Gross and histopathology
Enlarged livers were noted in two males administered 500 mg/kg/day and all animals administered 1000 mg/kg bw/day.
Minimal to marked hepatocellular hypertrophy was noted in almost all animals administered the test item. Minimal to moderate numbers of eosinophilic cytoplasmic inclusions were also noted in most animals administered the test item. A dose dependent decrease in hepatocellular glycogen content was also noted.

Table 3.12.1.7-03 Selected haematology and pathology findings among dogs (at termination)

	Males				Females			
Dosages (mg/kg bw)	**0**	**250**	**500**	**1000**	**0**	**250**	**500**	**1000**
N. in group	3	3	3	3	3	3	3	3
BLOOD CHEMISTRY								
Cholest Tot. (mmol/L)	3.41	2.75	1.65#	1.14#	3.19	1.86	1.87	1.28*
Phospholip. (mmol/L)	3.47	2.10	2.14	1.64*	3.77	3.17	2.12#	1.47#
ALAT (µkat/L)	0.53	0.40	0.44	1.25	0.63	0.69	0.60	0.75
ALP (µkat/L)	6.92	10.10	12.48	20.10*	6.39	14.46*	13.54	13.73
G-GT (ηkat/L)	50.5	66.9	69.2	90.9#	67.9	61.3	76.5	94.2
Alb (g/L)	1.36	1.33	1.47	1.36	1.35	1.45	1.47*	1.52*
Tot. prot. (g/L)	51.2	47.1*	46.8*	44.6#	54.3	50.0	46.7*	44.5#
Albumin (g/L)	26.56	20.52#	21.83#	21.27#	30.79	26.87	24.36*	23.70*
Calcium (mmol/L)	2.79	2.59*	2.61	2.57*	2.90	2.72	2.61*	2.58#
Magnesium (mmol/L)	0.81	0.75#	0.79	0.73#	0.87	0.81	0.79	0.81
Phosphorus (mmol/L)	2.62	2.26#	2.31#	2.14 #	2.25	2.05	2.09	2.22
HISTOPATHOLOGY (incidence)								
LIVER								
Hepat. hypertr	1	3	3	3	0	3	3	3
Grade 1	1	2	1	0	0	1	0	0
Grade 2	0	1	0	0	0	1	1	0
Grade 3	0	0	0	0	0	1	2	2
Grade 4	0	0	2	3	0	0	0	1
Mean sever.	1.0	1.3	3.0	4.0	0	2.0	2.7	3.3
Eosinoph. Incl.	*Total*	0	2	2	3	0	1	2
Grade 1	0	1	1	0	0	1	1	0
Grade 2	0	1	1	2	0	0	1	0
Grade 3	0	0	1	1	0	0	0	2
Mean sever.	0	1.5	2	2.3	0	1	1.5	3
Glycogen stor.	*Total*	2	3	3	0	3	3	3
Grade 1	0	0	1	0	0	2	0	1
Grade 2	1	1	1	0	1	1	3	0
Grade 3	1	2	1	0	1	0	0	0
Grade 4	0	0	0	0	1	0	0	0
Mean sever.	2.5	2.7	2.0	0	3.0	1.3	2.0	1.0
Single cell nec.	0	0	0	3	0	0	0	1
Grade 1	0	0	0	3	0	0	0	1
Mean sever.	0	0	0	1	0	0	0	1
Increas. apopt.	0	0	0	1	0	0	0	0
Grade 2	0	0	0	1	0	0	0	0
Mean sever.	0	0	0	2	0	0	0	0

* p<0.05 with respect to the control
\# p<0.01 with respect to the control

CONCLUSIONS

Following repeat administration of IR5885 to beagle dogs for 4 consecutive weeks, treatement-related changes were restricted to macroscopic and histological changes in the liver and enzymatic changes in blood chemistry. The single cell necrosis observed at 1000 mg/kg bw/day only and the accompanying changes in blood

chemistry were considered adverse effects. However, hepatocellular hypertrophy, decrease in hepatocellular glycogen content and eosinophilic cytoplasmic inclusions were considered to be an adaptive effect. Although changes in blood chemistry was also evident at 250 and 500 mg/kg bw/day, the mid dose level of 500 mg/kg bw/day was considered the NOAEL of IR5885 when administered by capsule to beagle dogs for 4 consecutive weeks.

3.12.1.8 Oral 90 toxicity study (dog)– IR5885

Reference Number: KII- Section 3, Volume 3, Annex IIA, point 5.3.3/01

Report: See annex conf. 12

Guidelines: OECD 409, OPPTS 870.3150.

Deviation: 1) Following a review of the results of the week 6 laboratory investigations (and subsequent repeat investigation) and the continued lack of bodyweight gain and poor food consumption, animal 808F was taken off-dose with effect from 20 January 2003 (having completed 7 weeks of treatment) and maintained on recovery for 6 weeks. Contrary to the final protocol, the tongue was taken at necropsy.
2) Triglycerides were not included in Protocol Amendment number 4 in error, however, triglyceride was measured.

These deviations were considered not to have affected the integrity of the study.

Reason for amendment: Amendment became necessary when it was found that the clinical signs section in the summary was incorrect.

GLP: Yes (certified laboratory)

Executive summary:

In 13-week toxicity study, 4 Beagle dogs/sex/dose were administered IR5885 in gelatine capsule at doses of 50, 250 or 750 mg/kg/day. A similarly constituted Control group received empty capsules at the same frequency. Animal No. 808F was taken off-dose on completion of 7 weeks of treatment following weight loss and laboratory results performed in Week 6 and repeated in Week 7. This animal was retained on recovery until the end of the study.

There were no deaths. White discoloured faeces and/or white/yellow powder in faeces were noted in several animals receiving 250 and 750 mg/kg bw/day. Bodyweight gains were affected, mainly at the highest dose where a decrease in food consumption was also noted. At haematology investigations, some parameters (higher platelet count, MCH and MCV values and lower RBC and reticulocyte counts) showed some differences from respective Controls values. Following blood chemistry analysis, AST, ALT, gGT and glucose values were, in some cases, higher than Controls whilst cholesterol, total protein and total albumin resulted decreased. Some organs showed differences versus Controls in their absolute and body-related weights (liver in both sexes and thyroid-parathyroids, prostate, epididymis and testes in males). Histopathological examination revealed generalised hepatocyte hypertrophy and slight alterations in thyroid glands at 250 or 750 mg/kg bw/day.
In this study, clear evidence of toxicity was noted at 750 mg/kg bw/day, whilst changes noted at 250 mg/kg bw/day were considered not of toxicological importance. Therefore, the NOAEL of IR5885, when administered by capsules to Beagle dogs for 13 consecutive weeks, was considered to be 250 mg/kg bw/day as the changes observed were minimal and/or within historical background ranges.

MATERIALS AND METHODS:

MATERIALS

Test Item:	IR5885
Description:	White powder
Lot/Batch #:	T025/02
Purity (w/w):	99.56 ± 0.14
CAS #:	283159-90-0
Stability of test item:	Stable in the dark at room temperature
Vehicle and/or positive control:	None
Test animals –	
Species:	Dog
Strain:	Beagle, pure-bred, dewormed and vaccinated against distemper, leptospirosis, contagious hepatitis, rabies and parvovirus
Age:	19 – 23 weeks
Weight at dosing:	6.0 – 9.6 kg for males and 6.0 – 8.3 for females
Source:	Harlan UK, Ltd.
Acclimation period:	4 weeks
Diet:	Standard dried pelleted diet (Teklad for dogs) (400 g) presented daily in the morning, approximately one hour after dosing during treatment. Any remaining diet was normally withdrawn approximately 2 hours later (or more in case of inappetance).
Water:	Tap water from the municipal water main system, *ad libitum* except during urine collection.
Housing:	Individually during treatment and feeding periods - 2/sex/dose during the rest part of the day in cages designed in accordance with UK requirements.
Environmental conditions -	
Temperature:	15-24 °C
Humidity:	Not specified
Air changes:	12 - 20 per hour
Photoperiod:	12-hour light : 12-hour dark cycle

STUDY DESIGN AND METHODS:

In life dates:	November 5, 2002 – March 4, 2003

Animal assignment and treatment

4 dogs of each sex per dose group were used. Animals were computer randomly allocated to cages and treatment groups. IR5885 was administered by gelatine capsule at the following doses – 0 (empty capsules), 50, 250, and 750 mg/kg/day (Table 3.12.1.8-01) to all males and females, for 13 weeks.

Table 3.12.1.8-01 Study design

Test group	Dosages (mg/kg bw/day)	Animals assigned	
		males	females
1	0	4	4
2	50	4	4
3	250	4	4
4	750	4	4

Dose preparation and analysis

The appropriate amount of test item was weighed directly into gelatine capsules without modifications. All capsules were prepared up to 8 days in advance of administration and stored at room temperature until used. The individual amounts of test item required for daily administrations were in accordance with the most recently recorded body weight of each animal. Control animals received empty gelatine capsule of the same size and number as those given to the highest dosage animals.

Statistics

All statistical analyses were carried out separately for males and females. The individual animal was the basic experimental unit. For categorical data, including treatment related histopathological findings, the proportion of animals was analysed using Fisher's exact test for each treated group versus control. For continuous data, Bartlett's test was first applied to test the homogeneity of variance between groups. Using tests dependent on the outcome of Bartlett's test, treated groups were then compared with Control group, incorporating adjustment for multiple comparisons where necessary. Significant differences were expressed at the 5% ($p<0.05$), 1% ($p<0.01$) or 0.1($p<0.001$) level.

Observations

Animals were visually inspected at least twice daily for any change in behaviour, reaction to treatment or ill-health. Daily during pre-test period and the first week of treatment, twice weekly during Week 2 to 4 and weekly thereafter, detailed observations were recorded.

Body weight, and Food consumption

Prior to feeding each animal was weighed weekly during the acclimatisation period, on the day that treatment commenced, weekly thereafter throughout the treatment period and before necropsy. The bodyweight of animal 808F receiving 750 mg/kg bw/day was excluded from calculation of the group means from Week 8 to 13 and from the overall mean gain.

Food consumption was measured daily from commencement of the acclimatisation period. The daily ration was weighed before and after feeding. From these data, the weekly consumption (g/dog/week) was calculated for each animal, since the last two weeks of the pre-test period. The food consumption of animal 808F receiving 750 mg/kg bw/day was excluded from calculation of the group means from Week 8 to 13.

Ophthalmic examination

Each animal was examined during pre-test and Week 13 for abnormalities of the eyes. Examinations were performed using a binocular indirect ophthalmoscope after the instillation of 1.0% tropicamide solution (Mydriacyl, Alcon Laboratories Ltd.). The observation area included the adnexae, anterior chamber, cornea, conjunctiva, sclera, iris, lens and fundus.

Haematology

Blood samples were collected during the morning (prior to dosing) from all animals before treatment commenced and during Week 6 and 13 of treatment. Animals were fastened overnight but allowed access to water, *ad libitum*. Blood samples were collected from the jugular vein into EDTA as anticoagulant and examined for the following parameters: haematocrit (Hct), haemoglobin concentration (Hb), erythrocyte count (RBC), total and differential leucocyte count (WBC), platelet count (Plt), mean cell haemoglobin concentration (MCHC), mean cell haemoglobin (MCH), mean cell volume (MCV). Additional blood sample were taken into citrate anticoagulant and examined in respect of prothrombin time (PT) and activated partial thromboplastin time (APTT). Additional samples were obtained during Weeks 8, 11 and 13 (Recovery Weeks 1, 4 and 6, respectively) for animal 808F.

Blood chemistry

At the same time as for peripheral haematology, further blood samples were taken and collected into lithium heparin as anticoagulant. After separation the plasma was analysed for: alkaline phosphatase (ALP), alanine amino-transferase (ALT), aspartate amino-transferase (AST), gamma glutamyl transpeptidase (gGT), glucose concentration (Gluc), total bilirubin concentration (Bili.), total cholesterol concentration (Chol.), total triglyceride concentration (Trig), creatinine concentration (Creat), urea concentration, total protein concentration (Total Prot), albumin (Alb), albumin/globulin ratio (A/G Ratio), sodium (Na), potassium (K), chloride (Cl), calcium concentration (Ca Total) and inorganic phosphorus (Phos). Additional samples were obtained in Weeks 8, 11, 12 and 13 (Recovery Weeks 1, 4, 5 and 6) for animal 808F.

Urinalysis

Urine samples were collected during the morning from all animals once before treatment commenced and during Week 6 and 13. Animals were deprived of water from approximately 12:00 and at approximately 17:00 they were then placed in an individual metabolism cage until approximately 09:00 of the following day. The individual samples were examined for the following characteristics: appearance (App), volume (Vol), pH, specific gravity (SG), protein (Prot), glucose (Gluc), ketones (Keto), bilirubin, blood. At microscopy, the sediment from centrifugation was examined for epithelial cells (Epi), polymorphonuclear leukocytes (Leuc), erythrocytes (RBC), crystals (Cryst), casts, spermatozoa and precursors (Sperm) or other abnormalities (Abn).

Sacrifice and pathology

At study termination, all surviving animals were anaesthetised by intravenous injection of sodium pentobarbitone and killed by exsanguination. All animals were subjected to a detailed necropsy.
Samples of adrenal glands, gall-bladder (empty), brain, heart, kidneys, liver, lungs (with bronchi), ovaries, pituitary gland, prostate gland, spleen, testes with epididymides, thymus, thyroid and parathyroid and uterus with cervix were weighed before fixation. Pair organ were weighed together. Organ weights were also adjusted for terminal bodyweight.
Samples of appropriate tissues were preserved and subsequently processed for histopathological examination where applicable.

RESULTS AND DISCUSSION

Clinical signs of toxicity and mortality

Infrequent observations of either discoloured faeces or white/yellow powder in faeces were noted throughout the study for 5 out of 8 and 7 out of 8 animals receiving 250 and 750 mg/kg bw/day, respectively. Other signs as watery faeces or vomiting occurred both in treated and control groups at similar incidence and therefore were considered not to be related to the treatment.
There were no deaths. From Week 8, female No. 808 (750 mg/kg bw/day) was taken off dose, as this animal was no longer tolerating the treatment.

Body weight and body weight gain

In the period Week 0-13, statistically significant lower group mean bodyweights gains were noted for both sexes receiving 750 mg/kg bw/day and, to a lesser extent, for both sexes given 250 mg/kg bw/day.

Animal 808F: In the period of Week 0-4 this animal showed a bodyweight gain comparable to other animals of the same group. However, in the period Week 5-7 it lost about 10% of its bodyweight and in Week 8 it weighed less than at the treatment start. In the period Week 8-13 (animal off-dose) a gain of 2.0 kg was seen and the terminal weight was similar to that of the other animals of the group given 750 mg/kg bw/day.

Food consumption

During the period from Week 1 to 13 the overall group mean food consumption for both sexes receiving 750 mg/kg bw/day was lower than concurrent Control values. From Week 3 these values showed a partial recovery but the mean food intakes remained consistently lower throughout the treatment period.

Animal 808F: Weekly food intake in the period Week 2 to 7 was, in general, notably lower than in Week 1. Since it was taken off-dose an improvement of food consumption was evident such that on Week 10 onwards, weekly intake was near to maximal.

Table 3.12.1.8-02 Overall group mean food consumption

Test group	Dosages (mg/kg bw/day)	Week 1-13 (g/week/animal)	
		males	females
1	0	2731	2490
2	50	2751	2673
3	250	2631	2513
4	750	2403**	2188*#

*p<0.05; ** p<0.01 at Williams test
n=3 (animal 808F off-dose from Week 8 excluded from calculation of mean and statistical analysis).

Ophthalmoscopy

There were no ophthalmic findings considered to be related to the treatment.

Blood analysis

Haematological findings

Investigation of haematology parameters revealed in Week 6 and 13 higher group mean platelet values for both sexes receiving 750 mg/kg bw/day and males given 250 mg/kg/ bw/day. In addition, in Week 6 only, higher group mean platelet values were noted for females at 250 mg/kg bw/day. A dosage-related shortening activated partial thromboplastin time (APTT) values were noted at Week 6 for males at 250 and 750 mg/kg bw/day. This finding was not evident for the females and was no longer present at Week 13 and, therefore, not considered to be of toxicological importance. Slightly lower group mean RBC counts were noted in Week 6 for males and from Week 13 for both sexes receiving the highest dose. Concomitantly higher MCH and MCV values were noted for males during Week 6 an 13; however, some overlap of individual values with controls and pre-treatment values was evident and therefore, these are not considered to be indicative of toxicity. Lower group mean reticulocyte counts were noted in high dosage-treated animals during Week 6, but a dosage-relationship was not apparent nor this effect was seen at Week 13.

Animal 808F: investigation in Week 6 and repeat investigation 5 days later showed higher WBC, neutrophils, platelet and prothrombin time when compared with this animal pre-treatment values. These parameters, after one week of recovery, were similar to those found during pre-treatment phase.

Clinical chemistry findings

Investigation of blood chemistry parameters in Week 6 and 13 revealed generalised higher group means hepatic enzymes values for animals treated with 250 and 750 mg/kg bw/day. In particular, progressively higher ALP concentration was evident in both sexes. Alanine amino-transferase (AST) values, which were progressive over time, were noted at Week 6 and 13 for both sexes receiving the highest dosage. In Week 6 and 13 higher gGT values were evident at the highest dosage for both males and females. In Week 13 only, higher than Control values were registered for AST in both sexes at the highest dose and in females receiving 250 mg/kg bw/day.

Animal 808F: Investigations at Week 6 revealed marked increases of ALP, ALT; AST, gGT and marked decreases in cholesterol, total protein, albumin and A/G ratio compared with pre-dose-value. By Week 4 of recovery, ALT, AST and gGT returned to values comparable to pre-dose whilst the other abnormal parameters did not show a complete recovery up to the end of the 6 week-recovery phase.

Table 3.12.1.8-03 Selected haematology and blood chemistry findings among male Beagle dogs

	Males							
Dosages (mg/kg bw/day)	**0**		**50**		**250**		**750**	
Week	**6**	**13**	**6**	**13**	**6**	**13**	**6**	**13**
Number in group	4	4	4	4	4	4	4	4
HAEMATOLOGY								
Plt (x10^9/L)	331	299	284	280	376	363	398	398#
APTT (sec)	23.4	26.7	24.0	30.6	20.7	29.9	19.8*	27.0
RBC (x10^{12}/L)	6.22	6.40	6.02	6.12	5.95	5.99	5.73	5.90
MCH (ρg)	21.2	21.9	22.3*	22.9*	22.7#	23.3#	23.2#	24.0#
MCV (fL)	65.0	67.5	67.6*	69.4	67.9*	70.4*	68.39#	71.9#
BLOOD CHEMISTRY								
ALP (U/L)	103	86	208	208#	366#	456#	450#	531#
ALT (U/L)	33	46	32	35	32	44	69	171
AST (U/L)	37	43	34	33	41	36	34	55
gGt (U/L)	3	3	4	3	4	5	5*	7#
Chol (mmol/L)	4.10	3.91	3.92	3.81	2.17#	2.36#	1.91#	1.66#
Urea (mmol/L)	2.79	3.09	3.49	3.56	4.24*	4.45	4.34*	4.63
A/G ratio	1.15	1.15	1.19	1.27	1.01	1.00	1.01	0.96

* $p<0.05$ with respect to the control; # $p<0.01$ with respect to the control

Table 3.12.1.8-04 Selected haematology and blood chemistry findings among female Beagle dogs

	Females							
Dosages (mg/kg bw/day)	**0**		**50**		**250**		**750**	
Week	**6**	**13**	**6**	**13**	**6**	**13**	**6**	**13**
Number in group	4	4	4	4	4	4	4	3
HAEMATOLOGY								
Plt (x10^9/L)	265	315	375	378	381	366	461*	448*
APTT (sec)	20.5	26.2	22.6	29.2	21.9	26.9	19.1	29.9
RBC (x10^{12}/L)	6.31	6.52	6.34	6.58	5.96	6.56	5.97	5.92
MCH (ρg)	22.1	22.5	22.3	22.6	22.1	23.0	22.4	23.1
MCV (fL)	66.7	68.4	66.6	68.8	66.9	69.6	68.0	70.6
BLOOD CHEMISTRY								
ALP (U/L)	122	110	211	257#	233	323#	574#	601#
ALT (U/L)	29	31	34	36	33	44	117	49*
AST (U/L)	33	34	42	35	44	44	42	42
gGt (U/L)	3	3	3	3	4	4*	7#	6#

Chol (mmol/L)	3.47	3.50	3.22	3.46	2.21#	2.43*	1.21#	1.09#
Urea (mmol/L)	3.38	3.76	3.70	3.98	3.65	3.97	3.93	3.93
A/G ratio	1.26	1.28	1.21	1.16	1.20	1.16	0.95#	0.94#

* p<0.05 with respect to the control; # p<0.01 with respect to the control

Urinalysis

Investigations of urinalysis parameters in Week 6 and 13 revealed lower group mean pH values for males at 250 and 750 mg/kg bw/day. However, in the absence of similar difference in females and the lack of any pathological findings, these differences were not considered to be of toxicological relevance.

Sacrifice and pathology

Organ weight

Analysis of terminal organ weights revealed statistically significant higher group mean bodyweight-adjusted liver weights for both sexes at 250 and 750 mg/kg bw/day and for females at 50 mg/kg bw/day. In addition, both sexes receiving 750 mg/kg bw/day and males receiving 250 mg/kg bw/day had absolute higher gall bladder weights, but in the absence of any associated histopathological findings, this was not believed to be of toxicological importance. Statistically significant higher group mean bodyweight-adjusted thyroid/parathyroid weights were noted for males given 250 or 750 mg/kg bw/day when compared with concurrent Control values. Marked lower group mean prostate and testis weights were noted for males receiving 750 mg/kg bw/day and higher group mean epididymis weights were found at the same dosage. In the absence of histopathological findings in testes and epididymides, the weight differences seen in these organs were not considered to be of toxicological importance.

Table 3.12.1.8-05 Selected organs absolute weights and weights adjusted for body weight

Group	**1M**	**2M**	**3M**	**4M**	**1F**	**2F**	**3F**	**4F**
Dosage (mg/kg)	**0**	**50**	**250**	**750**	**0**	**50**	**250**	**750**
Liver Absolute	381	410	477	500	300	418	393	488
Liver Adjusted	346	368	498**	555**	299	398**	400**	507**
Gall bladder Absolute	1.6473	1.7465	2.0795	2.2408*	1.5423	1.5900	1.6830	2.1427**
Thyroid Absolute	0.859	0.861	1.080	0.961	0.760	0.847	0.771	0.925
Thyroid Adjusted	0.722	0.693	1.162**	1.182**	nd	nd	nd	nd

* p<0.05; ** p<0.01 when compared to control groups
nd = not determined
M = males, F = females

Gross and histopathology

a) Liver: The macroscopic examination performed at termination revealed enlargement of the liver in all animals treated with 750 mg/kg bw/day and in some animals of both sexes treated with 250 and 50 mg/kg bw/day. Pallor of the liver was evident in 3 out of 4 males and 2 out of 3 females at 750 mg/kg bw/day.
Minimal to moderate hepatocellular hypertrophy, hepatocytes with pale cytoplasm and peripheral clumping and eosinophilic cytoplasmic inclusions were noted in all animals receiving test item. A dose-relationship was evident.

b) Thyroids: A higher incidence of follicular cell hypertrophy in thyroid glands was seen in males receiving 250 or 750 mg/kg bw/day and in all female treated groups.

c) Prostate: An increase in the incidence or degree of immaturity of the prostate gland was seen in all treated groups compared with controls.

Table 3.12.1.8-06 Selected pathology findings among Beagle dogs

	Males				**Females**			
	0	**50**	**250**	**750**	**0**	**50**	**250**	**750**
LIVER								
Hepat. hypertr	0	4*	4*	4*	0	4*	4*	3*
Minimal	0	3	0	0	0	2	1	0
Slight	0	1	2	0	0	2	1	0
Moderate	0	0	2	4	0	0	2	3
Eosinoph. Incl.	0	0	4*	4*	0	0	4*	3*
Minimal	0	0	4	0	0	0	3	0
Slight	0	0	0	2	0	0	1	1
Moderate	0	0	0	2	0	0	0	2
Pale Cytoplasm with periph. Incl.	0	4*	4*	4*	0	4*	4*	3*
Minimal	0	4	0	0	0	2	1	0
Slight	0	0	0	2	0	2	1	0
Moderate	0	0	0	2	0	0	2	3
THYROIDS								
Follicul. Cellular hypertrohpy	0	0	1	2	0	1	2	2
Minimal	0	0	1	1	0	0	0	2
Slight	0	0	0	1	0	1	2	2

Table 3.12.1.8-05 Selected pathology findings among Beagle dogs - continued

	Males				**Females**			
	0	**50**	**250**	**750**	**0**	**50**	**250**	**750**
PROSTATE								
Immature	**1**	**2**	**2**	**4**				
Minimal	**1**	**2**	**0**	**1**				
Slight	**0**	**0**	**1**	**0**				
Moderate	**0**	**0**	**1**	**3**				

* p<0.05 with respect to the control
\# p<0.01 with respect to the control

CONCLUSIONS

Following repeat administration of IR5885 to beagle dogs for 13 consecutive weeks, dosage up to 250 mg/kg bw/day was well tolerated. The treatment-related effects on liver and thyroids seen at 50 and 250 mg/kg bw/day were considered to be adaptive and thus 250 mg/kg bw/day was identified as the highest No Observed Adverse Effect Level (NOAEL) on this study. Clear evidence of toxicity was observed at 750 mg/kg bw/day, therefore, it was considered that longer term dosing at a level approaching 750 mg/kg bw/day may result in toxic changes in the liver that may not be tolerated and thus lead to the early termination of the animals. Based on this study, the proposed high level for the subsequent long-term 52-week toxicity study is 250 mg/kg bw/day.

3.12.1.9 Oral 1 year study on dog– IR5885

Reference Number: **KII- Section 3, Volume 4, Annex IIA, point 5.3.4/01**

Report:	M. D. Saunders (2005), IR5885 Toxicity study by oral capsule administration to Beagle Dogs for 52 weeks including assessment of recovery following 13 weeks administration. Huntingdon Life Sciences Ltd, Woolley Road, Alconbury, Huntingdon Cambridgeshire PE28 4HS, UK and Huntingdon Life Sciences Ltd, Eye, Suffolk, IP23 7PX, UK; unpublished report No. IGA 033/042508, June 17, 2005; dates of experimental work: March 18, 2003 to June 25, 2004.

Guidelines: OECD 452, OPPTS 870.4100, JMAFF (2-1-14), EEC B.30.

Deviation: 1) on one occasion in Week 13 animals in unit 35 were fed immediately following dosing.
2) on one occasion in Week 47, animal No. 770F (Control) received a capsule of 1 mg/kg/day in error.

These deviations were considered not to have affected the integrity of the study.

GLP: Yes (certified laboratory)

Executive summary:

In an 1 year toxicity study, 4 Beagle dogs/sex/dose were administered IR5885 in gelatine capsule at doses of 1, 7, 50, or 250 mg/kg/day. A similarly constituted Control group received empty capsules at the same frequency. Further 2 male and female dogs were assigned to each of the Control and high dosage groups for a 13-week treatment period followed by an 8-week recovery period. During the study, clinical conditions, bodyweight, food consumption, ophthalmic examination, haematology, blood chemistry, urinalysis, organ weight, macropathology and histipathology investigations were undertaken. In addition, thyroid hormones and testosterone measurements were added to aid interpretation of the present study, because of the presence of microscopic changes detected in thyroid, liver and prostate in the previous 90-d toxicity study.

There was one unscheduled death, unrelated to treatment. One female treated at 250 mg/kg/day failed to show oestrus during the treatment period. Bodyweight, food consumption, ophthalmic examination and urinalysis were unaffected. Higher platelet values were noted in males treated at 250 mg/kg/day from Week 13. Blood chemistry showed higher group mean ALP values from Wek 6 in both sexes receiving 250 mg/kg/day, and from Week 13 in both sexes at 50 mg/kg/day. Following 8 weeks of recovery, values for animals previously given 250 mg/kg/day showed a good degree of reduction. Higher ALP values were also noted from Week 13 in females at 7 mg/kg/day, and in Weeks 39 and 52 in animals treated at 1 mg/kg/day. Higher group mean gGT values were noted from Week 13 in both sexes receiving 250 mg/kg/day; following 4 weeks of recovery, values in previously treated animals returned to pre-treatment and/or control values. Recovery was also evident after 4-week off-dose for cholesterol values (decreased in Weeks 6 and 13 in both sexes) and for total protein and albumin values (reduced from Week 6). In Weeks 13 and 26, femal dogs treated at 50 mg/kg/day showed similar differences, compared to controls. Higher group mean triglyceride values were noted from Week 13 in males receiving 250 mg/kg/day, and in Week 39 and 52 in those given 50 mg/kg/day. Males receiving 250 mg/kg/day showed lower calcium values from Week 13. There was no clear consistent group correlation among the three thyroid parameters (TSH, T3 and T4), analysed in Weeks 13, 26, 39 and 52. The testosterone values recorded from Week 13 of treatment onwards were highly variable in all treated and control dogs, showing no treatment- related effect. Higher group mean absolute liver weights were noted at termination, as well as higher thyroids weights in both sexes receiving 250 or 50 mg/kg/day. At recovery, one male and one female showed higher than control thyroid weights while liver weights had returned to normal values. Lower prostate weights were recorded in males treated at 250 mg/kg/day. Lower absolute ovary and

uterus/cervix were noted at termination in females treated at 250 or 50 mg/kg/day, but after an 8-week recovery period, ovary weights for females at the highest dose were similar to controls. Generalised hepatocyte hypertrophy in the liver was observed at histopathology, with animals dosed at 250 mg/kg/day and a single female at 50 mg/kg/day showing hepatocytes with pale cytoplasm and peripheral clumping. One male dog given 250 mg/kg/day showed also eosinophilic concentric intracytoplasmatic inclusion in hepatocytes. Histopathological examination at the end of recovery period revealed minimal cantrilobular rarefaction of hepatocytes. Minimal or slight follicular cell hypertrophy of thyroid glands was observed in some dogs of both sexes given 250 mg/kg/day and in one mal and one female dog given 50 mg7kg/day. Full recovery was seen after 8 weeks off-dose in dogs previously given 250 mg/kg/day for 13 consecutive weeks. Absence of corpora lutea was seen in three females given 250 mg/kg/day.

The general health of the animals treated at 250 mg/kg/day fro 52 weeks was not adversely affected however, the prostate weight changes and the ovary/lack of oestrus in females at this dose level were considered to represent adverse effects. Treatment-related effects recorded at 50 mg/kg/day were confined to the liver and thyroid. The microscopic thyroid changes at this dose level were confined to single animals of each sex and consisted of follicular cell hypertrophy: due to the low severity and incidence of these findings, the lack of clear concomitant effects on thyroid hormone levels, and the fact that no microscopic thyroid changes were detected at the end of an 8-week recovery period (following 13-week treatement at a higher dose), the thyroid changes were not considered to be of toxicological importance. Microscopic changes seen in the liver were mainly adaptive hepatocyte hypertrophy of no more than a slight degree. The blood chamistry changes recorded were considered to be consistent with the microscopically evident adaptive changes. On this basis, the NOAEL of the study was set at 50 mg/kg/day.

MATERIALS AND METHODS:

MATERIALS

Test Item:	IR5885
Description:	White powder
Lot/Batch #:	T025/02
Purity (w/w):	99.56 ± 0.14 and 99.63 ± 0.03
CAS #:	283159-90-0
Stability of test item:	Stable in the dark at room temperature
Vehicle and/or positive control:	None
Test animals –	
Species:	Dog
Strain:	Beagle, pure-bred, dewormed and vaccinated against distemper, leptospirosis, contagious hepatitis, rabies and parvovirus
Age:	21 – 30 weeks at beginning of treatment
Weight at dosing:	7.9 – 10.9 kg for males and 7.1 – 10.1 kg for females
Source:	Harlan UK, Ltd.
Acclimation period:	at least 4 weeks (approx. 35 days)
Diet:	Standard dried pelleted diet (Teklad for dogs; Harlan UK) (400 g) presented daily in the morning, approximately one to 1.5 hours after dosing during treatment. Any remaining diet was normally withdrawn approximately 2 hours later (or more in case of inappetance).
Water:	Tap water from the municipal water main system, *ad libitum* except during urine collection.
Housing:	Individually during treatment and feeding periods - 2/sex/dose during the rest part of the day in cages designed in accordance with UK requirements.

Environmental conditions -
Temperature: 15-24 °C
Humidity: Not specified
Air changes: approx. 12 per hour
Photoperiod: 12-hour light : 12-hour dark cycle

STUDY DESIGN AND METHODS:

In life dates: **March 18, 2003 – April 23, 2004**

Animal assignment and treatment

4 dogs of each sex per dose group were used for the main study (52 weeks consecutive weeks). Further two dogs of each sex wer assigned to the highest and control groups, treated for 13 consecutive weeks and allowed to a recovery period of up to 8 weeks. Before the start of pre-treatment investigations, the animals were weighed and 48 dogs allocated to five groups employing a pseudo-eandom bodyweight stratification procedure to yeld groups with approximately equal mean bodyweight, while avoiding inclusion of litter-mates within the same group where possible. IR5885 was administered by gelatine capsule at the following doses – 0 (empty capsules), 1, 7, 50, and 250 mg/kg/day (Table 3.12.1.9-01) to all males and females, for 52 weeks. Recovery animals were treated for 13 consecutive weeks and then maintained off-dose for 8 weeks.

Table 3.12.1.9-01 Study design

Test group	Dosages (mg/kg bw/day)	Animals assigned	
		males	females
1	0	6	6
2	1	4	4
3	7	4	4
4	50	4	4
	250	6	6

Dose preparation and analysis

The appropriate amount of test item was weighed directly into gelatine capsules without modifications. Capsules were prepared up to 8 days in advance of administration and the quantity of test substance was in accordance with the most recently recorded body weight of each animal. Control animals received empty gelatine capsule of the same size and number as those given to the highest dosage animals.

Statistics

All statistical analyses were carried out separately for males and females. The individual animal was the basic experimental unit. Data for Recovery study animals was not analysed due to the low numbers of animals (n = 2).

The following data types were analysed at each timepoint separately:
- Bodyweight, using gain over appropriate study periods
- Food consumption, over appropriate study periods
- Blood chemistry, haematology and urinalysis
- Organ weights, both absolute and adjusted for terminal bodyweight and relative to body weight
- Pathological findings, for the number of animals with and without each finding

For continuous data, Bartlett's test was first applied to test the homogeneity of variance between groups. Using tests dependent on the outcome of Bartlett's test, treated groups were then compared with Control group, incorporating adjustment for multiple comparisons where necessary.

The following sequence of statistical tests was used for bodyweight, food consumption, and clinical pathology data:

If 75% of the data (across all groups) were the same value, for example c, then a frequency analysis was applied. Treatment groups were compared using a Mantel test for a trend in proportions and also parwise Fisher's Exact test for each dose group against the control for both i) values <c versus values >=c, and for ii) values <=c versus values >c, as applicable.

If Barlett's test for variance homogeneity was not significant at the 1% level, then parametric analysis was applied. If the F1 test for monotonicity of dose-response was not significant at the 1% level, Williams' test for a monotic trend was applied. If the F1 test was significant, suggesting that the dose-response was not monotone, Dunnett's test was performed instead.

If Barlett's test was significant at the 1% level, then logarithmic and square-root transformations were tried. If Barlett's test was still significant, then non-parametric test was applied. If the H1 test for monotonicity of dose-response was not significant at the 1% level, Shirley's test for a monotonic trend was applied. If the H1 test was significant, suggesting that the dose-response was not monotone, Steel's test was performed instead.

For organ weight data, analysis of covariate was initially performed using terminal bodyweight as covariate. If the within group relationship between organ weight and bodyweight was significant at the 10% level, then the treatment comparisons were made on **adjusted** group means in order to allow for differences in bodyweight which may influence the organ weights.

Significant differences were expressed at the 5% ($p<0.05$), or 1% ($p<0.01$) level.

Observations

Animals were visually inspected at least twice daily for any change in behaviour, reaction to treatment or ill-health. Daily during the first week of treatment, twice weekly during Week 2 to 4 and weekly during Weeks 5 to 13 and once every two weeks from Week 14 onwards detailed observations were recorded immediately before dosing, between half and hour and two hours after dosing, and as late as possible in the working day. During the acclimatisation and recovery periods observations of the animals and their kennels were recorded at least once per day.

Body weight, and Food consumption

Prior to feeding each animal was weighed weekly during the acclimatisation period, on the day that treatment commenced, weekly thereafter throughout the treatment and recovery periods and before necropsy.

The weight of food supplied to each animal, that remaining and an estimate of any spilled was recorded daily during the acclimatisation period and throughout the study. From these records the weekly consumption (g/dog/week) was calculated for each dog for the last two weeks of the acclimatisation period and for each week throughout the treatment and recovery periods.

Ophthalmic examination

Each animal was examined during pre-test and Week 52 for abnormalities of the eyes. The eyes of Recovery animals were examined during Week 13. Examinations were performed using a binocular indirect ophthalmoscope after the instillation of 1.0% tropicamide solution (Mydriacyl, Alcon Laboratories Ltd.). The observation area included the adnexae, anterior chamber, cornea, conjunctiva, sclera, iris, lens and fundus.

Haematology

Before treatment commenced and during Week 13, 26, 39 and 52 for all animals and during Week 6 for Recovery animals only, blood samples were collected from the jugular or other suitable veins during the

morning (prior to dosing). Blood samples were also obtained from animals assigned to recovery in Week 4 of recovery to check platelets counts, PT and APTT. Using EDTA as anticoagulant, the following parameters were determined using a Bayer-Technicon H*1E Haematology Analyser: haematocrit (Hct), haemoglobin concentration (Hb), erythrocyte count (RBC), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), mean cell volume (MCV), total and differential leucocyte count (WBC – N, L, E, B, M and Large unstained cells LUC), platelet count (Plt). Additional blood sample were taken into citrate anticoagulant and examined in respect of prothrombin time (PT) and activated partial thromboplastin time (APTT). Repeat investigations were performed as considered necessary by the Study Director although new results were not used for mean value calculation.

Blood chemistry

At the same time as for peripheral haematology – and in Weeks 4, 6 and 8 for Recovery animals for selected parameters only, further blood samples were taken and collected into lithium heparin as anticoagulant. After separation the plasma was analysed using a Hitachi 917 Clinical Chemistry Analyser for: alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (gGT), creatinine phosphokinase (CPK), total bilirubin (Bili.), urea (Urea), creatinine (Creat), glucose (Gluc), total cholesterol (Chol.), sodium (Na), potassium (K), chloride (Cl), triglycerides (Trig), total protein (Total Prot), albumin (Alb), calculated albumin/globulin ratio (A/G Ratio), calcium (Ca) and inorganic phosphorus (Phos). From Week 13the following hormone concentrations were also examined, using radio immunoassay: tri-iodothyronine (T3), thyroxine (T4), thyroid stimulating hormone (TSH) and testosterone. Repeat investigations were performed as considered necessary by the Study Director although new results were not used for mean value calculation.

Urinalysis

Urine samples were collected during the morning from all animals once before treatment commenced and during Week 6 (recovery animals only), 13, 26, 39 and 52. Animals were deprived of water from approximately 12:00 and at approximately 16:30 they were placed in an individual metabolism cage without food or water. Urine was then collected until approximately 08:30 of the following day. The individual samples were examined for the following characteristics: appearance (App), volume (Vol), pH, specific gravity (SG), protein (Prot), glucose (Gluc), ketones (Keto), bilirubin, blood. At microscopy, the sediment from centrifugation was examined for epithelial cells (Epi), polymorphonuclear leukocytes (Leuc), erythrocytes (RBC), crystals (Cryst), casts, spermatozoa and precursors (Sperm) or other abnormalities (Abn). Urinalysis was not conducted during the recovery period because of the lack of any treatment-related findings.

Sacrifice and pathology

Animals were killed by intravenous overdose injection of sodium pentobarbitone and subsequent exsanguination. All animals were subjected to a detailed necropsy.
Adrenals, brain, epididymides, gall-bladder, heart, kidneys, liver, ovaries, pituitary gland, prostate, spleen, testes, thyroid with parathyroid and uterus with cervix were weighed before fixation. Pair organ were weighed together. Organ weights were also adjusted for terminal bodyweight and relative to terminal weight, using weight recorded immediately before necrospy.
Samples of appropriate tissues were preserved and subsequently processed for histopathological examination where applicable.

RESULTS AND DISCUSSION

Clinical signs of toxicity and mortality

One female receiving 250 mg/kg/day did not show evidence of oestrus throughout the study. The time of first onset (in terms of age in weeks) and number of oestrus cycle of all the other females were similar among the groups.

There was one unscheduled death: male dog No. 783 (1 mg/kg/day) was sacrificed in Week 34 due to poor clinical condition. This death was not considered to be related to treatment. The weight losses recorded from Week 30 and the clinical signs (underreactivity, hunched posture, stiff hind leg gait and increased body temperature) noted from Week 31 were not seen in any other animal. Both macroscopic examination and microscopic pathology indicated that the factor contributing to death was a bacterial infection in the thoracic region. In addition, a slight generalised hepatocyte hypertrophy was also noted in the liver.

Body weight and body weight gain

There was no effect of treatment on bodyweight gain.
During the 13-week treatment period and the 8-week recovery period, gains for animals treated at 250 mg/kg/day were similar or slightly higher than those of the relevant control animals.

Food consumption

There was no effect of treatment on food consumption, either in the main study dogs or in the recovery ones.

Ophthalmoscopy

The ophthalmic examination in Weeks 13 and 52 did not reveal any treatment-related effect.

Blood analysis

Haematological findings

From Week 13, main group male dogs at 250 mg/kg/day showed higher than control mean platelet values, attaining statistical significance by Week 26 onwards. There was, however, no indication that the difference from control increased in magnitude over time. In Weeks 13, 26 and 39 main group females at 250 or 50 mg/kg/day also showed higher than control platelet values, but with no dose-relationship and no statistical significance, with individual values, especially at 250 mg/kg/day, within concurrent control values: for these reasons these differences from control were not considered related to treatment.
Platelets in Weeks 6 and 13 in male dogs assigned to recovery were similar to controls and/or their pre-dose value; in females, there was and increase, compared to control, in Week 6 but in Week 13 only one value was higher than control and both were lower than those in Week 6. At the recovery investigations platelet values were similar to controls.

Clinical chemistry findings

From Week 13, dose-related higher than control group mean ALP values were noted in both sexes at 250 or 50 mg/kg/day. The magnitude of the differences from control showed an increase over time between Week 13 and 26, but values for both sexes receiving 50 mg/kg/day decreased between Week 26 and 52 (control values remaining consistent). From Week 13 females receiving 7 mg/kg/day had a tendency to higher ALP values but not progressing over time. Between Weeks 26 and 39 animals receiving 1 mg/kg/day showed an increase in APL values. In Week 52, increases compared to control were mainly due to a single male in this group, and to two females those values were higher both than control and values recorded at the previous time-points. In Weeks 6 and 13 ALP values for treated animals at 250 mg/kg/day assigned to recovery were higher than controls, although consistent between the two timepoints. After 4 weeks of recovery, a notable lowering in ALP was observed, compared to values recorded at Week 13. At the Week 6 and 8 recovery investigations, ALP for one male and one female previously treated were comparable with controls; values for the remaining one male and one female continued to be higher than control, but below pre-dose values, indicating recovery.
From Week 13 onwards, main group animals at 250 mg/kg/day had higher than control gGT values. These were, however, generally attributable to high individual values (males 801 and 807, females 804 and to a lesser extent 808). In Week 52 all treated male groups showed higher than control mean group gGT values, but without any dose-relationship and individual values within concurrent control values. In addition, the high

group mean value recorded at 1 mg/kg/day was mainly attributable to a high outlying value of animal No. 779, which was not confirmed at repeated investigation.
In Week 13 one male and one female assigned to recovery showed higher than control gGT values. After 4 week of recovery gGT values were similar to pre-treatment and/or control values.
In Week 13, a statistically significant decrease in group mean total cholesterol was noted in animals dosed at 250 mg/kg/day. At subsequent time-points, total cholesterol remained lower than control, but statistical significance was not attained, there was no progression over time and individual values tended to be within concurrent control values. For these reasons the effect on total cholesterol was considered to be limited to Week 13. A similar reduction in total cholesterol, not progressing over time, was also observed in animals assigned to recovery: by Week 4 of recovery this parameter recovered.
From Week 13, males receiving 250 mg/kg/day showed higher mean triglyceride values compared to control, though the higher values in Week 13 and 39 were mainly due a single animal (801). In Weeks 39 and 52 also males at 50 mg/kg/day showed higher triglyceride values, but again single animals at each single time-point were considered to be responsible for such increases. In Week 39 only females treated at 250 mg/kg/day also showed higher mean triglyceride value, but values obtained at Week 52 were similar to controls. No effect on triglycerides was observed either in Week 6 or 13 in animals assigned to recovery.
From Week 13 male dogs treated at 250 mg/kg/day showed lower than control calcium value; the difference from control was similar at all time-points and similar differences were not apparent in females of the same group.
From Week 13 animals receiving 250 mg/kg/day showed lower mean total albumin levels, resulting in lower protein in both sexes, lower A/G ratios in males and in females only in Week 26. Lower albumin levels were also noted in females dosed at 50 mg/kg/day in Weeks 13 and 26. The values of albumin recorded in both Weeks 6 and 13 in animals assigned to recovery were consistently lower over time. The parameter recovered by Week 4 of recovery.
There were some differences in thyroid hormone values between controls and some treated animals, however, there were no clear consistent group correlations among the three thyroid parameters measured.
The testosterone values recorded from Week 13 of treatment onwards were highly variable in all treated and control animals, as normally seen at the performing laboratory in case of single samples in dogs of similar age and strain. There were no differences between treated and control animals.

Table 3.12.1.9-02 Selected haematology findings among male Beagle dogs

		Males				
Dosage (mg/kg/day)						
Parameter	**Time-point**	**0**	**1**	**7**	**50**	**250**
HAEMATOLOGY						
Plt (x10^9/L)	pre-dose	332 ± 31.1 n = 5	347 ± 60.7 n = 3	322 ± 164.6 n = 3	358 ± 76.4 n = 4	327 ± 69.2 n = 4
	Week 6	357 & 283 indiv. val.	-	-	-	299 & 398 indiv. val.
	Week 13	272 ± 54.2 n = 6	307 ± 54.2 n = 4	268 ± 60.0 n = 4	307 ± 76.0 n = 4	344 ± 79.1 n = 6
	Week 4 R	309 & 253 indiv. val.	-	-	-	269 & 368 indiv. val
	Week 26	232 ± 49.0 n = 4	300 ± 72.6 n = 4	262 ± 46.2 n = 4	283 ± 55.8 n = 4	365 ± 87.5* n = 4
	Week 39	219 ± 30.0 n = 4	306 ± 33.4 n = 3	239 ± 73.3 n = 4	283 ± 47.4 n = 4	381 ± 61.7# n = 4
	Week 52	276 ± 42.4 n = 4	349 ± 49.7 n = 3	300 ± 27.2 n = 4	278 ± 36.9 n = 4	396 ± 84.2# n = 4

* p<0.05 with respect to the control; # p<0.01 with respect to the control at Williams'test
n = number of animals examined: this number may be lower than the number of animals in the group at the sample point when e.g. results could not be obtained for some animals
R: recovery period; indiv. val.: individual values

Table 3.12.1.9-03 Selected haematology findings among female Beagle dogs

		Females				
Dosage (mg/kg/day)						
Parameter	**Time-point**	**0**	**1**	**7**	**50**	**250**
HAEMATOLOGY						
Plt (x10^9/L)	pre-dose	278 ± 56.9 n = 5	291 ± 85.2 n = 4	345 ± 47.8 n = 3	291 ± 75.2 n = 3	263 ± 99.3 n = 3
	Week 6	277 & 274 indiv. val.	-	-	-	307 & 449 indiv. val.
	Week 13	296 ± 30.2 n = 6	277 ± 61.5 n = 4	306 ± 51.7 n = 4	384 ± 88.0 n = 4	357 ± 60.4 n = 6
	Week 4 R	358 & 246 indiv. val.	-	-	-	251 & 297 indiv. val
	Week 26	291 ± 23.5 n = 4	268 ± 44.8 n = 4	329 ± 62.6 n = 4	388 ± 66.7 n = 4	344 ± 49.6 n = 4
	Week 39	322 ± 62.2 n = 4	280 ± 57.4 n = 3	295 ± 46.8 n = 4	377 ± 81.8 n = 4	377 ± 41.5 n = 4
	Week 52	369 ± 78.5 n = 4	259 ± 93.2 n = 3	316 ± 74.7 n = 4	408 ± 66.6 n = 4	349 ± 62.7 n = 4

* p<0.05 with respect to the control; # p<0.01 with respect to the control at Williams'test
n = number of animals examined: this number may be lower than the number of animals in the group at the sample point when e.g. results could not be obtained for some animals
R: recovery period; indiv. val.: individual values

Table 3.12.1.9-04 Selected blood chemistry findings among male Beagle dogs

		Males				
Dosage (mg/kg/day)						
Parameter	**Time-point**	**0**	**1**	**7**	**50**	**250**
BLOOD CHEMISTRY						
ALP (U/L)	pre-dose	110 ± 23.8 n = 5	127 ± 34.4 n = 3	118 ± 9.0 n = 3	106 ± 24.4 n = 4	149 ± 23.1$ n = 6
	Week 6	85 & 124 indiv. val.	-	-	-	471 & 237 indiv. val.
	Week 13	77 ± 14.0 n = 6	88 ± 17.6 n = 4	98 ± 24.5 n = 4	178 ± 85.8* n = 4	505 ± 433.7# n = 6
	Week 4 R	68 & 95 indiv. val.	-	-	-	179 & 88 indiv. val
	Week 6 R	56 & 73 indiv. val.	-	-	-	145 & 70 indiv. val
	Week 8 R	51 & 60 indiv. val.	-	-	-	159 & 64 indiv. val
	Week 26	66 ± 3.7 n = 4	94 ± 30.0 n = 4	97 ± 25.5 n = 4	209 ± 101.7* n = 4	915 ± 821.1# n = 4
	Week 39	67 ± 5.7 n = 4	122 ± 31.3 n = 3	108 ± 25.4 n = 4	202 ± 77.4# n = 4	971 ± 739.8# n = 4
	Week 52	67 ± 27.8 n = 4	106 ± 25.9 n = 3	94 ± 27.8 n = 4	160 ± 133.5 n = 4	925 ± 868.7# n = 4
gGT (U/L)	pre-dose	3 ± 0.5 n = 5	2 ± 0.6 n = 3	2 ± 0.6 n = 3	2 ± 0.5 n = 4	3 ± 0.8 n = 6
	Week 6	3 & 2 indiv. val.	-	-	-	3 & 2 indiv. val.
	Week 13	2 ± 0.8 n = 6	2 ± 0.8 n = 4	3 ± 0.6 n = 4	3 ± 0.* n = 4	4 ± 1.6# n = 6
	Week 4 R	3 & 3 indiv. val.	-	-	-	3 & 3 indiv. val
	Week 26	3 ± 0.6 n = 4	3 ± 0.6 n = 4	3 ± 0.5 n = 4	3 ± 0.5* n = 4	5 ± 2.8* n = 4
	Week 39	2 ± 1.3 n = 4	3 ± 0.6 n = 3	3 ± 0.6 n = 4	3 ± 1.0 n = 4	5 ± 2.4* n = 4
	Week 52	3 ± 0.0 n = 4	5 ± 2.0 n = 3	4 ± 1.0 n = 4	4 ± 1.0 n = 4	5 ± 3.1 n = 4
Total cholesterol (mmol/L)	pre-dose	4.27 ± 0.382 n = 5	4.04 ± 0.853 n = 3	3.92 ± 0.535 n = 3	4.69 ± 0.685 n = 4	3.98 ± 0.347 n = 6
	Week 6	4.17 & 3.91 indiv. val.	-	-	-	3.57 & 2.60 indiv. val.
	Week 13	4.11 ± 0.446 n = 6	3.64 ± 0.237 n = 4	3.76 ± 0.231 n = 4	3.76 ± 0.450 n = 4	2.97 ± 0.593# n = 6
	Week 4 R	4.17 & 3.91 indiv. val.	-	-	-	3.57 & 2.60 indiv. val
	Week 26	3.57 ± 0.467 n = 4	2.73 ± 0.572 n = 4	3.16 ± 0.606 n = 4	3.63 ± 0.225 n = 4	3.21 ± 0.716 n = 4
	Week 39	3.16 ± 0.462 n = 4	3.10 ± 0.386 n = 3	3.07 ± 0.117 n = 4	2.97 ± 0.354 n = 4	2.95 ± 0.769 n = 4
	Week 52	3.31 ± 0.825 n = 4	2.67 ± 0.314 n = 3	2.77 ± 0.296 n = 4	2.64 ± 0.378 n = 4	2.52 ± 0.461 n = 4

Table 3.12.1.9-04 Selected blood chemistry findings among male Beagle dogs - continued

		Males				
Dosage (mg/kg/day)						
Parameter	**Time-point**	**0**	**1**	**7**	**50**	**250**
BLOOD CHEMISTRY						
Triglycerides (mmol/L)	pre-dose	0.28 ± 0.036 n = 5	0.26 ± 0.130 n = 3	0.24 ± 0.050 n = 3	0.24 ± 0.043 n = 4	0.27 ± 0.031 n = 6
	Week 6	0.19 & 0.25 indiv. val.	-	-	-	0.27 & 0.23 indiv. val.
	Week 13	0.32 ± 0.089 n = 6	0.28 ± 0.055 n = 4	0.29 ± 0.085 n = 4	0.27 ± 0.032 n = 4	0.36 ± 0.099 n = 6
	Week 26	0.23 ± 0.034 n = 4	0.29 ± 0.022 n = 4	0.26 ± 0.038 n = 4	0.23 ± 0.057 n = 4	0.44 ± 0.111# n = 4
	Week 39	0.28 ± 0.051 n = 4	0.27 ± 0.096 n = 3	0.28 ± 0.043 n = 4	0.34 ± 0.246 n = 4	0.35 ± 0.240 n = 4
	Week 52	0.37 ± 0.075 n = 4	0.39 ± 0.176 n = 3	0.33 ± 0.022 n = 4	0.45 ± 0.098 n = 4	0.48 ± 0.088 n = 4
Total protein (g/L)	pre-dose	53 ± 2.6 n = 5	55 ± 6.6 n = 3	52 ± 1.5 n = 3	55 ± 2.5 n = 4	52 ± 1.2 n = 6
	Week 6	54 & 50 indiv. val.	-	-	-	51 & 47 indiv. val.
	Week 13	55 ± 2.1 n = 6	57 ± 1.7 n = 4	56 ± 3.1 n = 4	54 ± 2.9 n = 4	50 ± 2.3# n = 6
	Week 4 R	56 & 53 indiv. val.	-	-	-	57 & 55 indiv. val
	Week 26	56 ± 2.9 n = 4	56 ± 1.3 n = 4	57 ± 3.1 n = 4	56 ± 1.8 n = 4	52 ± 2.1* n = 4
	Week 39	57 ± 1.3 n = 4	58 ± 2.0 n = 3	55 ± 1.5 n = 4	57 ± 4.7 n = 4	53 ± 3.9 n = 4
	Week 52	60 ± 1.7 n = 4	61 ± 4.0 n = 3	57 ± 3.5 n = 4	55 ± 1.5* n = 4	52 ± 1.3# n = 4
Albumin (g/L)	pre-dose	26 ± 1.8 n = 5	28 ± 4.4 n = 3	26 ± 1.5 n = 3	28 ± 1.0 n = 4	28 ± 1.2 n = 6
	Week 6	28 & 25 indiv. val.	-	-	-	26 & 22 indiv. val.
	Week 13	30 ± 1.5 n = 6	31 ± 1.5 n = 4	31 ± 1.8 n = 4	28 ± 1.5 n = 4	26 ± 2.6# n = 6
	Week 4 R	29 & 29 indiv. val.	-	-	-	33 & 30 indiv. val
	Week 26	30 ± 0.0 n = 4	29 ± 1.0 n = 4	30 ± 1.9 n = 4	29 ± 1.4 n = 4	26 ± 1.9# n = 4
	Week 39	31 ± 0.8 n = 4	31 ± 2.5 n = 3	30 ± 1.7 n = 4	30 ± 2.0 n = 4	27 ± 3.5* n = 4
	Week 52	32 ± 2.9 n = 4	32 ± 3.6 n = 3	30 ± 2.5 n = 4	29 ± 1.3 n = 4	26 ± 3.4* n = 4

$ p<0.05 Students t test
* p<0.05 with respect to the control; # p<0.01 with respect to the control at Williams'test
n = number of animals examined: this number may be lower than the number of animals in the group at the sample point when e.g. results could not be obtained for some animals
R: recovery period; indiv. val.: individual values

Table 3.12.1.9-05 Selected blood chemistry findings among female Beagle dogs

		Females				
Dosage (mg/kg/day)						
Parameter	**Time-point**	**0**	**1**	**7**	**50**	**250**
BLOOD CHEMISTRY						
ALP (U/L)	pre-dose	107 ± 23.7 n = 5	129 ± 37.9 n = 4	127 ± 18.6 n = 3	124 ± 25.4 n = 3	138 ± 19.3 n = 3
	Week 6	134 & 133 indiv. val.	-	-	-	300 & 572 indiv. val.
	Week 13	86 ± 21.7 n = 6	100 ± 23.8 n = 4	158 ± 60.9 n = 4	240 ± 79.2# n = 4	379 ± 101.3# n = 6
	Week 4 R	120 & 112 indiv. val.	-	-	-	156 & 163 indiv. val
	Week 6 R	100 & 96 indiv. val.	-	-	-	133 & 113 indiv. val
	Week 8 R	65 & 98 indiv. val.	-	-	-	120 & 89 indiv. val
	Week 26	65 ± 17.6 n = 4	89 ± 23.0 n = 4	172 ± 72.9# n = 4	324 ± 158.7# n = 4	550 ± 71.3# n = 4
	Week 39	66 ± 21.3 n = 4	102 ± 6.1* n = 3	165 ± 40.7# n = 4	249 ± 85.6# n = 4	439 ± 186.9# n = 4
	Week 52	78 ± 23.3 n = 4	167 ± 85.2 n = 3	170 ± 29.8 n = 4	220 ± 54.2* n = 4	569 ± 149.8# n = 4
gGT (U/L)	pre-dose	2 ± 0.4 n = 5	3 ± 0.6 n = 4	3 ± 1.2 n = 3	1 ± 0.6 n = 3	2 ± 0.8 n = 5
	Week 6	2 & 2 indiv. val.	-	-	-	3 & 4 indiv. val.
	Week 13	2 ± 0.8 n = 6	2 ± 0.0 n = 4	3 ± 0.5 n = 4	3 ± 0.0 n = 4	4 ± 1.5# n = 6
	Week 4 R	4 & 1 indiv. val.	-	-	-	2 & 4 indiv. val
	Week 26	3 ± 0.5 n = 4	3 ± 0.5 n = 4	3 ± 1.0 n = 4	3 ± 0.0 n = 4	5 ± 2.4 n = 4
	Week 39	3 ± 1.0 n = 4	4 ± 1.0 n = 4	3 ± 0.5 n = 4	3 ± 0.5 n = 4	4 ± 2.4 n = 4
	Week 52	3 ± 1.0 n = 4	2 ± 1.3 n = 4	3 ± 0.8 n = 4	3 ± 1.0 n = 4	5 ± 2.2 n = 4
Total cholesterol (mmol/L)	pre-dose	3.84 ± 0.567 n = 5	3.88 ± 0.396 n = 4	4.11 ± 0.430 n = 3	4.48 ± 0.855 n = 3	4.10 ± 0.328 n = 5
	Week 6	4.38 & 3.55 indiv. val.	-	-	-	2.99 & 2.39 indiv. val.
	Week 13	4.32 ± 0.910 n = 6	4.03 ± 0.648 n = 4	4.23 ± 0.710 n = 4	3.94 ± 0.576 n = 4	3.25 ± 0.633* n = 6
	Week 4 R	6.55 & 2.48 indiv. val.	-	-	-	4.02 & 5.33 indiv. val
	Week 26	4.00 ± 1.377 n = 4	4.46 ± 1.316 n = 4	5.04 ± 1.503 n = 4	4.48 ± 1.221 n = 4	3.39 ± 0.932 n = 4
	Week 39	3.52 ± 0.659 n = 4	4.62 ± 1.399 n = 4	4.60 ± 1.664 n = 4	4.24 ± 1.556 n = 4	3.10 ± 0.910 n = 4
	Week 52	3.63 ± 1.232 n = 4	5.48 ± 2.332 n = 4	4.19 ± 1.014 n = 4	4.25 ± 1.608 n = 4	2.85 ± 0.575 n = 4

Table 3.12.1.9-05 Selected blood chemistry findings among female Beagle dogs – continued

		Females				
Dosage (mg/kg/day)						
Parameter	**Time-point**	**0**	**1**	**7**	**50**	**250**
BLOOD CHEMISTRY						
Triglycerides (mmol/L)	pre-dose	0.27 ± 0.087 n = 5	0.34 ± 0.044 n = 4	0.23 ± 0.040 n = 3	0.32 ± 0.083 n = 3	0.27 ± 0.048 n = 5
	Week 6	0.41 & 0.30 indiv. val.	-	-	-	0.28 & 0.28 indiv. val.
	Week 13	0.48 ± 0.174 n = 6	0.41 ± 0.129 n = 4	0.45 ± 0.211 n = 4	0.40 ± 0.052 n = 4	0.36 ± 0.054 n = 6
	Week 26	0.31 ± 0.057 n = 4	0.46 ± 0.156 n = 4	0.33 ± 0.139 n = 4	0.38 ± 0.117 n = 4	0.35 ± 0.087 n = 4
	Week 39	0.25 ± 0.080 n = 4	0.46 ± 0.108* n = 4	0.44 ± 0.130* n = 4	0.40 ± 0.075* n = 4	0.31 ± 0.044* n = 4
	Week 52	0.58 ± 0.190 n = 4	0.48 ± 0.082 n = 4	0.53 ± 0.272 n = 4	0.35 ± 0.044 n = 4	0.50 ± 0.109 n = 4
Total protein (g/L)	pre-dose	55 ± 1.8 n = 5	53 ± 1.5 n = 4	57 ± 2.6 n = 3	56 ± 2.1 n = 3	54 ± 1.6 n = 5
	Week 6	55 & 54 indiv. val.	-	-	-	50 & 47 indiv. val.
	Week 13	55 ± 1.9 n = 6	55 ± 2.6 n = 4	57 ± 0.8 n = 4	53 ± 2.5 n = 4	50 ± 1.5# n = 6
	Week 4 R	61 & 52 indiv. val.	-	-	-	56 & 55 indiv. val
	Week 26	58 ± 3.8 n = 4	60 ± 3.1 n = 4	59 ± 3.3 n = 4	56 ± 2.1 n = 4	54 ± 1.8 n = 4
	Week 39	56 ± 2.5 n = 4	58 ± 2.2 n = 4	58 ± 2.4 n = 4	56 ± 4.2 n = 4	51 ± 0.5* n = 4
	Week 52	59 ± 4.3 n = 4	61 ± 5.0 n = 4	59 ± 2.9 n = 4	56 ± 2.2 n = 4	53 ± 1.0* n = 4
Albumin (g/L)	pre-dose	29 ± 1.1 n = 5	28 ± 0.8 n = 4	30 ± 1.5 n = 3	28 ± 1.2 n = 3	29 ± 0.5 n = 5
	Week 6	30 & 32 indiv. val.	-	-	-	27 & 25 indiv. val.
	Week 13	32 ± 0.8 n = 6	31 ± 1.7 n = 4	33 ± 2.1 n = 4	29 ± 3.3* n = 4	28 ± 1.4# n = 6
	Week 4 R	309 & 31 indiv. val.	-	-	-	33 & 33 indiv. val
	Week 26	33 ± 1.3 n = 4	32 ± 0.8 n = 4	31 ± 2.5 n = 4	29 ± 1.9* n = 4	28 ± 2.5# n = 4
	Week 39	32 ± 3.2 n = 4	32 ± 1.0 n = 4	32 ± 1.0 n = 4	30 ± 3.3 n = 4	27 ± 1.3# n = 4
	Week 52	31 ± 2.1 n = 4	31 ± 1.4 n = 4	31 ± 1.7 n = 4	30 ± 2.1 n = 4	29 ± 1.9 n = 4

$ p<0.05 Students t test
* p<0.05 with respect to the control; # p<0.01 with respect to the control at Williams'test
n = number of animals examined: this number may be lower than the number of animals in the group at the sample point when e.g. results could not be obtained for some animals
R: recovery period; indiv. val.: individual values

Urinalysis

There were no effect of treatment on urinalysis parameters in Weeks 13, 26, 39 or 52.

Sacrifice and pathology

Organ weight

Analysis of terminal organ weights revealed higher group mean absolute liver weights for for all treated groups compared to controls. However, there was no dose-relationship between groups receiving 1 or 7 mg/kg/day. In both sexes treated at 250 mg/kg/day liver weight relative to bodyweight was also increased.
Analysis of organ weights of animals which underwent recovery revealed revealed absolute liver weight for both males and a female comparable to controls. As the remaining female was actually the heaviest (bodyweight) among recovery females, its liver weight relative to bodyweight was comparable to controls.
After 52 weeks of treatment, both sexes at 250 and 50 mg/kg/day showed higher than control dose-related thyrodi weights. After 13 weeks of treatment followed by an 8-week recovery period, one male and one female showed higher than control thyroid weights, also having the higher liver weight at termination.
After 52 weeks of treatment, lower group mean prostate weights were note in dogs treated at 250 mg/kg/day, not attaining statistcal significance. Recovery males also showed lower prostate weights, compared to concurrent controls. Lower group mean absolute ovary and uterus/cervix weights were noted in females receiving 250 or 50 mg/kg/day, though statistical significance was attained for the mean ovary weight only. After an 8-week recovery period following 13 weeks of treatment, ovary weights for previously treated females were similar to control values.

Table 3.12.1.9-06 Selected organ weights in Beagle dogs treated for 52 consecutive weeks (g)

Dose level (mg/kg/day)		**0**	**1**	**7**	**50**	**250**
Male						
Terminal bodyweight		12475 ± 1050 n = 4	11700 ± 1100 n = 3	13025 ± 1189.9 n = 4	13925 ± 1037.2 n = 4	13525 ± 1571.4 n = 4
LIVER	abs.	352 ± 50	391 ± 27	375 ± 36	439 ± 40*	601 ± 84#
	rel.	*2.8 ± 0.2*	*3.4 ± 0.3*	*2.9 ± 0.2*	*3.2 ± 0.4*	*4.5 ± 0.8#*
THYROID (+ parath.)	abs.	0.866 ± 0.141	0.776 ± 0.063	0.761 ± 0.062	1.044 ± 0.157	1.245 ± 0.205#
	rel.	*0.0071 ± 0.0018*	*0.0066 ± 0.0004*	*0.0059 ± 0.0006*	*0.0075 ± 0.0008*	*0.0093 ± 0.0016**
PROSTATE	abs.	12.296 ± 3.011	12.770 ± 2.487	13.524 ± 1.190	10.924 ± 1.598	9.250 ± 0.511
	adj.	12.662	13.682	13.503	10.269	8.877*
	rel.	*0.0979 ± 0.0186*	*0.1086 ± 0.0125*	*0.1043 ± 0.0112*	*0.0786 ± 0.0104*	*0.0692 ± 0.0104#*
SPLEEN	abs.	157.4 ± 29.7	106.5 ± 26.0	114.4 ± 12.9	123.2 ± 40.7	106.8 ± 35.7
	adj.	166.7	129.8#	113.9#	106.5#	97.3#
	rel.	*1.26 ± 0.18*	*0.90 ± 0.14**	*0.88 ± 0.10**	*0.87 ± 0.26**	*0.78 ± 0.22#*
Females						
Terminal bodyweight		11875 ± 797.4 n = 4	13025 ± 1748.1 n = 4	12200 ± 867.9 n = 4	12150 ± 591.6 n = 4	13125 ± 2146.9 n = 4
LIVER	abs.	334 ± 28	424 ± 57	402 ± 74	419 ± 87	496 ± 47#
	rel.	*2.8 ± 0.2*	*3.3 ± 0.5*	*3.3 ± 0.6*	*3.5 ± 0.9*	*3.8 ± 0.5**
THYROID (+ parath.)	abs.	0.792 ± 0.093	0.810 ± 0.167	0.794 ± 0.108	0.971 ± 0.153	1.199 ± 0.301#
	rel.	*0.0067 ± 0.0010*	*0.0063 ± 0.0014*	*0.0065 ± 0.0005*	*0.0080 ± 0.0012*	*0.0094 ± 0.0032*
OVARIES	abs.	2.69 ± 1.03	1.94 ± 0.43	1.85 ± 0.76	142 ± 0.34*	1.34 ± 0.21#
	rel.	*0.023 ± 0.009*	*0.015 ± 0.004*	*0.015 ± 0.006*	*0.012 ± 0.003**	*0.010 ± 0.003#*
UTERUS & CERVIX	abs.	17.8 ± 6.4	21.5 ± 10.0	13.5 ± 10.3	11.7 ± 5.9	8.5 ± 2.4
	rel.	*0.15 ± 0.06*	*0.17 ± 0.09*	*0.11 ± 0.08*	*0.10 ± 0.05*	*0.07 ± 0.03*
SPLEEN	abs.	101.1 ± 38.6	116.8 ± 33.9	102.7 ± 44.9	69.6 ± 17.5	114.5 ± 23.0
	rel.	*0.86 ± 0.34*	*0.89 ± 0.18*	*0.83 ± 0.30*	*0.58 ± 0.16*	*0.90 ± 0.28*

* p<0.05 with respect to the control; # p<0.01 with respect to the control at Williams'test
abs.: absolute organ weight; adj.: adjusted (from analysis of covariance); *rel.*: organ weight relative to bodyweight

Statistically significant lower group mean and dosage-related (relative to and adjusted for terminal bodyweight) spleen weights were noted for all treated male groups compared to concurrent control values. However, this was not evident in the females and there was no corroborative haematology or histopathology findings, thus this observtion was not considered to be of toxicological importance. There were no clear differences from controls in spleen weights in animals assigned to recovery.

Table 3.12.1.9-07 Selected organ weights in Beagle dogs treated for 13 consecutive weeks followed by an 8-week recovery period (individual values, g)

		Males		Females	
Dose level (mg/kg/day)		**0**	**250**	**0**	**250**
Terminal bodyweight		14000; 12700	13700; 13800	12100; 10000	11800; 13600
LIVER	abs.	438; 367	416; 444	372; 261	378; 444
	rel.	*3.1; 2.9*	*3.0; 3.2*	*3.1; 2.6*	*3.2; 3.3*
THYROID (+ parath.)	abs.	0.801; 0.574	0.841; 1.135	0.728; 0.486	0.738; 0.903
	rel.	*0.0057; 0.0045*	*0.0061; 0.0082*	*0.0060; 0.0049*	*0.0063; 0.0066*
PROSTATE	abs.	10.106; 10.818	5.633; 4.360		
	rel.	*0.0722; 0.0852*	*0.0411; 0.0316*		
OVARIES	abs.			1.69; 1.21	1.19; 1.221
	rel.			*0.014; 0.012*	*0.010; 0.009*
UTERUS & CERVIX	abs.			6.3; 4.1	3.7; 4.0
	rel.			*0.05; 0.04*	*0.03; 0.03*
SPLEEN	abs.	86.3; 96.0	104.8; 73.0	124.3; 69.1	139.0; 78.8
	rel.	*0.62; 0.76*	*0.77; 0.53*	*1.03; 0.69*	*1.18; 0.58*

abs.: absolute organ weight; *rel*.: organ weight relative to bodyweight

Gross and histopathology

a) Liver: the macroscopic examination performed at termination revealed enlargement of the liver 3 out of 4 males and 2 out of 4 females at 250 mg/kg bw/day and 1 out of 4 females at 50 mg/kg/day. The necropsy performed at termination of the 8-week recovery period reveal no lesions attributable to treatment.
Generalised hepatocyte hypertrophy was seen in animals from all treated groups. Hepatocytes with pale cytoplasm and peripheral clumping were seen in all males given 250 mg/kg/day and in females given 250 or 50 mg/kg/day. In addition, eosinophilic concentric cytoplasmic inclusions in hepatocyte were seen in one male at 250 mg/kg/day
Minimal rerefaction of hepatocytes, compared to controls, was seen in recovery animals.

b) Thyroids: hypertrophy of the follicular epithelium was seen in both sexes receiving 250 and in a single male and female given 50 mg/kg/day.
No thyroid changes were observed in recovery animals.

c) Prostate: minimal immaturity was noted in recovery male dogs. No abnormality was observed at histopathology in main study animals.

d) Ovaries: no corpora lutea were present in the ovaries of three out four females given 250 mg/kg/day.

Table 3.12.1.9-08 Selected pathology findings among Beagle dogs

		Males				
Dose level (mg/kg/day)		**0**	**1**	**7**	**50**	**250**
LIVER		n = 4	n = 3	n = 4	n = 4	n = 4
Hepatocyte hypertrophy generalised	tot.	0	2	1	4	4
	min.	0	2	1	2	0
	slight	0	0	0	2	3
	mod.	0	0	0	0	1
Hepatocytes with pale citoplasm with periferal clumping	tot.	0	0	0	0	4
	min.	0	0	0	0	4
THYROIDS		n = 4	n = 3	n = 4	n = 4	n = 4
Hypertrophy of follicular hepithelium	tot.	0	0	0	1	3
	min.	0	0	0	1	1
	slight	0	0	0	0	2
		Females				
LIVER		n = 4	n = 4	n = 4	n = 4	n = 4
Hepatocyte hypertrophy generalised	tot.	0	2	2	4	4
	min.	0	2	1	3	0
	slight	0	0	1	1	3
	mod.	0	0	0	0	1
Hepatocytes with pale citoplasm with periferal clumping	tot.	0	0	0	1	3
	min.	0	0	0	1	3
THYROIDS		n = 4	n = 4	n = 4	n = 4	n = 4
Hypertrophy of follicular hepithelium	tot.	0	0	0	1	2
	min.	0	0	0	1	2
	slight	0	0	0	0	0

tot.: total number; min.: minimal; slight: slight; mod.: moderate, degree of severity (ascending)

CONCLUSIONS

The general health of the animals treated at 250 mg/kg/day for 52 weeks was not adversely affected however, the prostate weight changes and the ovary/lack of oestrus in females at this dose level were considered to represent adverse effects. Treatment-related effects recorded at 50 mg/kg/day were confined to the liver and thyroid. The microscopic thyroid changes at this dose level were confined to single animals of each sex and consisted of follicular cell hypertrophy: due to the low severity and incidence of these findings, the lack of clear concomitant effects on thyroid hormone levels, and the fact that no microscopic thyroid changes were detected at the end of an 8-week recovery period (following 13-week treatement at a higher dose), the thyroid changes were not considered to be of toxicological importance. Microscopic changes seen in the liver were mainly adaptive hepatocyte hypertrophy of no more than a slight degree. The blood chamistry changes recorded were considered to be consistent with the microscopically evident adaptive changes. On this basis, the NOAEL of the study was set at 50 mg/kg/day.

3.12.2 Human data

No human data available on specific target organ toxicity – repeated exposure

3.12.3 Other data

No other data available on specific target organ toxicity – repeated exposure

3.13 Aspiration hazard

No data available on aspiration hazard

3.13.1 Human data

No human data available on aspiration hazard

3.13.2 Other data

No other data available on aspiration hazard

4 ENVIRONMENTAL HAZARDS

4.1 Degradation

4.1.1 Ready biodegradability

4.1.1.1 Effects on the activity of sludge micro-organisms

Reference Number:	KII - **Post submission 03**, Section 6, Volume 3, Annex IIA, point 8.15/01
Report:	**See annex conf. 40**
Guidelines:	OECD Guideline 209
Deviations:	none
GLP:	Yes; certified laboratory

Executive Summary:

The purpose of this study was to determine potential effects of the test item on the activity of micro-organisms of activated sludge from a sewage treatment plant.
Based on the results of a range-finding test (non-GLP, not covered by this study plan) and agreed with the sponsor/study monitor one test item treatment (5 replicates), one control treatment (two replicates) and one solvent control (two replicates) were tested. The test concentration of the test item is chosen on the basis of the range finding test and of the solubility of the test item in water (that is 2.4 x 10^{-2} mg/L at room temperature).
The method is based on the measurement of the respiration rate of micro-organisms (measured as oxygen consumption) after a contact time of three hours with the test item. The respiration rate is measured over a period of ten minutes.
No adverse effects of the test item IR5885 tech. on the activity of the micro-organisms of activated sludge were observed at the tested concentration of 100 mg/L (limit test) compared to the solvent control. The organic solvent (methanol) did not show significant inhibition of the activity of the micro-organisms (measured as O_2-consumption).
Therefore it is concluded that the EC_{50} is higher than 100 mg/L (i.e. higher than the limit of solubility that is 2.4 x 10^{-2} mg/L).

MATERIALS AND METHODS

MATERIALS

Test Material	IR5885
Description	white powder
Lot/Batch #	G005/07
Purity	97.97 ± 0.20 % w/w (analysed)
CAS #	283159-90-0

Test Medium:	
Species:	Activated sludge
Source:	Municipal sewage treatment plant in D-04451 Borsdorf, Germany.
Test Water:	Tap water

Incubation period: The incubation of the test solution was performed at a temperature of 20 ± 2 °C over a time span of three hours (contact time).
Test Units: Reaction flask

Test Conditions:
Light Regime: dark
pH Values: 6.7 (of the activated sludge suspension)
Temperature: 20 ± 2 °C

STUDY DESIGN AND METHODS

In life dates: Experimental Start: May 7, 2007
Experimental Termination: May 9, 2007

Dosage Preparation and Dosing

- Preparation of the stock solution:
Because of its low water solubility, the weighed amount of the test item was mixed with methanol as organic solvent to prepare a stock solution immediately before application.
- Preparation of the control solutions:
16 mL of synthetic sewage (prepared in accordance with OECD guideline 209), 284 mL tap water and 200 mL sludge suspension were pipetted into an incubation vessel (2 L beaker).
- Preparation of the solvent control solutions:
2 mL methanol, 16 mL of synthetic sewage (prepared in accordance with OECD guideline 209), 282 mL tap water and 200 mL sludge suspension were pipetted into an incubation vessel (2 L beaker).
- Preparation of the test item solutions:
2 mL of the test item stock solution, 16 mL of synthetic sewage (prepared in accordance with OECD guideline 209), 282 mL tap water and 200 mL sludge suspension were pipetted into an incubation vessel (2 L beaker).
Afterwards, the total mixture (500 mL) was aerated with oil-free air with an air flow of 0.5 to 1 L per minute. The incubation of the test solution was performed at a temperature of 20 ± 2 °C over a time span of three hours (contact time).

Experimental Design

One test item treatment (5 replicates), two control replicates (one at the start and the other one at the end of the test series) and two solvent control replicates were tested in the sequence control (first replicate), solvent control (first replicate), test item replicates, solvent control (second replicate) and control (second replicate).
The use of the organic solvent methanol was necessary to achieve an equal distribution of the poorly water soluble test item in the test solution. To check if methanol affects the activity of the micro-organisms a solvent control was included in the test.
After three hours of incubation, the content (500 mL) of the incubation vessel was poured into a reaction flask (1 L wide-mouth glass flask). Afterwards, a stirrer bar and a small vessel containing 18 mL 1 m NaOH solution were placed into the reaction flask which was then tightly closed.
To determine the respiration rate, the reaction flask was then connected with a respirometer (“BSB digi SELUTEC”, Mössingen-Öschingen, Germany).
The respiration of the micro-organisms leads to O_2 consumption and formation of CO_2 which is absorbed by the NaOH solution. The absorption of CO_2 caused low-pressure in the reaction flask which is compensated by delivering of O_2 by the respirometer.
The respirometer records the O_2 consumption of the micro-organisms of the test solution over a period of 10 minutes, in a rhythm of 30 seconds cumulatively.

RESULTS AND DISCUSSION

No adverse effects of the test item IR5885 tech. on the activity of the micro-organisms of activated sludge were observed at the tested concentration of 100 mg/L (limit test) compared to the solvent control. Only a negligible deviation from the solvent control of -2.6 % was measured.
The organic solvent (methanol) did not show significant inhibition of the activity of the micro-organisms (measured as O_2-consumption). The results are summarised in Table 4.1.1.1-01.

Table 4.1.1.1-01: Effects on activated sludge after treatment with IR5885 tech.

Control		Solvent control			100 mg test item/L		
O_2 consumption [mg/L /h]	CV [%]	O_2 consumption [mg/L/h]	CV [%]	Inhibition compared to control [%][1]	O_2 consumption [mg/L/h]	CV [%]	Inhibition compared to solvent control [%][1]
33.00	0.00	34.50	1.02	+4.5	33.60	0.86	-2.6

CV [%] = Coefficient of Variation
[1)] based on O_2 consumption; - = % inhibition; + = % stimulation

CONCLUSIONS

It is concluded that the EC_{50} is higher than 100 mg/L (i.e. higher than the limit of solubility that is 2.4 x 10^{-2} mg/L).

To verify the sensitivity of the test system the reference item 3,5-dichlorohenol was tested on the same batch of activated sludge like the test item at concentrations of 10.0, 20.0 and 40.0 mg/L.
The EC_{50} was calculated to be 21.9 mg/L (required 5 – 30 mg/L).

4.1.1.2 Ready biodegradability of the active substance – IR5885

Reference Number: KII-Section 5, Volume 2, Annex IIA, point 7.7/01

Report: See annex conf. 15

Guidelines: EEC method C.4-D (1992) Manometric Respirometry Test; OECD 301 F "Ready biodegradability: Manometric Respirometry Test (1992).

Deviations: none

GLP: Yes; certified laboratory

Executive Summary:

The purpose of this study was to determine the ready biodegradability of the test item IR 5885. The test item was exposed to activated sludge from the aeration tank of a domestic waste water treatment plant for 28 days. The biodegradation was followed by the oxygen uptake of the micro organisms during exposure. As a reference item Aniline was tested simultaneously under the same conditions as the test item, and functioned as a procedure control. This study is recognized by the OECD and EEC guidelines and should provide a rational basis to assess the ready biodegradation properties of the test item when incubated with activated sludge. Under the test conditions the percentage biodegradation of IR5885 reached 3 % after 28 days of incubation, based on ThODNH4. If the calculation is based on ThODNO3, a mean of 2 % biodegradation was found after

28 days of incubation.The test item can therefore be considered to be not readily biodegradable.

MATERIALS AND METHODS

MATERIALS

Test Material	IR5885
Description	white, solid
Lot/Batch #	T 025/02
Purity	99.56 ± 0.14 % (w/w)
Stability of the test compound:	Stable under test conditions
Vehicle and /or positive control:	Deionised water /Aniline p.A.
Test Animals:	Activated sludge, micro organisms from a domestic waste water treatment plant
Source:	Supplied by the sewage plant Groß-Zimmern, Germany
Conditioning:	The activated sludge used for this study was washed by centrifugation and the supernatant liquid phase was decanted. The solid material was resuspended in tap water and again centrifuged. This procedure was repeated twice. An aliquot of the final sludge suspension was weighed, dried and the ratio of wet sludge to its dry weight was determined. Based on this ratio, calculated aliquots of washed sludge suspension, corresponding to 1.5 g dry material per litre were mixed with test water (see 6.5) and then aerated until use.
Test Units:	Manometric Test System with test flasks containing a volume of 500 mL
Environmental conditions:	
Temperature:	21°C
pH-value of Test solution:	7.6 (measured at the start of the test)
Air changes:	Climatic chamber
Photoperiod:	Darkness

STUDY DESIGN AND METHODS

Preparation of the Test Solutions

Test Item (flasks 1 and 2):	25.0 and 25.2 mg IR 5885, respectively and activated sludge at a concentration of 30 mg suspended solids per litre were filled up with test water to a volume of 244 mL.
Inoculum Control (flasks 3 and 4):	activated sludge at a concentration of 30 mg suspended solids per litre was filled up with test water to a volume of 244 mL.
Procedure Control (flask 5):	25.4 mg Aniline and activated sludge at a concentration of 30 mg suspended solids per litre were filled up with test water to a volume of 244 mL. The procedure control was also used for other projects which ran in parallel.
Abiotic Control (flask 6):	25.3 mg IR 5885 filled up with test water (sterile filtered, 0.2 - 0.45 µm filter) to a volume of 244 mL.
Toxicity Control (flask 7):	25.4 mg IR 5885, 25.1 mg Aniline and activated sludge at a concentration of 30 mg suspended solids per litre were filled up with test water to a volume of 244 mL.

Study design

The test item was exposed to activated sludge for 28 days. The amounts of test item and reference item were directly weighed into the test flasks. No emulsifiers or solvents were used, but the solutions were dispersed by stirring to achieve a homogeneous solution of the test item. The closed test flasks were incubated in a climatic chamber under continuous stirring. The consumption of oxygen was determined by measuring the change of pressure in the flasks. Evolved carbon dioxide was absorbed in an aqueous solution (45 %) of potassium hydroxide. The change of pressure in the test flasks was measured by means of a manometric method each day. Temperature was measured each working day in the climatic chamber. pH-values were measured in all flasks at the start and end of the test using a pH-electrode ECM-Multi.

RESULTS AND DISCUSSION

Biodegradation of Test Item

Under the test conditions the percentage biodegradation of IR 5885 reached 3 % after 28 days of incubation, based on $ThOD_{NH4}$. The results are represented in Tables 4.1.1.2-01 and 4.1.1.2-02. If the calculation is based on $ThOD_{NO3}$, a mean of 2 % biodegradation was found after 28 days of incubation.
The test item can therefore be considered to be not readily biodegradable.

Biodegradation of Reference Item Aniline

The reference item Aniline was sufficiently degraded to 82 % after 14 days, and to 92 % after 28 days of incubation, based on $ThOD_{NH4}$. The results are represented in Tables 4.1.1.2-01 and 4.1.1.2-02.
The percentage biodegradation of the reference item confirms the suitability of the used activated sludge inoculum.

Biodegradation in the Toxicity Control

In the toxicity control containing both, the test item and the reference item Aniline, 43 % biodegradation was noted within 14 days and 50 % biodegradation was determined after 28 days of incubation, based on $ThOD_{NH4}$. The results are represented in Tables 4.1.1.2-01 and 4.1.1.2-02.
According to the test guidelines the test item can be assumed to be not inhibitory on the activated sludge micro organisms because degradation was > 25 % within 14 days.

Abiotic Control

The oxygen demand in the abiotic control was less than in the two control flasks (Table 4.1.1.2-01).

Table 4.1.1.2-01 Cumulative biochemical oxygen demand (mgO_2/L) in test flasks (28 days)

Time				Flask No.			
(days)	1 Test item	2 Test item	3 Inoculum control	4 Inoculum control	5 Aniline (Proc.cont.)	6 Abiotic control	7 Toxicity control
1	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0
4	0	0	0	5	5	0	5
5	5	0	5	5	25	0	30
6	5	5	5	5	85	0	95
7	15	10	10	10	165	5	160
8	15	15	10	10	170	5	165
9	15	15	10	10	185	5	175
10	20	15	10	10	195	5	180
11	20	15	10	10	200	5	180

Time				Flask No.			
(days)	1 Test item	2 Test item	3 Inoculum control	4 Inoculum control	5 Aniline (Proc.cont.)	6 Abiotic control	7 Toxicity control
12	20	15	10	10	205	5	185
13	20	20	10	10	210	5	190
14	25	20	10	10	215	5	195
15	25	20	10	10	215	5	195
16	25	20	10	10	220	5	200
17	25	20	10	10	225	5	205
18	25	20	10	10	225	5	210
19	25	25	10	15	230	5	215
20	25	25	15	15	230	5	220
21	25	25	15	15	235	5	220
22	25	30	15	20	235	5	220
23	25	30	15	20	240	5	225
24	30	30	20	20	240	5	225
25	30	30	20	20	245	5	230
26	30	30	20	20	250	5	235
27	30	30	20	25	250	5	235
28	30	30	25	25	255	5	240

Tables 4.1.1.2-02 Percentage biodegradation (BOD/ThOD$_{NH4}$)

Time	Percentage BOD			
(days)	IR 5885		Aniline	Toxicity control
	flask 1	flask 2	flask 5	flask 7
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4	-1	-1	1	1
5	0	-3	8	6
6	0	0	32	21
7	3	0	62	35
8	3	3	64	36
9	3	3	70	39
10	6	3	74	40
11	6	3	76	40
12	6	3	78	41
13	6	6	80	42
14	9	6	82	43
15	9	6	82	43
16	9	6	84	45
17	9	6	86	46
18	9	6	86	47
19	7	7	87	47
20	6	6	86	48
21	6	6	88	48
22	4	7	87	47
23	4	7	89	49
24	6	6	88	48
25	6	6	90	49
26	6	6	92	50
27	4	4	91	50
28	3	3	92	50

$ThOD_{NH4}$ of IR 5885: 1.72 mg O_2/mg test item
$ThOD_{NH4}$ of Aniline: 2.41 mg O_2/mg Aniline

CONCLUSIONS

According to the test guidelines the test item can be assumed to be not inhibitory on the activated sludge micro organisms because degradation was > 25 % within 14 days.

4.1.2 BOD_5/COD

No data available.

4.1.3 Hydroysis

4.1.3.1 Hydrolytic degradation – IR5885

Reference Number:	KII Section 5, Volume 2, Annex IIA, point 7.5/01 Cross Reference, Section 1, Annex IIA, point 2.9.1
Report:	See annex conf. 35
Guidelines:	EEC method C7; OPPTS 835.2110 "Hydrolysis as a function of pH" (U.S. EPA Fate, Transport and Transformation Test Guidelines).
Deviations:	none
GLP:	Yes; certified laboratory

Executive Summary:

The hydrolysis rate of IR5885 was determined in three buffered aqueous solutions (pH 4, 7 and 9) at a concentration of 1 µg/mL.
The study was carried out in the absence of light, under sterile conditions.
The Preliminary Test was performed at 50°C; at each pH value six samples were analyzed after an incubation period of 0, 1, and 3 hours and 1, 2, and 5 days.
At pH 4 ^{14}C-IR5885 was substantially stable. After 5 days its amount accounted for 97.99% of the applied radioactivity (AR) so showing a hydrolysis degradation significatively lower than 10% (value corresponding to a half-life higher than 1 year at field conditions, according to EEC method C7, 1.6.5.1). At this pH value no more investigation was carried out.
After 5 days of incubation at pH 7 and 9 the amount of test substance accounted for 64.18% and 0.31% AR, respectively. As the hydrolyzed amount was higher than 10%, at these pH values further tests (Test No.1 and No.3) were performed.

The Test No.1 was carried out at 50°C. At each pH value ten samples were analyzed: after 0, 2, 3, 4, 5, 7, 8, 9, 10 and 11 days of incubation at pH 7, and after 0, 2, 3, 4, 5, 6, 7, 8, 9 and 10 hours at pH 9. The amount of ^{14}C-IR5885 accounted for 32.51% AR after 11 days at pH 7, and 40.39% after 10 hours at pH 9.

The Test No.3 was carried out at 55°C and 65°C at pH 7 and at 25°C at pH 9.
At pH 7 two set of five samples were analyzed after 0, 3, 5, 7 and 10 days (55°C) and after 0, 31, 55, 79 and 103 hours (65°C).
At pH 9 five samples were analyzed after 0, 2, 3, 4 and 7 days.

The amount of ^{14}C-IR5885 accounted for 26.24% AR after 10 days at 55°C and pH 7, and for 22.98% after 103 hours at 65°C and pH 7.
The amount of ^{14}C-IR5885 accounted for 30.47% AR after 7 days at 25°C and pH 9.
At pH 7 and 9 two main compounds were found in samples: H1 (unchanged ^{14}C-IR5885) and H3 which reached levels higher than 10% and was identified as ^{14}C-IR5885-acid. Its amount increased rapidly during incubation in every experimental condition: at last sampling time, at pH 7 it reached 65.67%, 72.48%, and 75.46% AR at 50°C, 55°C, and 65°C, respectively; while at pH 9 it reached 57.45% and 67.77% AR at 50°C and 25°C, respectively.
In all experimental conditions the compounds H2, H4, and H5 were found as traces and their amount never reached 2%.
The ^{14}C-mass balance was always higher than 95% and ranged from 95.44% to 105.37% AR.
The results showed that the hydrolysis degradation of ^{14}C-IR5885 follows a pseudo first-order kinetic.
The values of k_{obs} and of DT_{50} and DT_{90} (Disappearance Time of 50% and 90%, respectively, of the test substance) are summarized in the table 4.1.4.1-01:

Table 4.1.3.1-01 - k_{obs} and of DT_{50} and DT_{90} Values

pH	T (°C)	k_{obs} (days^{-1})	DT_{50} (days)	DT_{90} (days)
7	65	0.331	2.09	6.95
	55	0.133	5.21	17.32
	50	0.0909	7.62	25.34
	25 *	0.00762 *	90.94 *	302.23 *
9	50	2.1	0.33	1.1
	25	0.167	4.15	13.79

* The values of k_{obs} and of DT_{50} and DT_{90} at 25°C and pH 7 were calculated by Arrhenius relationship.

HPLC analysis showed that all over the incubation time for both the compound H1 (unchanged IR5885) and the main degradation compound H3 (IR5885-acid) the diastereoisomeric ratio (S,R/S,S) was about 1.

MATERIALS AND METHODS

MATERIALS

Unlabelled Test Material Batch 20071/77
Chemical purity (by HPLC): > 99%

[^{14}C-U-phenyl] IR5885 Lot # 192
Specific activity: 5.089 MBq/mg
137.536 µCi/mg
305331 dpm/µg
Radiochemical purity (by TLC and HPLC): > 97%

Study Design and methods

The hydrolysis study was carried out under sterile conditions. All the glassware and the materials necessary for the study were sterilized at 121°C for 20 minutes by autoclaving.
Handling of sterile materials and sample preparation were performed by using a bacteriologic hood equipped with an UV lamp.

Preparation of buffer solutions: the following buffr solutions were prepared:

Buffer solution at pH 4: 0.01M acetate, Buffer solution at pH 7: 0.01M citrate, Buffer solution at pH 9: 0.01M borate.

Each buffered solution was bubbled with gaseous nitrogen to remove dissolved oxygen, and sterilized. The buffered solutions were then made up to the mark with sterilized distilled water (carbon dioxide and oxygen free). The final pH was measured on 50-mL aliquot of each buffered solution.

Preparation and analysis of the stock solution

An aliquot of ^{14}C-IR5885 was dissolved into CH_3CN to yield a *ca*. 100 µg/mL Stock Solution.
The radiochemical purity of the test substance was determined by Thin Layer Chromatography (TLC) by using two kinds of stationary phase:

- *normal phase*: silica gel
- *reversed phase*: RP-18

and three solvent systems:

❑ *solvent system 1*:	acetonitrile-water	(85-15)	for silica gel
❑ *solvent system 2*:	acetonitrile-water	(60-40)	for RP-18
❑ *solvent system 3*:	methanol-1N acetic acid	(65-35)	for RP-18

The diastereoisomeric ratio of the test substance was determined by HPLC.

Preparation and analysis of the test solutions

Suitable aliquots of the Stock Solution were diluted hundred times with the sterilized buffer solutions at pH 4, 7 and 9, to obtain the Test Solutions (approximately 1 µg/mL).
The percentage of acetonitrile was 1% of the total volume.
Six 0.1-mL aliquots of each Test Solution were analyzed by LSC to determine the actual concentration of the test substance.

Preparation of samples

Aliquots of 1 mL of each Test Solution were dispensed under sterile conditions into 10-mL glass tubes which were then sealed with glass plugs.
Each sample contained approximately 1 µg of ^{14}C-IR5885 corresponding to 0.14 µCi.

Study Design

A Preliminary Test was carried out in the dark, at 50°C, and at pH 4, 7 and 9 to establish a coarse hydrolysis rate. Samples were analyzed at time intervals of 0, 1 and 3 hours and 1, 2 and 5 days.

Test No.1

On the basis of Preliminary Test results, Test No.1 was carried out in the dark, at 50°C, and at pH 7 and 9.

pH 7: the samples were withdrawn and analyzed at time intervals of 0, 2, 3, 4, 5, 7, 8, 9, 10, and 11 days;
pH 9: the samples were withdrawn and analyzed at time intervals of 0, 2, 3, 4,5, 6, 7, 8, 9, and 10 hours.

Test No.3

On the basis of Preliminary and No.1 Test results, Test No.3 was carried out in the dark at pH 7 (55 and 65°C) and at pH 9 (25°C).

pH 7: at 55°C, the samples were withdrawn and analyzed at time intervals of 0, 3, 5, 7, and 10 days;
at 65°C, the samples were withdrawn and analyzed at time intervals of 0, 31, 55, 79, and 103 hours;

pH 9: the samples were withdrawn and analyzed at time intervals of 0, 2, 3, 4, and 7 days.

During all the Tests the pH values were checked on a set of samples prepared with unlabelled test substance (at experimental concentration) for each buffered solution.

Additional sample

An additional sample at a very high concentration of test substance was also prepared to assist to the identification of the hydrolysis products.

Analytical procedure

At chosen sampling times each sample was directly analyzed by LSC to establish the radioactivity content and by TLC and HPLC to profile the radiolabelled hydrolysis compounds.
The samples incubated at pH 7 and 9 were previously acidified to pH 3-4 with 4N HCl.

The identification of unchanged IR5885 was carried out by co-TLC of some representative samples with ^{14}C-IR5885 reference standard and by LC-MS analysis of the methanolic solution obtained from the additional sample.
In the same way the identification of hydrolysis product H3 was carried out by LC-MS.
Furthermore representative samples were co-chromatographed (TLC) to set a comparison among hydrolysis compounds generated at the three pH values (4, 7 and 9).

^{14}C-Mass balance

The ^{14}C-mass balance was obtained by calculating the percentage of the radioactivity content of each sample with respect to the radioactivity content of an equal volume of the Test Solution.

RESULTS AND DISCUSSION

Concentration and radiochemical purity of test substance

The results of LSC analysis of the Test Solutions showed that the concentration of the test substance in the samples was about 1 µg/mL for each Test. All the values are summarized below:

Experimental Phase		Concentration of IR5885 (µg/mL)		
		pH 4	pH 7	pH 9
Preliminary Test		1.023	1.019	0.982
Test No.1			0.995	0.998
Test No.3	25°C			1.042
	55°C		1.060	
	65°C		1.051	

TLC analysis of the Stock Solution showed that the radiochemical purity of the test substance was always > 97% while HPLC analysis showed that the diastereoisomeric ratio (S,R to S,S) was about 1.
All the values are summarized below::

Experimental Phase		Analytical system		Radiochemical purity of IR5885 (%)	Diastereoisomeric characteristics of IR5885		
					S,R	S,S	ratio[a]
Preliminary Test		TLC	solvent system 1	98.21			
			solvent system 2	97.80			
			solvent system 3	97.99			
		HPLC			47.55	50.60	0.94

Test No.1		TLC	solvent system 1	98.42			
			solvent system 2	97.75			
			solvent system 3	97.79			
		HPLC			47.82	50.27	0.95
Test No.3		TLC	solvent system 1	98.30			
			solvent system 2	97.40			
			solvent system 3	97.66			
		HPLC			46.04	51.28	0.90

a = (S,R) / (S,S) ratio

^{14}C-Mass Balance

The recovered radioactivity ranged from 95.44% to 105.37% of the applied radioactivity (AR).

Degradation rate of IR5885

The results of the Preliminary Test (50°C) showed that the degradation rate of the test substance varied depending on the pH. At pH 4 no degradation was found. After 5 days of incubation the amount of test substance accounted for 97.99% AR while the trigger value to perform further work is <90%.
On the other hand, at pH 7 and 9 IR5885 hydrolyzed very quickly. After 5 days the amount of the test substance was 64.18% AR and 0.31% AR at pH 7 and 9, respectively.
The degradation rate of IR5885 at pH 7 and 9 was confirmed in Test No.1 at 50°C (Tables 4.1.4.1-01and 4.1.4.1-02). In the Test No.3 at 55°C and 65°C for pH 7 (Table 4.1.4.1-03) and at 25°C for pH 9 (Table 4.1.4.1-04) the degradation rate progressively increased.
On the basis of the data obtained from the Tests No. 1 and 3, a regression analysis was carried out by plotting the logaritm of the remaining IR5885 (as percentage) *versus* time, according to Slide Write plus software (Advanced Graphics Software Inc). The decline of the test substance followed a pseudo first order kinetic.

The DT_{50} values (Disappearance Time for 50% of the starting concentration) were as follows:
- pH 7 7.62 days (50°C, Test No. 1), 5.21 days (55°C, Test No. 3), 2.09 days (65°C, Test No. 3)
- pH 9 0.33 days (50°C, Test No.1), 4.15 days (25°C, Test No. 3).

The DT_{90} values (Disappearance Time for 90% of the starting concentration) were as follows:
- pH 7 25.34 days (50°C, Test No.1), 17.32 days (55°C, Test No.3), 6.95 days (65°C, Test No.3)
- pH 9 1.10 days (50°C, Test No.1), 13.79 days (25°C, Test No. 3).

The DT_{50} and DT_{90} values at 25°C and pH 7 were obtained by the Arrhenius plot and resulted 90.94 and 302.23 days, respectively.

Table 4.1.3.1-02 Test No.1. Radioactivity distribution at pH 7.
Solvent system 1: CH_3CN/H_2O (85/15) 1 run (data are expressed as percentage of the applied radioactivity)

at 50°C

sampling time (days)	compound				
	H1 (IR5885)	H2	H3 (IR5885-acid)	H4	H5
0	98.34	1.30	0.36	n.d.	n.d.
2	86.67	1.52	11.81	n.d.	n.d.
3	77.31	1.05	20.83	0.38	0.43
4	69.54	0.85	28.76	0.38	0.47
5	62.38	0.75	36.18	0.40	0.30
7	55.58	0.81	42.67	0.59	0.35
8	55.34	0.53	43.20	0.53	0.39
9	48.11	0.62	50.12	0.63	0.52
10	41.36	0.55	57.02	0.69	0.37
11	32.51	0.36	65.67	1.04	0.42

n.d. = not detected

Table 4.1.3.1-03 Test No.1. Radioactivity distribution at pH 9.
Solvent system 1: CH_3CN/H_2O (85/15) 1 run (data are expressed as percentage of the applied radioactivity)

at 50°C

sampling time (hours)	compound				
	H1 (IR5885)	H2	H3 (IR5885-acid)	H4	H5
0	98.33	1.24	0.43	n.d.	n.d.
2	84.95	1.23	13.49	0.33	n.d.
3	80.05	0.99	18.58	0.37	n.d.
4	73.57	1.00	24.96	0.48	n.d.
5	68.23	0.86	30.21	0.47	0.22
6	61.55	0.92	36.61	0.55	0.38
7	59.27	0.92	38.93	0.57	0.31
8	51.74	0.76	46.36	0.79	0.35
9	45.49	0.69	52.74	0.79	0.30
10	40.39	0.65	57.45	1.02	0.49

n.d. = not detected

Table 4.1.3.1-04 Test No.3. Radioactivity distribution at pH 7.
Solvent system 1: CH_3CN/H_2O (85/15) 1 run (data are expressed as percentage of the applied radioactivity)

at 55°C

sampling time (days)	compound				
	H1 (IR5885)	H2	H3 (IR5885-acid)	H4	H5
0	98.50	1.22	0.28	n.d.	n.d.
3	62.46	0.88	35.77	0.49	0.40
5	49.77	0.55	48.79	0.59	0.29
7	36.55	0.34	62.08	0.77	0.26
10	26.24	n.d.	72.48	0.96	0.32

n.d. = not detected

at 65°C

sampling time (hours)	compound				
	H1 (IR5885)	H2	H3 (IR5885-acid)	H4	H5
0	97.51	1.76	0.73	n.d.	n.d.
31	59.42	1.02	38.58	0.57	0.41
55	48.48	0.83	49.61	0.67	0.41
79	35.58	0.49	62.54	0.90	0.48
103	22.98	0.47	75.46	0.80	0.29

n.d. = not detected

Table 4.1.3.1-05 Test No.3. Radioactivity distribution at pH 9.
Solvent system 1: CH_3CN/H_2O (85/15) 1 run (data are expressed as percentage of the applied radioactivity)

at 25°C

sampling time (days)	compound				
	H1 (IR5885)	H2	H3 (IR5885-acid)	H4	H5
0	97.86	1.66	0.48	n.d.	n.d.
2	69.60	0.84	28.76	0.48	0.32
3	54.23	0.84	43.97	0.62	0.34
4	46.53	0.44	51.90	0.76	0.37
7	30.47	0.39	67.77	1.05	0.32

n.d. = not detected

Appearance rate of the hydrolysis compounds at different pH values

The rate of hydrolysis varied depending on pH values: at pH 4 no hydrolysis occurred while at pH 7 and 9 a main degradation compound reaching amounts higher than 10% AR was found and identified.

pH 4: The compound H1 (unchanged IR5885) was substantially stable while none of the three trace compounds H2, H3, and H4 ever reached amount higher than 2%.

pH 7: The main hydrolysis compound was H3 which reached the maximum amounts of 65.67%, 72.48%, and 75.46% after 11 days (50°C), 10 days (55°C), and 103 hours (65°C), respectively.
Trace compounds (H2, H4, and H5) were observed but their amount never reached 2%.

pH 9: The main hydrolysis compound was H3, that increased quickly to reach the maximum amounts of 57.45%, and 67.77% after 10 hours (50°C), and after 7 days (25°C), respectively.
Trace compounds (H2, H4, and H5) were observed but their amount never reached 2%.

Comparison of samples obtained at different pH values

Representative samples incubated at different pH values were analyzed by co-TLC. The results showed that the hydrolysis products found at the different experimental conditions were the same.

HPLC analysis of diasteroisomeric composition

All over the incubation time both compound H1 (unchanged IR5885) and the main degradation compound H3 (IR5885-acid) showed a constant diastereoisomeric ratio (S,R/S,S), amounting approximately to 1.

Identification of IR5885 and its hydrolysis compound

The identification of compound H1 (unchanged IR5885) was performed by co-TLC with reference standard and by LC-MS. The identification of compound H3 was performed by LC-MS.
The MS spectra of compound H1 showed the same molecular fragmentation of IR5885 reference standard
On the basis of co-TLC and MS analyses, the compound H1 was identified as IR5885.
The MS spectra of compound H3 showed the same molecular fragmentation of IR5885-acid reference standard.
On the basis of MS analyses, the compound H3 was identified as RS-β-alanine, N-[(1-methylethoxy)carbonyl]-L-valyl-3-(4-chlorophenyl) acid (IR5885-acid).

CONCLUSIONS

Study results showed that no significant degradation of ^{14}C-IR5885 occurred in buffered solution at pH 4, while at pH 7 and 9 a pseudo-first order kinetic hydrolysis reaction was observed.
The values of k_{obs}, DT_{50} and DT_{90} were determined for pH 7 and 9 at different temperatures:

pH	T (°C)	k_{obs} (days^{-1})	DT_{50} (days)	DT_{90} (days)
7	65	0.331	2.09	6.95
	55	0.133	5.21	17.32
	50	0.0909	7.62	25.34
	25 *	0.00762 *	90.94 *	302.23 *
9	50	2.1	0.33	1.1
	25	0.167	4.15	13.79

At pH 7 and 9 two main compounds were found in samples: H1 (unchanged ^{14}C-IR5885) and H3 which reached levels higher than 10% and was identified as ^{14}C-IR5885-acid. In all experimental conditions trace compounds (H2, H4, and H5) were observed but their amount never reached 2%.
The ^{14}C-mass balance ranged from 95.44% and 105.37% of the applied radioactivity.
HPLC analysis showed that all over the incubation time for both the compound H1 (unchanged IR5885) and the main degradation compound H3 (IR5885-acid) the diastereoisomeric ratio (S,R/S,S) was about 1.

4.1.4 Aquatic simulation tests

4.1.4.1 Water / sediment study – IR5885

Reference Number: **KII- Section 5, Volume 2, Annex IIA, point 7.8.3/01**

Report:	See annex conf. 38

Guidelines: OECD Guideline 308. "Aerobic and Anaerobic Transformation in Aquatic Sediment Systems" - 24th April 2002.
(SETAC-Europe), Part 1 - Section 8.2 "Aerobic aquatic degradation" - March, 1995.

GLP: Yes; Certified Laboratory

Executive Summary:

The degradation of ^{14}C-IR5885 has been studied in two aquatic systems, named Pond system and River system. The study was conducted in compliance with SETAC and OECD guidelines.
Samples of each aquatic system were dispensed into glass cylinders to obtain incubation units containing a 2.5 cm soil layer flooded with associated water to a depth of 10 cm. The incubation units were gently agitated on an orbital shaker. Moistened carbon dioxide-free air was drawn over the water surface and the units were maintained in the dark at 20±2°C for 32 days to allow the attainment of the equilibrium.
Following the acclimation period, 14C-IR5885 was applied to each unit at the maximum recommended field application (240 g a.i./ha). Each unit was connected to a glass drechsel containing KOH solution to trap evolved carbon dioxide.
Duplicate incubation units were collected and analyzed 0, 1, 2, 4, 6, 8, 14, and 22 days after the application for both systems.

The surface water and the corresponding sediment were analyzed separately. The surface water was separated from the soil by pipette and the radioactivity content determined by Liquid Scintillation Counting (LSC). Suitable aliquots of water were concentrated and analyzed by Thin Layer Chromatography (TLC) and, for representative samples, also by High Performance Liquid Chromatography (HPLC).

Sediments were extracted with different solvent mixtures and the extractable radioactivity was determined by LSC. Suitable aliquots of soil extracts were combined, concentrated, and analyzed by TLC and, for representative samples, also by HPLC. The radioactivity content in KOH solution was determined by LSC. The non-extractable radioactivity was determined by LSC after oxidation by means of biological oxidizer.

The radioactivity in the surface water decreased during all the study and it was 40.84% and 43.74% of applied radioactivity (AR) at the end of incubation period in Pond and River systems, respectively.
The radioactivity in the sediment increased during all the study reaching 50.64% AR and 45.51% AR at the end of incubation period in Pond and River systems, respectively.

IR5885 degraded in both aquatic systems: after 22 days it accounted for 5.92% AR and 5.51% AR in Pond and River systems, respectively. The $DT_{50\ lab}$ and $DT_{90\ lab}$ values, in days, are listed in table 4.1.4.1-01:

Table 4.1.4.1-01 Summary of the results

Systems	Surface water		Whole system	
	$DT_{50\ lab}$	$DT_{90\ lab}$	$DT_{50\ lab}$	$DT_{90\ lab}$
Pond	4.87	16.19	5.30	17.61

River	5.04	16.75	5.19	17.24

Six compounds were found in the surface water and in the sediment extracts.
The main degradation compounds were S2 and S3: S2 reached 52.80% AR and 56.34% AR in Pond and River systems, respectively.
It was identified as 3-(4-chlorophenyl)-3-({(2S)-2-[(isopropoxycarbonyl) amino]-3-methylbutanoyl}amino) propanoic acid (named IR5885 acid).
The compound S3, that increased till to 13.77% AR and 8.16% AR (in Pond and River systems, respectively), was identified as 4-chlorobenzoic acid (named PCBA).
The fraction S6 slowly increased reaching 8.93% AR and 8.04% AR. It was represented by a pool of 4 compounds and none of these reached values higher than 3.13% AR.
None of the other compounds, S4 and S5, ever reached levels higher than 5% AR.

The non-extractable radioactivity (bound residue) increased till to 8.99% and 16.24% AR in Pond and River systems, respectively. The radioactivity in the ^{14}C-CO_2 traps was always lower than the detection limit in both the systems except at the last two-three sampling times when it reached values ranging between 0.77% AR and 1.24% AR. The ^{14}C-Mass Balance was always higher than 90% AR and ranged from 90.61% to 104.12% AR for Pond system and from 90.49% to 107.96% AR for River system.

MATERIALS AND METHODS

MATERIALS

Unlabelled Test Material	IR5885
Batch	20071/77
Chemical purity:	> 99%
Labelled Test Material	
[^{14}C-U-phenyl] IR5885	Lot # 199
Specific activity:	4.435 MBq/mg
	119.858 µCi/mg
	266,084 dpm/µg
Radiochemical purity (by TLC and HPLC):	> 98%

Aquatic systems

Collection and storage: Two unchallenged sediment-water systems were collected in the territory of the "Natural Park of Ticino Valley" (near Novara, Piemonte - North-Western Italy). The sediments differed in the following parameters:

- grain size (particle size distribution)
- microbial biomass
- organic carbon content

Both sediments and the accompanying water were carefully collected from natural sites not treated with more common chemicals. Water temperature, oxygen content, pH, and redox potential were measured at the site immediately prior to the collection.
Prior to the start of the study, the water was passed through a 0.2 mm filter and the sediment was passed through a 2 mm sieve.
The water-sediment systems were stored at 4°C before starting the incubation.

Characterization: Sediments and associated waters were characterized both at the origin (before starting) and at the end of the study for the parameters reported in Tables 4.1.4.1-02 and 4.1.4.1-03. Due to the fast degradation rate and the consequent short duration of the study, the microbial biomass was determined on soil sediment only at the starting of the study.
The microbial biomass was determined according to Vance et al..

Table 4.1.4.1-02 Characteristics of the Pond system.

Sediment			
		start	end
Particle size distribution	50-2000 µm (sand)	32.25	-
	2-50 µm (silt)	40.75	-
	< 2 µm (clay)	27.00	-
USDA classification		clay loam	-
pH (in water 1:2.5)		6.07	6.26
pH (in 0.01 M $CaCl_2$)		5.46	5.32
Organic Carbon (%)		6.12	5.98
Cation Exchange Capacity (meq/100 g)		29.20	-
Water content at pF = 2.5 = 0.33 bar (g/100 g)		35.11	-
Disturbed bulk density (g/cm^3)		1.14	-
Dry mass (%)		72.13	-
Microbial biomass C (mg C/100 g dry weight)		107.68	-
Microbial biomass C (% referred to total organic carbon)		1.76	-
Surface water			
		start	end
pH		5.88	6.86
Organic carbon (µg/L)		490	25100
Total nitrogen (µg/L)		2870	4330
Total phosphorus (µg/L)		23.4	121
Water hardness (µg/L)		110000	146000

Table 4.1.4.1-03 Characteristics of the River system.

Sediment			
		start	end
Particle size distribution	50-2000 µm (sand)	96.00	-
	2-50 µm (silt)	2.50	-
	< 2 µm (clay)	1.50	-
USDA classification		sand	-
pH (in water 1:2.5)		6.65	7.24
pH (in 0.01 M $CaCl_2$)		6.12	6.50
Organic Carbon (%)		1.01	0.83
Cation Exchange Capacity (meq/100 g)		1.70	-
Water content at pF = 2.5 = 0.33 bar (g/100 g)		5.38	-
Disturbed bulk density (g/cm^3)		1.61	-
Dry mass (%)		78.50	-
Microbial biomass C (mg/100 g)		80.93	-
Microbial biomass C (% referred to total organic carbon)		8.01	-
Surface water			
		start	end
pH		6.97	7.36
Organic carbon (µg/L)		882	2040
Total nitrogen (µg/L)		2860	1560
Total phosphorus (µg/L)		30.0	348
Water hardness (µg/L)		134000	149000

TEST DESIGN AND METHODS

Preparation of water-sediment samples

Sediments were added to individual glass cylinders (4.50 cm internal diameter; 15.90 cm^2 area) to a depth of 2.5 cm (ca. 50 g dry weight of both systems). Associated water was added to each bottle to a depth of 10 cm above the sediment. The level was maintained throughout the study by additions of purified water as necessary.
The oxygen content in water was assured by passing pre-moistened carbon dioxide-free air over the surface. Air was not passed directly into the water.

Each unit was connected to a glass drechsel containing 100 mL of 2N KOH solution to trap evolved carbon dioxide.
The water-sediment systems (hereafter named as incubation units) were slightly agitated on an orbital shaker.

The following series of incubation units were prepared for each system:

- 18 samples to be treated with ^{14}C-IR5885
- 4 samples to be used as "additional samples"
- 2 samples as "control" to monitor pH, redox potential, and oxygen content
- 1 sample to be used as "blank"

Prior to application of the Test Item, the incubation units were kept for 32 days in the dark at 20±2°C to allow the complete separation of phases and the attainment of the equilibrium (pH and redox potential in water and soil, and oxygen content in water). Redox potential, pH, and oxygen content values were determined on the control samples during all the incubation period and on the samples taken at each sampling interval after Test Substance application.

Preparation of soil samples for biomass

Eight sediment samples of 50 g dry weight were placed into 250 mL glass cylinders and associated water (depth layer of 10 cm) was added to each sample. The level was maintained throughout the study by addition of purified water as necessary. The glass cylinders were sealed with cotton wool stopper. The samples were incubated in the dark at 20±2°C and slightly agitated on an orbital shaker.

Treatment rate

This study was carried out at the rate of 240 g/ha. This rate was converted to an applied dose by assuming that water surface is equivalent to a soil surface and that there is a homogeneous distribution of Test Item down to the depth of 30 cm according to the following calculation:

application rate = 240 g/ha is equivalent to 2.40 µg/cm^2
surface area = 15.90 cm^2
total µg = 2.40 µg/cm^2 · 15.90 cm^2 = 38.16 µg/sample
water column depth = 10 cm
adjust quantity in relation to a depth of 30 cm:

38.16 µg · 10 cm : 30 cm = 12.72 µg/sample

Preparation of radiolabelled test solutions

Three Test Solutions were prepared: No.1 just before the treatment of the samples incubated for 0, 1, 2, 6, and 14 days; No.2 for 4 and 8 days, and No.3 for 22 days.
Aliquots of ^{14}C-IR5885 (Lot#199) were dissolved in a 9:1 water-acetonitrile mixture to give approximately a 13 µg/mL solution corresponding to 0.058 MBq/mL (1.558 µCi/mL).

Analysis of the test solutions

LSC analysis: Three 0.5 mL aliquots of the Test Solutions were diluted to 10 mL with acetonitrile, and four 100 µL aliquots of these diluted solutions were checked by LSC to determine the actual concentration of the Test Item.
Radiochemical purity: The radiochemical purity of the Test Item was determined both by TLC and HPLC.

Application of test solutions

0.95 mL aliquots of the IR5885 Test Solutions were dropwise applied to each incubation unit.
The final concentration of acetonitrile was <0.01% of the water quantity (about 115-120 mL for each unit).

Sampling times

Sampling was carried out at 0 (immediately after dose application), 1, 2, 4, 6, 8, 14, and 22 days after treatment for both the systems.

Additional samples

To assist to the identification of the degradation compounds, eight samples (four for each system) were set up as above described but treated with a solution containing a higher concentration of IR5885 (7.90 µg of ^{14}C-IR5885 + 316 µg of unlabelled IR5885 for each sample).

Analytical procedures

At chosen time intervals (T0 included) duplicate incubation units for each system were taken for analysis. Water and sediment were analyzed separately.

Surface water analysis: The surface water (approx. 115-120 mL) was removed by pipette, evaporated to about 100 mL, collected in a volumetric flask, acidified with a suitable aliquot of 4N HCl solution and made up to the mark (100 mL) with CH_3CN.
The radioactivity present in the water was quantified by LSC.
All the water samples were stored at 1-7°C.

The blanks were subjected to the same procedure.

Sediment analysis: Each sediment was extracted by shaking (1 hour at 300 strokes/min) with 250 mL of the following solvent mixtures:

- acetone – 0.1 N HCl 5 - 5 by volume **extract A**
- acetone – 0.1 N HCl 2 - 8 by volume **extract B**

After each extraction the sediment residue was separated by centrifugation, the clear liquid collected in a volumetric flask and made up to the mark (250 mL) with acetonitrile.
The Extracts A and B were analyzed by LSC.
All the extracts were stored at 1-7°C.

The blank was subjected to the same procedure.

Bound residue: The radioactivity remaining in sediment after extraction was considered as bound residue. After extraction the sediment was allowed to dry at room temperature and then three aliquots were oxidized (3 minutes) by means of a biological oxidizer.

Determination of evolved ^{14}C-CO_2: At each sampling time, except at 0 day, KOH solution contained in the drechsel was transferred into volumetric flask, made up to the mark (100 mL) and the radioactivity content quantified by LSC.

^{14}C-mass balance: ^{14}C-Mass Balance was determined by summing the radioactivity contained in the surface water, in sediment extracts A and B, in bound residue, and in KOH traps for ^{14}C-CO_2.

RESULTS AND DISCUSSION

Concentration and radiochemical purity of test solutions

The results of LSC analysis showed that the concentration of ^{14}C-IR5885 in the Test Solutions was 13.61 and 13.62 µg/mL corresponding to 0.06 MBq/mL. Samples were treated with 0.95 mL of Test Solutions. These amounts corresponded to 12.93 and 12.94 µg/sample, and to a dose of 244 g a.i./ha.
The radiochemical purity, determined by TLC and HPLC, resulted higher than 98%.

Distribution of applied radioactivity

Radioactivity in the surface water

***Pond system*.** The amount of applied radioactivity in the surface water decreased progressively from 101.71% AR at 0 day to 40.84% AR at 22 days (Table 4.1.4.1-04).

***River system*.** The amount of applied radioactivity in the surface water decreased progressively from 106.45% AR at 0 day to 43.74% AR at 22 days (Table 4.1.4.1-05).

Table 4.1.4.1-04 Pond system. ^{14}C-Mass Balance
(data are expressed as percentage of applied radioactivity)

Time point (days)	Surface water	Sediment			CO_2	Total
		extracts A+B	bound residue	total		
0	101.71	2.11	<dl	2.11	*na*	103.82
1	95.73	8.39	<dl	8.39	<dl	104.12
2	91.53	11.61	<dl	11.61	<dl	103.14
4	80.61	16.99	0.85	17.84	<dl	98.45
6	83.30	15.44	1.19	16.63	<dl	99.93
8	75.47	19.47	2.53	22.00	<dl	97.47
14	66.21	20.18	3.17	23.35	1.05	90.61
22	40.84	41.65	8.99	50.64	1.04	92.52

na = not analyzed dl = detection limit = 0.55% (bound residue), 0.13% (CO_2)

Table 4.1.4.1-05 River system. ^{14}C-Mass Balance
(data are expressed as percentage of applied radioactivity)

Time point (days)	Surface water	Sediment			CO_2	Total
		extracts A+B	bound residue	total		
0	106.45	1.51	<dl	1.51	na	107.96
1	96.03	9.16	0.28	9.44	<dl	105.47
2	91.03	11.09	0.69	11.78	<dl	102.81
4	84.16	14.42	0.86	15.28	<dl	99.44
6	86.45	14.72	1.49	16.21	<dl	102.66
8	78.68	15.01	2.79	17.80	0.17	96.65
14	70.90	15.57	6.15	21.72	0.51	93.13
22	43.74	29.27	16.24	45.51	1.24	90.49

na = not analyzed dl = detection limit = 0.41% (bound residue), 0.10% (CO_2)

Radioactivity in the sediments

***Pond system*.** The radioactivity in the sediment increased from 2.11% AR at 0 day to 50.64% AR at 22 days (Table 4.1.4.1-04).
This radioactivity was constituted by extractable (sediment extracts) and not-extractable (bound residue) radioactivity. The extractable radioactivity increased from 2.11% AR at 0 day to 41.65% AR at 22 days. The non-extractable radioactivity increased during all the study to 8.99% AR.

***River system*.** The radioactivity in the sediment increased from 1.51% AR at 0 day to 45.51% AR at 22 days (Table 4.1.4.1-05).
This radioactivity was constituted by extractable (sediment extracts) and not-extractable (bound residue) radioactivity. The extractable radioactivity increased from 1.51% AR at 0 day to 29.27% AR at 22 days. The non-extractable radioactivity increased during all the study to 16.24% AR.

Volatile radioactivity

***Pond system*.** The volatile radioactivity, evolved as $^{14}C\text{-}CO_2$ and found in the KOH traps, was always lower than detection limit except at the last two sampling time (14 and 22 days) when it amounted to 1.05% AR and 1.04% AR (Table 4.1.4.1-04).

***River system*.** The volatile radioactivity, evolved as $^{14}C\text{-}CO_2$ and found in the KOH traps, was always lower than detection limit except at the last three sampling times (8, 14, and 22 days) when it amounted to 0.17%, 0.51%, and 1.24% AR (Table 4.1.4.1-05).

^{14}C-mass balance

The ^{14}C-Mass Balance was determined by summing the radioactivity contained in the surface water, in the sediment extracts A and B, in the bound residue, and in the KOH solutions. Recovered radioactivity, at each sampling time and for both systems, was always higher than 90% AR and ranged from 90.61% to 104.12% AR for Pond system and from 90.49% to 107.96% AR for River system (Tables 4.1.4.1-04 and 4.1.4.1-05).

Profiling of the compounds

The chromatographic pattern of the surface water and of the sediment extracts showed a progressive degradation of IR5885 and the formation of several degradation compounds (Tables 4.1.4.1-06 to10). The main of these, were S2 and S3 and were identified.
Other six minor compounds were found: S4, S5, and a pool of 4 substances constituting fraction S6.

***Surface water*:** Six substances were found: unchanged IR5885 (S1) and five degradation compounds.

***Pond system*.** The TLC analyses showed that 14C-IR5885 decreased from 97.39% AR at 0 day to 4.57% AR at 22 days.
The main degradation compound, S2, increased from 1.39% AR at 0 day to 35.32% AR at 8 days, then it decreased to 26.77% AR at 22 days.
The compound S3 increased from 1.98% AR at 0 day to 12.18% AR at 14 days, then it decreased to 3.17% AR at 22 days.
Fraction S6 (maximum amount of 5.60% at 8 days) was represented by a pool of 4 substances.
None of the minor compounds, S4 and S5, ever reached amounts higher than 4% AR (Table 4.1.4.1-06).

***River system*.** The TLC analyses showed that ^{14}C-IR5885 decreased from 101.04% AR at 0 day to 4.94% AR at 22 days.
The main degradation compound, S2, increased from 2.26% AR at 0 day to 44.03% AR at 14 days, then it decreased to 28.85% AR at 22 days.
The compound S3 increased from 2.14% AR at 0 day to 7.25% AR at 14 days, then it decreased to 2.34% AR at 22 days.
Fraction S6 (maximum amount of 4.95% AR at 22 days) was represented by a pool of 4 substances.
None of the minor compounds, S4 and S5, ever reached amounts higher than 3% AR (Table 4.1.4.1-09).

Sediment extracts
Six substances were found: unchanged IR5885 (S1) and five degradation compounds.

Pond system. ^{14}C-IR5885 kept always lower than 2% AR for all the study duration.
The main degradation compound, S2, increased from 5.39% AR at 1 day to 26.03% AR at 22 days. The compound S3 increased to 5.04% AR at 22 days .
Fraction S6 (maximum amount of 5.54% AR at 22 days) was represented by a pool of 4 substances .
None of the minor compounds, S4 and S5, ever reached levels higher than 2% AR (Table 4.1.4.1-07).

River system. ^{14}C-IR5885 kept always lower than 2% AR for all the study duration.
The main degradation compound, S2, increased from 8.21% AR at 1 day to 22.26% AR at 22 days. The compound S3 reached the maximum amount of 1.90% AR at 22 days.
Fraction S6 (maximum amount of 3.09% AR at 22 days) was represented by a pool of 4 substances .
The minor compound S4 never reached levels higher than 2% AR. S5 was always under detection limit (Table 4.1.4.1-10).

Whole system

Pond system. ^{14}C-IR5885 decreased from 97.39% AR at 0 day to 5.92% AR at 22 days.
The main degradation compound, S2, increased from 1.39% AR at 0 day to 52.80% AR at 22 days. The compound S3 increased from 1.98% AR at 0 day to 13.77% AR at 14 days, then it decreased to 8.22% AR at 22 days.
Fraction S6 reached a maximum amount of 8.93% AR at 22 days and was represented by a pool of 4 substances.
None of the minor compounds, S4 and S5, ever reached levels higher than 5% AR (Table 4.1.4.1-08).

River system. ^{14}C-IR5885 decreased from 101.04% AR at 0 day to 5.51% AR at 22 days.
The main degradation compound, S2, increased from 2.26% AR at 0 day to 56.34% AR at 14 days, then it decreased to 51.11% AR at 22 days.
The compound S3 increased from 2.14% AR at 0 day to 8.16% AR at 14 days, then it decreased to 4.25% AR at 22 days.
Fraction S6 reached a maximum amount of 8.04% AR at 22 days) and it was represented by a pool of 4 substances .
None of the minor compounds, S4 and S5, ever reached levels higher than 4% AR (Table 4.1.4.1-11).

Table 4.1.3.1-06 Pond system. Distribution of ^{14}C-IR5885 and degradation compounds in *surface water*
(data are expressed as percentage of IR5885 equivalents referred to applied radioactivity)

Time point (days)	Compounds					
	S1 (IR5885)	S2	S3	S4	S5	S6
0	97.39	1.39	1.98	<dl	<dl	0.96
1	90.23	2.45	2.41	<dl	<dl	0.64
2	77.82	10.02	2.46	0.65	<dl	0.58
4	49.48	25.78	2.60	1.11	<dl	1.64
6	46.61	26.56	3.19	3.88	<dl	3.06
8	26.43	35.32	2.96	2.87	2.29	5.60
14	12.59	31.92	12.18	1.35	2.79	5.38

dl = detection limit = 0.46%

Table 4.1.3.1-07 Pond system. Distribution of ^{14}C-IR5885 and degradation compounds in *sediment*.
(data are expressed as percentage of IR5885 equivalents referred to applied radioactivity)

Time point (days)	Compounds					
	S1 (IR5885)	S2	S3	S4	S5	S6
0	na	na	na	na	na	na
1	1.79	5.39	0.32	0.32	<dl	0.57
2	0.70	9.93	0.35	0.21	<dl	0.43
4	0.95	14.27	0.49	0.33	<dl	0.95
6	1.32	11.87	0.46	0.60	<dl	1.19
8	0.64	16.22	0.46	0.50	0.45	1.20
14	1.76	12.86	1.59	1.04	0.83	2.10
22	1.34	26.03	5.04	1.75	1.95	5.54

dl = detection limit = 0.12%
na = not analysed

Table 4.1.3.1-08 Pond system. Distribution of ^{14}C-IR5885 and degradation compounds in *whole system*.
(data are expressed as percentage of IR5885 equivalents referred to applied radioactivity)

time point (days)	Compounds					
	S1 (IR5885)	S2	S3	S4	S5	S6
0	97.39	1.39	1.98	<dl	<dl	0.96
1	92.03	7.83	2.73	0.32	<dl	1.21
2	78.52	19.95	2.82	0.86	<dl	1.01
4	50.44	40.05	3.09	1.44	<dl	2.59
6	47.93	38.43	3.65	4.49	<dl	4.26
8	27.07	51.55	3.42	3.38	2.74	6.80
14	14.35	44.78	13.77	2.40	3.62	7.48
22	5.92	52.80	8.22	3.20	3.46	8.93

dl = detection limit

Table 4.1.3.1-09 River system. Distribution of ^{14}C-IR5885 and degradation compounds in *surface water*.
(data are expressed as percentage of IR5885 equivalents referred to applied radioactivity)

time point (days)	Compounds					
	S1(IR5885)	S2	S3	S4	S5	S6
0	101.04	2.26	2.14	<dl	<dl	1.00
1	88.75	3.49	2.82	<dl	<dl	0.98
2	78.13	8.85	2.75	0.73	<dl	0.57
4	53.19	24.63	2.91	1.81	<dl	1.62
6	47.17	30.78	2.97	2.29	1.40	1.85
8	31.63	38.80	3.29	2.78	<dl	2.18
14	14.53	44.03	7.25	1.89	1.09	2.10
22	4.94	28.85	2.34	1.14	1.53	4.95

dl = detection limit = 0.46%

Table 4.1.3.1-10 River system. Distribution of ^{14}C-IR5885 and its degradation compounds in *sediment*. (data are expressed as percentage of IR5885 equivalents referred to applied radioactivity)

time point (days)	Compounds					
	S1 (IR5885)	S2	S3	S4	S5	S6
0	na	na	na	na	na	na
1	0.12	8.21	0.19	0.14	<dl	0.49
2	<dl	9.46	0.31	0.29	<dl	1.03
4	1.15	11.09	0.39	0.38	<dl	1.42
6	0.35	11.90	0.39	0.52	<dl	1.55
8	<dl	12.70	0.24	0.45	<dl	1.62
14	<dl	12.30	0.91	0.54	<dl	1.83
22	0.57	22.26	1.90	1.46	<dl	3.09

dl = detection limit = 0.12%
na = not analyzed

Table 4.1.3.1-11 River system. Distribution of ^{14}C-IR5885 and degradation compounds in *whole system*. (data are expressed as percentage of IR5885 equivalents referred to applied radioactivity)

time point (days)	Compounds					
	S1(IR5885)	S2	S3	S4	S5	S6
0	101.04	2.26	2.14	<dl	<dl	1.00
1	88.87	11.70	3.02	0.14	<dl	1.47
2	78.13	18.31	3.06	1.03	<dl	1.60
4	54.34	35.72	3.30	2.19	<dl	3.04
6	47.52	42.68	3.37	2.81	1.40	3.41
8	31.63	51.51	3.53	3.23	<dl	3.79
14	14.53	56.34	8.16	2.43	1.09	3.93
22	5.51	51.11	4.25	2.60	1.54	8.04

dl = detection limit

Degradation rate of ^{14}C-IR5885

Surface Water

The disappearance of ^{14}C-IR5885 from surface water followed a first order kinetic. Plotting the logarithms of concentrations (as percentage) versus time, a straight line was obtained.
The $DT_{50\ lab}$ and $DT_{90\ lab}$ values (Disappearance Time for 50% and 90% of the starting concentration) and the determination coefficients r^2 were calculated by regression analysis according to Slide Write Plus software (Advanced Graphics Software Inc).

Whole system

The disappearance of ^{14}C IR5885 from the whole system followed a first order kinetic.
The $DT_{50\ lab}$ and $DT_{90\ lab}$ values and the determination coefficients r^2 were calculated by regression analysis (Table 4.1.4.1-12) according to Slide Write Plus software (Advanced Graphics Software Inc).

Table 4.1.3.1-12 Disappearance of ^{4}C IR5885

Systems	Surface water			Whole system		
	$DT_{50\ lab}$	$DT_{90\ lab}$	r^2	$DT_{50\ lab}$	$DT_{90\ lab}$	r^2
Pond	4.87	16.19	0.9915	5.30	17.61	0.9861
River	5.04	16.75	0.9978	5.19	17.24	0.9970

Comparison of degradation compounds

The comparison of surface water and sediment extracts, carried out by co-TLC, shows that the compounds found in the two systems and in the two phases are the same.
On the basis of these results only the water surface of additional samples can be used for the identification.

Identification of IR5885 and of main degradation compounds

Unchanged IR5885 (compound S1)
The compound S1 and the reference ^{14}C-IR5885 standard had the same chromatographic properties. On the basis of co-TLC and LC-MS analyses, the compound S1 was identified as unchanged IR5885.

Compound S2
The compound S2 and the ^{14}C-IR5885 acid reference standard had the same chromatographic properties.
On the basis of co-TLC and LC-MS analyses, the compound S2 was identified as IR5885 acid.

Compound S3
The compound S3 and the authentic ^{14}C-PCBA (4-chlorobenzoic acid) reference standard had the same chromatographic properties.
On the basis of co-TLC and LC-MS analyses, the compound S3 was identified as PCBA: 4-chlorobenzoic acid.

Diastereoisomeric composition
Diastereoisomeric ratio (S,R/S,S) of IR5885 (S1) and of IR5885 acid (S2) did not change in all the samples.

CONCLUSIONS

IR5885 progressively degraded in both the systems; its decline was represented by a first order kinetic.
The $DT_{50\ lab}$ (in days) were 4.87 and 5.04 in surface water of Pond and River systems, respectively, and 5.30 and 5.19 in whole system.
The $DT_{90\ lab}$ (in days) were 16.19 and 16.75 in surface water of Pond and River systems, respectively, and 17.61 and 17.24 in whole system.

The non-extractable radioactivity (bound residue) gradually increased during all the incubation period increasing to 8.99% and 16.07% AR in Pond and River systems, respectively.

Traces of ^{14}C-CO_2 were only found at the last two-three sampling times.

The Mass Balance was always higher than 90%AR.

The radioactivity in the surface water decreased during all the study to 40.84% and 43.74% of applied radioactivity (AR) at the end of incubation in Pond and River systems, respectively.
The radioactivity in the sediment increased during all the study to 50.64% AR and 45.51% AR at the end of incubation in Pond and River system, respectively.

Six degradation compounds were found in the surface water and in the sediment extracts.
The main degradation compounds, S2 and S3, were identified as IR5885 acid and as 4-chlorobenzoic acid (PCBA).

None of the other 6 minor compounds ever reached levels higher than 5% AR.

DT_{50} and DT_{90} whole system values were recalculated by the RMS during review and are:
DT50 whole system: 4.5 days (pond) and 4.71 days (river)
DT90 whole system: 14.9 days (pond) and 15.64 days (river).

4.1.5 Other degradability studies

No other studies available. Photochemical degradation studies not performed as no absorption at wave lengths > 290 nm (IR5885 and metabolites)

4.1.6 Environmental fate and other relevant information

4.1.6.1 Adsorption and desorption - five soils – IR5885

Reference Number: KII- Section 5, Volume 1, Annex IIA, point 7.4.1/01

Report: See annex conf. 39

Guidelines: OECD Guideline 106

GLP: Yes; certified laboratory

Executive Summary:

The adsorption-desorption characteristics of [^{14}C-U-phenil]*IR5839* were determined on three different soils: loamy sand (AR-1), clay (Stirone) and loam (G-2).
This study, designed according to OECD Guideline 106, was carried out with three different tiers at the incubation temperature of 20°C.
As IR5839 in soil degrades very quickly, it was necessary to operate under sterile condition. The soils were sterilized by γ-ray irradiation while glassware, equipment and $CaCl_2$ solutions were sterilized by autoclaving at 121°C.
The adsorption kinetics were carried out at the nominal concentration of 5 µg/cm^3.
The **Tier 1** was carried out with AR-1 (low clay and high organic carbon content) and Stirone (high clay and low organic carbon content) soils.
The better soil/solution ratio was 1/10 and the equilibration time was within 8 hours for both soils.
The analyses of supernatants and soil extracts showed that the Test Item was stable during the study time.
The Mass Balance was 99.70% and 99.33% of the applied mass (AM) for AR-1 and Stirone soils, respectively.
The **Tier 2** data showed that the equilibration time also for Cal, G-2 and SP-2.1 soils was reached within 4 hours. The Mass Balance was 103.48, 102.04, 102.13, 101.07 and 103.00% of the applied mass (AM) for AR-1, Stirone, Cal, G-2 and SP-2.1 soils, respectively.
The Test Item was substantially stable during the study ranging from 87.46 to 94.52% AM.
The K_d and K_{oc} values at equilibrium are reported in the table below:

Table 4.1.6-01

Soil	K_d (cm^3/g)	K_{oc} (cm^3/g)
AR-1	54	375
Stirone	15	1686
Cal	9	472
G-2	9	400
SP-2.1	8	834

TheT**tier 3** (carried out at five nominal concentration of 5, 1.5, 0.5, 0.15, and 0.05 µg/cm^3) showed that adsorption and desorption data obtained fitted well with Freundlich equation. Its parameters are shown in the table Table 4.1.6-02.

Table 4.1.6-02 Tier 3 (carried out at five nominal concentration of 5, 1.5, 0.5, 0.15, and 0.05 µg/cm^3)

Soil	Adsorption isotherms			Desorption isotherms		
	1/n	K_F^{ads}	r^2	1/n	K_F^{des}	r^2
AR-1	0.958	73	0.998	0.998	135	0.998
Stirone	0.954	19	0.998	1.169	91	0.959
Cal	0.973	9	0.999	0.955	49	0.996
G-2	0.905	8	0.992	1.038	124	0.978
SP-2.1	1.017	7	0.999	1.031	87	0.986

The amount of adsorbed Test Item, referred to the total applied mass, was substantially constant for all concentrations (5, 1.5, 0.5, 0.15 and 0.05 µg/cm^3) in most of soils except in G-2 soil; it ranged from 87.16 to 89.80% AM in AR-1 soil, from 63.34 to 70.33% AM in Stirone soil, from 46.33 to 51.47% AM in Cal soil, from 38.84 to 43.19% AM in SP-2.1 soil, and from 40.26 to 56.21% AM in G-2 soil.
The amount of desorbed Test Item, referred to the adsorbed one was substantially constant in all the soils for all concentrations: it ranged from 6.42% to 7.58% AM in AR-1 soil, from 7.54 to 19.43% AM in Stirone soil, from 13.29% to 17.87% AM in Cal soil, from 5.97% to 11.05% AM in G-2 soil, and from 9.57% to 14.15% AM in SP-2.1 soil.

MATERIALS AND METHODS

MATERIALS

Labelled Test Material

[^{14}C-U-phenyl]-IR5885 Lot # 192
Specific Activity: 5.089 MBq/mg
137.536 µCi/mg
305.331 dpm/µg

Radiochemical purity (TLC): 99.21%
Diasteroisomeric Ratio S,R/S,S (HPLC): 0.99

Unlabelled Test Material

IR5885
Batch #20071/77
Chemical purity (HPLC) >99%

Soils

The study was carried out with the following five different, fully characterized, soils:

- AR-1 loamy sand
- Stirone clay
- Cal clay
- G-2 loam
- SP-2.1 sand

Fresh soil samples were supplied by:

- Metabolism & Environmental Fate - Isagro Ricerca, (Novara-Italy): AR-1 and Stirone soils;
- “Consulenza secondo natura per la salute e l’ambiente” (Novara-Italy): G-2 soil;

- Excel Research (California-USA): Cal soil;
- Agricultural Research Institute in Speyer (Germany): SP-2.1 soil.

All the soils were taken from their natural location, and collected from the upper 20 cm layer.
Before using, the test soils were passed through a 2 mm sieve, to remove coarse components, dried at room temperature and sterilized by γ-rays.
The sterilization of soils was carried out by Gammatom (Guanzate-Como-Italy).
The water content was determined by means of a gravimetric method (12 hours at 105°C).
Soil characteristics are reported in Table 4.1.6-03.

Table 4.1.6-03 Characteristics of the soils

Characteristics		AR-1	Stirone	Cal	G-2	SP-2.1
	50-2000 µm (sand)	83.50	5.25	18.00	35.00	87.2
Particle size distribution	2-50 µm (silt)	13.75	34.50	22.00	49.75	9.00
	< 2 µm (clay)	2.75	60.25	60.00	15.25	3.80
USDA classification		loamy sand	clay	clay	loam	sand
pH (0.01 $CaCl_2$)		3.38	7.55	5.6	5.16	5.2
Organic Carbon (%)		14.42	0.89	1.80	2.13	0.90
C.E.C. (meq/100 g)		33.53	28.69	47.50	13.57	6.00
Dry Mass (%)		95.24	93.46	90.09	98.04	99.01

STUDY DESIGN AND METHODS

This study included three tiers.

Tier 1 – preliminary test: carried out with two soils to determine:

- the optimal soil/solution ratio;
- the equilibration time for adsorption and the amount of Test Item adsorbed at equilibrium;
- ^{14}C-Mass Balance on the samples with the optimal soil/solution ratio;
- the adsorption of the Test Item on the surface of the test vessels and the stability of the Test Item during the test period.

Tier 2 – screening test: carried out to determine:

- the adsorption equilibrium in a further different soil type by means of adsorption kinetic only at the optimal soil/solution ratio;
- the distribution coefficient K_d and the organic carbon normalized adsorption coefficient K_{oc} of all the five soils.

Tier 3 – adsorption and desorption isotherms: carried out with all the five soils to determine:

- the parameters of Freundlich equation for adsorption and desorption isotherms to study the influence of concentration on the extent of adsorption and desorption from the soils;
- the distribution coefficient K_{des} at desorption equilibrium.

The adsorption-desorption study was conducted under sterile conditions. All the glassware and the materials necessary for the study were sterilized at 121°C for 20 minutes by autoclaving. Handling of sterile materials and sample preparation were performed by using a bacteriological hood equipped with a UV lamp.

1. Preparation of soil samples:

1 g dry weight aliquots of soils were placed into polypropylene centrifuge tubes (50 cm^3) and pre-equilibrated with an aqueous 0.01 M $CaCl_2$ solution: to each sample was added a $CaCl_2$ volume of 4, 10, or 25 cm^3 so to give a soil/solution ratio of 1/4, 1/10 and 1/25, respectively.
Pre-equilibration was carried out for at least 12 hours by stirring the soil suspensions at 20±2°C.

2. Preparation and analysis of the Test Solutions:

The Test Solutions were prepared immediately before each Tier.

Tier 1:
As the number of samples of Tier 1 was very high, it was performed in two phases and the Test Solution was prepared before each one.
Aliquot of approximately 200 µg of ^{14}C-IR5885 (lot#192) equivalent to 1.018 MBq (27.5 µCi) and 1800 µg of cold IR5885 (batch 20071/77) were dissolved into 400 µL of acetonitrile to give 5,000 µg/cm^3 (nominal concentration) Test Solutions (TS-A and TS-B).

Tier 2:
Aliquot of approximately 180 µg of ^{14}C-IR5885 (lot#192) equivalent to 0.916 MBq (25 µCi) and 1575 µg of cold IR5885 (batch 20071/77) were dissolved into 350 µL of acetonitrile to give a 5,000 µg/cm^3 (nominal concentration) Test Solution (TS-C).

Tier 3:
Five Test Solutions at different Test Item concentrations were prepared immediately before each adsorption step as described below:

Aliquots of approximately 1000, 300, 100, 30 and 10 µg of ^{14}C-IR5885 (lot#192) were dissolved into 200 µL of acetonitrile to give a 5000, 1500, 500, 150 and 50 µg/cm^3 (nominal concentration) Test Solutions (TS-D, TS-E, TS-F, TS-G and TS-H).

Two 0.02 cm^3 aliquots of unlabelled Test Item (Batch 20687/52) before adding ^{14}C-IR5839 (TS-A, TS-B, and TS-C) were analyzed by HPLC.

3. Analysis of the Test Solutions:

LSC analysis*:*
Tiers 1 and 2, three 0.01 cm^3 aliquots of Test Solutions TS-A, TS-B and TS-C were diluted into 1 cm^3 of acetonitrile and four 0.05 cm^3 of each diluted solution were checked by LSC to determine the actual concentration of the Test Item.
Tier 3, three 0.01 cm^3 aliquots of each Test Solution were diluted into 10 cm^3 with acetonitrile.
Four 0.1-0.5 cm^3 aliquots of each diluted solution were checked by LSC to determine the actual concentration of Test Item.

TLC analysis

Two 10 µL aliquots of diluted solutions were analysed by using two stationary phases and two solvent systems:

HPLC analysis
The diastereoisomeric ratio (*S,R/S,S*) of the Test Item was determined by HPLC analysis.

A. Tier 1 – preliminary test

Two soil types, AR-1 (low clay and high organic carbon content) and Stirone (high clay and low organic carbon content), and three soil/solution ratios (1/4, 1/10, and 1/25) were used. The following sets of samples for each soil were prepared and numbered in progressive order.

Samples: twelve couples, treated with the Test Item, one for each soil/solution ratio and for each sampling time.

Blanks: a couple for each soil/solution ratio set up like the above, but not treated with the Test Item.

Controls: a couple of samples constituted by 10 cm^3 of $CaCl_2$ 0.01 M without soil.

After pre-equilibration period aliquots of 4, 10, and 25 µL of the Test Solution TS-A or TS-B were added to the samples (1/4, 1/10, and 1/25 soil/solution ratio, respectively).
An aliquot of 10 µL of the Test Solution TS-A was added to the controls.
The total volume V_0 in contact with the soil varied depending on the water content of the dried soil.
As the final volume of the added Test Solution represented less than 0.1% of V_0, it was disregarded.
The final nominal concentration of the Test Item for each sample was 5 µg/cm^3.
The final concentration of acetonitrile in each sample was lower than 0.1%.

Adsorption kinetic: The pH value of the 0.01 M $CaCl_2$ solution was measured on the blank samples before and after contact with the soil.
All the samples were incubated at 20±2°C under agitation to maintain soil in suspension.
The analyses were performed at the following time intervals:

- 30 min, 1, 2, and 4 hours (samples);
- 4 hours (blanks);
- 0 and 4 hours (controls).

At the defined time intervals each couple of *samples* was centrifuged to separate the phases, and the aqueous supernatants were recovered as much as possible. The volumes V_r were recorded and the radioactivity content determined by LSC on two aliquots.
The *blanks* were taken and centrifuged as above described after 4 hours.
The radioactivity content on the *control* samples was determined at 0 and 4 hours to check whether any adsorption of the Test Item occurred to the tube walls. All the supernatants were stored at 4-6°C until analysis.

B. Tier 2 – screening test

The adsorption characteristics of Cal, G-2 and SP-2.1 soils were studied at the optimal soil/solution ratio (1/10) determined in Tier 1. Four couples of samples and a couple of blanks for each soil were prepared and equilibrated. All the samples were numbered in progressive order.
After pre-equilibration period 10 µL of the Test Solution TS-C were added to the samples.
The final nominal concentration of the Test Item for each sample was 5 µg/cm^3.
The final concentration of acetonitrile in each sample was lower than 0.1%.

Adsorption kinetic: The pH value of the 0.01 M $CaCl_2$ solution was measured on the blanks before and after contact with the soil.

All the samples were incubated at 20±2°C under agitation to maintain soil in suspension.
The analyses were performed at the following time intervals:

- 30 minutes, 1, 2 and 4 hours (samples);
- 4 hours (blanks).

At the defined time intervals each couple of samples was centrifuged (20 minutes at 17,200 RCF) to separate the phases, and the aqueous supernatants were recovered as much as possible. The volumes V_r were recorded and the radioactivity content determined by LSC on two aliquots.

The blanks were taken and centrifuged as above described after 4 hours. All the supernatants were stored at 4-6°C until analysis.

Soil extraction: At the end of the adsorption step, the *samples* referred to the optimal soil/solution ratio 1/10 of Tier 1, the *samples* of Tier 2 and the relevant *blanks* were extracted by sonication (30 min. at 40°C) and by stirring (30 min. at 20°C) with 10 cm^3 of the following solvent mixtures:

- ❑ acetone-0.5N HCl (2-8 by volume)**extract A**
- ❑ acetone-0.5N HCl (2-8 by volume)**extract B**
- ❑ acetone-0.5N HCl (2-8 by volume)**extract C**

After each extraction the soil residue was separated by centrifugation, the supernatant was recovered in a volumetric flask and made up to the mark (10 cm^3). The radioactivity content was determined by LSC on two aliquots.
All the extracts were stored at 4-6°C until analysis. The non-extracted radioactivity (bound residue) remaining in soil samples was determined by LSC after combustion by means of a biological oxidizer.

^{14}C-mass balance: The ^{14}C-Mass Balance was calculated only on the samples with the optimal soil/solution ratio, by summing the percentage of applied mass (% AM) found in the adsorption supernatants, in the soil extracts and in the bound residue.

Stability of the test item: The adsorption supernatants and the organic extracts (pooled extracts A+B and C) of the *samples* (referred to 1/10 soil/solution ratio) were analyzed by TLC to check the stability of the Test Item at the end of adsorption kinetic. The *controls* were analyzed, after 4 hours of incubation, by TLC to check the Test Item stability in $CaCl_2$ solution.

C. Tier 3 – adsorption desorption isotherms

Adsorption: The five soils AR-1, Stirone, Cal, G2 and SP-2.1 were used at the optimal soil/solution ratio 1/10 for the determination of adsorption and desorption isotherms. The test was carried out at five Test Item concentrations.
Duplicate samples (1 gram dry weight) of each soil were placed into polypropylene centrifuge tubes and pre-equilibrated with a 10 cm^3 of 0.01 M $CaCl_2$ solution. The samples were numbered in progressive order.
After equilibration, 10 µL aliquots of Test Solutions TS-D, TS-E, TS-F, TS-G and TS-H were added to the samples to give the following nominal concentrations: 5.00, 1.50, 0.5, 0.15 and 0.05 µg/cm^3.
The final concentration of acetonitrile in each sample was lower than 0.1%.
The samples were incubated at 20±2°C under agitation to maintain the soil in suspension during a period of 1 hour (equilibration time).
At equilibrium the samples were centrifuged to separate the phases; the supernatants were recovered as much as possible and the volumes V_r recorded. Radioactivity content of supernatants was determined by LSC. All the supernatants were stored at 4-6°C.

Desorption At the end of adsorption step the volume V_r removed was replaced by an equal volume of 0.01 M $CaCl_2$ solution.
The samples were agitated for 1 hour at 20±2°C, then centrifuged to separate the phases. The radioactivity content of supernatants was determined by LSC on two aliquots and the samples were agitated again. This procedure was repeated until the desorption equilibrium was reached (four cycles for all the soils).

The total volume of the two aliquots taken for LSC analysis at each desorption cycle (two 0.05 cm^3 aliquots) was equal to 1% of the total solution in contact with the soil. This volume was replaced with an equal volume of 0.01 M $CaCl_2$ to maintain the soil/solution ratio.

D. Data processing

The radioactivity found in supernatants and in bound residues was expressed as micrograms (µg) and as percentage. Experimental data were used to determine:

- the amount of the Test Item adsorbed at equilibrium A (% of AM);
- the amount the Test Item desorbed at equilibrium D (% of adsorbed);
- the distribution coefficients K_d and the organic carbon normalized adsorption coefficient K_{oc};
- the apparent desorption coefficients $K_{des;}$
- the parameters of Freundlich equation for adsorption isotherms K_F^{ads} and 1/n;
- the parameters of Freundlich equation for desorption isotherms K_F^{des} and 1/n.

Statistical analysis, included arithmetic mean and standard deviation, where appropriate.

RESULTS AND DISCUSSION

Test solutions

Tiers 1 and 2:
the actual concentration and the calculated specific activity of ^{14}C-IR5885 in the Test Solutions were accounted in the Table 4.1.6-04:

Table 4.1.6-04

TIER	Test Solution	Test Solution concentration (µg/cm^3)			specific activity (dpm/µg)
		^{14}C-IR5885	cold IR5885	^{14}C-IR5885 + cold IR5885	
1	TS-A	521.447	4,500	5,021.447	31,707
	TS-B	532.989	4,500	5,032.989	32,334
2	TS-C	519.751	4,500	5,019.751	31,614

The radiochemical purity of IR5885 resulted higher than 99%.
The diastereoisomeric ratio as *S,R/S,S*, determined by HPLC, resulted 0.99.

Tier 3:
the actual concentration of ^{14}C-IR5885 in the Test Solutions was accounted in the Table 4.1.6-05:

Table 4.1.6-05

Test Solution	nominal concentration (µg/cm^3)	Test Solution		actual concentration (µg/cm^3)
		concentration (µg/cm^3)	volume added (µL)	
TS-D	5.00	5,126.371	10	5.126
TS-E	1.50	1,624.401		1.624
TS-F	0.50	517.930		0.518
TS-G	0.15	150.918		0.151
TS-H	0.05	50.280		0.050

The radiochemical purity of IR5885 resulted higher than 99%.
Adsorption kinetic at 1/4, 1/10 and 1/25 soil/solution ratio - Tier 1

Concentrations and total amounts of the Test Item in the samples are reported in Table.4.1.6-06.

Table 4.1.6-06

soil/solution ratio	V_0 (cm^3)		Test Solution Added (cm^3)	amount of IR5885 (µg/sample)		concentration of IR5885 (µg/cm^3)			
	AR-1	Stirone		AR-1 and Stirone		AR-1		Stirone	
				*	**	*	**	*	**
1/4	4.05	4.07	0.004	20.086	20.132	4.960	4.971	4.935	4.946
1/10	10.05	10.07	0.010	50.214	50.330	4.996	5.008	4.986	4.998
1/25	25.05	25.07	0.025	125.536	125.825	5.011	5.023	5.007	5.019

* samples treated with Test Solution TS-A
** samples treated with Test Solution TS-B

The adsorption kinetics carried out at the three different soil/solution ratio showed that the adsorption equilibrium was reached within 4 hours for both the soil.

At equilibrium the amount of the Test Item adsorbed, as percentage of the applied mass (% AM) was: in AR-1 soil 93.69% (1/4 ratio), 84.28% (1/10 ratio), and 63.70% AM (1/25 ratio); in Stirone soil 74.67% (1/4 ratio), 59.58% (1/10 ratio), and 36.54% AM (1/25 ratio). Data are shown in Table 4.1.6-08.

As optimal soil/solution ratio was chosen 1/10 in both the soils. At this ratio the Kd values at equilibrium were 54 and 15 for AR-1 and Stirone soil, respectively (Table 4.1.6-10). The corresponding Koc values were 375 and 1686.

The LSC analysis of the controls showed that no adsorption to tube walls occurred. The radioactivity found in solution accounted for 99.56% AM at the start of incubation and 99.58% AM after 4 hours of incubation.

Tier 2 – Screening test

The adsorption kinetic of soils Cal, G-2 and SP-2.1 was carried out at the previously chosen soil/solution ratio of 1/10.
Concentrations and total amounts of the Test Item in the samples were reported in the Table 4.1.6-07.

Table 4.1.6-07

soil	soil/solution ratio	V_0 (cm^3)	Test Solution added (cm^3)	amount of IR5885 (µg/sample)	concentration of IR5885 (µg/cm^3)
Cal	1/10	10.11	0.010	50.198	4.965
G-2		10.02			5.010
SP-2.1		10.01			5.015

The equilibrium was reached within 4 hours of incubation. At equilibrium the amount of the Test Item adsorbed on soils was: 45.42% AM in Cal soil, 45.59% AM in G-2 soil and 42.61% AM in SP-2.1 soil (Table 4.1.6-09).

The K_d value at equilibrium was 9, 9 and 8 in Cal, G-2 and SP-2.1 soils, respectively. The corresponding K_{oc} values were: 472, 400 and 834 (Table 4.1.6-10).

Table 4.1.6-08 **Tier 1.** Kinetic of adsorption to AR-1 and Stirone soils
(data are expressed as percentage of ^{14}C adsorbed in soil referred to total applied mass (m_0))

Time	AR-1 soil			Stirone soil		
	soil/solution ratio					
	1/4	1/10	1/25	1/4	1/10	1/25
30 min	93.46	78.39	58.41	75.64	40.04	18.85
	92.57	80.62	55.67	68.82	31.12	15.50
mean	93.02	79.51	57.04	72.23	35.58	17.18
±S.D.	0.629	1.577	1.937	4.822	6.307	2.369
1 hour	92.76	79.43	61.62	69.01	53.21	29.03
	91.31	84.02	65.80	73.39	50.89	28.70
mean	92.04	81.73	63.71	71.20	52.05	28.87
±S.D.	1.025	3.246	2.956	3.097	1.640	0.233
2 hours	93.03	83.92	61.97	72.27	59.62	31.48
	93.66	81.53	66.33	81.29	57.16	32.49
mean	93.35	82.73	64.15	76.78	58.39	31.99
±S.D.	0.445	1.690	3.083	6.378	1.739	0.714
4 hours	93.23	84.09	60.80	75.06	56.00	32.83
	94.14	84.47	66.59	74.27	63.15	40.24
mean	93.69	84.28	63.70	74.67	59.58	36.54
±S.D.	0.643	0.269	4.094	0.559	5.056	5.240

Table 4.1.6-09 Tier 2. Kinetic of adsorption to Cal, G-2 and SP-2.1 soils at soil/solution ratio of 1/10 (data are expressed as percentage of ^{14}C adsorbed in soil referred to total applied mass (m_0))

Time	Cal	G-2	SP-2.1
30 min	37.01	44.05	40.16
	39.88	48.09	40.83
mean	38.45	46.07	40.50
±S.D.	2.029	2.857	0.474
1 hour	39.97	43.44	48.38
	39.65	43.69	39.03
mean	39.81	43.57	43.71
±S.D.	0.226	0.177	6.611
2 hours	45.01	42.98	39.77
	39.78	42.31	46.06
mean	42.40	42.65	42.92
±S.D.	3.698	0.474	4.448
4 hours	47.08	47.53	40.20
	43.76	43.65	45.02
mean	45.42	45.59	42.61
±S.D.	2.348	2.744	3.408

Table 4.1.6-10 **Tiers 1 and 2**. C_{aq}^{ads}, C_s^{ads}, % A, K_d, and K_{oc} at equilibrium
(data obtained at soil/solution ratio 1/10)

Soil	Equil. time	C_{aq}^{ads}	C_s^{ads}	% A	K_d	% of organic carbon	K_{oc}
AR-1	4 hours	0.795	42.226	84.09	53	14.42	368
		0.778	42.511	84.47	55		381
mean		0.787	42.369	84.28	54		375
± S.D.		0.012	0.202	0.269	1.414		9.19
Stirone	4 hours	2.194	28.119	56.00	13	0.89	1461
		1.842	31.784	63.15	17		1910
mean		2.018	29.952	59.58	15		1686
± S.D.		0.249	2.592	5.056	2.828		317.49
Cal	4 hours	2.627	23.634	47.08	9	1.80	500
		2.792	21.966	43.76	8		444
mean		2.710	22.800	45.42	9		472
± S.D.		0.117	1.179	2.348	0.707		39.598
G-2	4 hours	2.628	23.861	47.53	9	2.13	423
		2.823	21.910	43.65	8		376
mean		2.726	22.886	45.59	9		400
± S.D.		0.138	1.380	2.744	0.707		33.23
SP-2.1	4 hours	2.999	20.181	40.20	7	0.90	778
		2.757	22.600	45.02	8		889
mean		2.878	21.391	42.61	8		834
± S.D.		0.171	1.710	3.408	0.707		78.49

C_{aq}^{ads} = concentration of the Test Item found in adsorption supernatant (µg/cm^3)
C_s^{ads} = concentration of the adsorbed Test Item in soil (µg/g)
A = percentage of Test Item adsorbed on soil, referred to total applied mass
K_d = distribution coefficient
K_{oc} = organic carbon normalized adsorption coefficient

^{14}C-Mass Balance

The ^{14}C-Mass Balance was determined in Tier 1, at the optimum soil/solution ratio 1/10, and in Tier 2 and was calculated by summing the % AM found in supernatants, in soil extracts, and in the bound residue at the end of adsorption step.

Supernatants. The amount of radioactivity found was 13.69, 38.29, 51.54, 47.72 and 47.61% AM in AR-1, Stirone, Cal, G-2, and SP-2.1 soils, respectively.

Soil extracts. The amount of radioactivity extracted was 79.93, 57.84, 42.30, 50.17 and 52.21% AM in AR-1, Stirone, Cal, G-2, and SP-2.1 soils, respectively.

Bound residue. The amount of non-extracted radioactivity was 9.86, 5.92 8.30, 3.18 and 3.19% AM in AR-1, Stirone, Cal, G-2, and SP-2.1 soils, respectively.

The ^{14}C-Mass Balance was 103.48, 102.04, 102.13, 101.07 and 103.00% AM in AR-1, Stirone, Cal, G-2, and SP-2.1 soils, respectively.

Test Item stability

Samples

At the end of adsorption kinetic the Test Item was substantially stable.

The amount of total analysed radioactivity (supernatant and soil extracts) accounted for 93.62, 96.12, 93.84, 97.89 and 99.82% AM for AR-1, Stirone, Cal, G-2, and SP-2.1 soils, respectively.
Total amounts of IR5885 found in analysed solutions were 87.46, 89.53, 89.15, 91.79 and 94.52% AM for the five soils.

Control
Total amount of IR5885 found in the *controls* accounted for 95.93% AM after 4 hours of incubation.

Tier 3 – Adsorption and desorption isotherms

Adsorption isotherms: The added volume of the Test Solution, the actual concentration and the amount of the Test Item applied are reported in the table 4.1.6-11:

Table 4.1.6-11

Nominal concentration (µg/cm^3)	Test Solution added (cm^3)	µg/sample	actual concentration (µg/cm^3)				
			AR-1	Stirone	Cal	G-2	SP-2.1
5.00	0.010	51.264	5.101	5.091	5.071	5.116	5.121
1.50		16.244	1.616	1.613	1.607	1.621	1.623
0.50		5.179	0.515	0.514	0.512	0.517	0.517
0.15		1.509	0.150	0.150	0.149	0.151	0.151
0.05		0.503	0.050	0.050	0.050	0.050	0.050

The actual concentration varied depending on the water content of each soil.

From the values of C_{aq}^{ads} (eq) and of C_{s}^{ads} (eq) the following Freundlich parameters were obtained (Table 4.1.6-12):

Table 4.1.6-12

Soil	Adsorption isotherms		
	1/n	K_F^{ads}	r^2
AR-1	0.958	73	0.998
Stirone	0.954	19	0.998
Cal	0.973	9	0.999
G-2	0.905	8	0.992
SP-2.1	1.017	7	0.999

The values of 1/n (near to 1) showed that the percentage of adsorbed Test Item kept substantially stable by increasing concentration in all the tested soils except in G-2, which showed a 1/n value of 0.905.
The amount of adsorbed Test Item showed little differences in the soils; it ranged from 87.16 to 89.80% AM in AR-1 soil, from 63.34 to 70.33% AM in Stirone soil, from 46.33 to 51.47% AM in Cal soil, from 40.26 to 56.21% AM in G-2 soil, and from 38.84 to 43.19% AM in SP-2.1 soil.

Desorption isotherms

From the values of C_{aq}^{des} (eq) and of C_{s}^{des} (eq) the following values of Freundlich parameters were obtained (Table 4.1.6-13):

Table 4.1.6-13

Soil	Desorption isotherms		
	1/n	K_F^{des}	r^2
AR-1	0.998	135	0.998
Stirone	1.169	91	0.959
Cal	0.955	49	0.996
G-2	1.038	124	0.978
SP-2.1	1.031	87	0.986

The amount of desorbed Test Item referred to the adsorbed one was substantially constant in the most soils for all concentrations: it ranged from 6.42 to 7.58% AM in AR-1 soil, from 7.54 to 19.43% AM in Stirone soil, from 13.29 to 17.87% AM in Cal soil, from 5.97 to 11.05% AM in G-2 soil and from 9.57 to 14.15% AM in SP-2.1 soil.

CONCLUSIONS

The adsorption-desorption characteristics of ^{14}C-IR5885 were determined on five different soils: loamy sand (AR-1), clay (Cal), loam (G-2), sand (SP-2.1). and clay (Stirone). The soils differed for texture, pH, organic carbon, and the clay content.

Tier 1, carried out with the AR-1 and Stirone soils, showed that the best soil/solution ratio was 1/10, and the equilibration time was reached within 4 hours.

Tier 2, carried out with the Cal, G-2 and SP-2.1 soils, showed again that the equilibration time was reached within 4 hours.

The Mass Balance was 103.48, 102.04, 102.13, 101.07 and 103.00% AM for AR-1, Stirone, Cal, G-2 and SP-2.1 soils, respectively.

Test Item was stable during the study.

The K_{oc} values were 375, 1686, 472, 400 and 834 for AR-1, Stirone, Cal, G-2 and SP-2.1 soils, respectively. Tier 3 data of adsorption and desorption isotherms fitted well with Freundlich equation and the K_F^{ads} values were 73, 19, 9, 8, and 7; the K_F^{des} were 135, 91, 49, 124 and 87, respectively.

4.2 Bioaccumulation

4.2.1 Bioaccumulation test on fish

4.2.1.1 Flow-through fish test in Rainbow trout

Reference Number:	KII- Section 6, Volume 1, Annex IIA, point 8.2.6/01
Report:	See annex conf. 21
Guidelines:	OECD Guideline for testing of Chemicals, No. 305, "Bioconcentration: flow-through fish test", proposal adopted June 14, 1996. OPPTS 850.1730, Fish BCF, "Public draft". EPA 712-C-96-129, April, 1996
Deviations:	none.
GLP:	Yes; certified laboratory

Executive Summary:

The bioconcentration and depuration of IR5885 in Rainbow Trout was investigated in edibles and non-edibles by means of a dynamic flow through system. Based on the results in edibles and non-edible fish parts bioconcentration in whole fish was calculated.

The fish were continuously exposed to ^{14}C-IR5885 at an average high dose concentration of 893.5 µg-eq/L and an average low dose concentration of 93.5 µg-eq/L for 14 days at a temperature ranging from 13.3 to 14.8°C, a pH ranging from 8.0 to 8.2 and an oxygen concentration ranging from 8.2 to 9.5 mg/L. Thereafter, the fish were transferred to flowing untreated water and the depuration of radioactivity was followed for 14 days.

Due to the extremely low accumulation of IR5885 in fish at both dose levels, no relevant plateau levels and consequently no half-lives as well as accumulation/depuration kinetics could be determined.

At the high dose level, the residual radioactivity found in fish during the whole exposure period amounted to 1160 ± 355, 2685 ± 325 and 1940 ± 367 µg-eq/kg for edibles, non-edibles and whole fish, respectively being about 2 fold higher than the high dose exposure concentration. Thereafter, radioactivity was depurated from fish during 14 days. At the end of the depuration period, concentrations ranged from 428 to 474 µg-eq/kg.

At the low dose level, the residual radioactivity found in fish during the whole exposure period amounted to 142 ± 36, 283 ± 53 and 215 ± 46 µg-eq/kg for edibles, non-edibles and whole fish, respectively being about 2 fold higher than the low dose exposure concentration. Thereafter, radioactivity was depurated from fish during 14 days. At the end of the depuration period, concentrations ranged from 48 to 60 µg-eq/kg.

Based on the total radioactivity concentration in the exposure water and the residual radioactivity found in fish parts, ratios between fish and water (BCF) amounted to 1.3, 3.0 and 2.3 for edibles, non-edibles and whole fish, respectively, indicating lack of bioconcentration at both dose levels.

Analyses of radioactivity in the exposure water showed mainly parent item at both dose levels throughout the entire exposure period. Besides the constant levels of parent item ranging on average from 96.2 to 98.0% of the radioactivity recovered, three unknown radioactive fractions W0, W2 and W3/4 were found in minor amounts (< 3% of the radioactivity recovered).

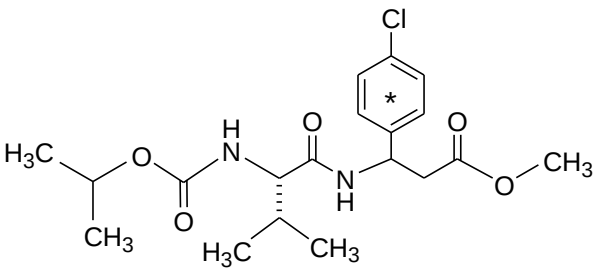
In conclusion, IR5885 showed lack of bioconcentration (BCF < 4) in rainbow trout during the whole exposure period.

MATERIALS AND METHODS

MATERIALS

Test Material IR5885 (Unlabelled)
Description white powder
Log Pow 3.1
Lot/Batch # T 025/02
Purity 99.56% (w/w)
CAS # 283159-90-0

Test Material (Labelled) ^{14}C-IR5885
Position of labelling *



Lot/Batch # 199 (RCC substance 146574/A)
Spec. Radioactivity 119.8µCi/mg or 4.435 MBq/mg or 266084 dpm/µg
Radiochemical purity 97.1%

Test Animals:
Species: *Oncorhynchus mykiss* (Rainbow trout)
Weights: for the study, a total of 205 fish was used, taking into account accidental fish mortality. The target average fish weight was about 1-2 g.
Source: Forellenzucht Hohler, Zeiningen, Switzerland
Acclimation period: the fish were allowed to acclimatise to the laboratory environment in tap water for at least two weeks
Diet: the fish were fed daily (H.U. Hofmann AG, HOKOVIT, "Forellenfutter", diet of known lipid and total protein content), based on about 2% of the average fish body weight during acclimation and during the study, taking into account increasing body weights and the decreasing number of fish per sampling interval**.**
Apparatus-test water: flow-through system with tap water (preferred for bioconcentration test) comprising three aerated and temperature controlled tanks each containing 75 L. During accumulation, one tank served as the control (receiving an identical amount of solvent without test item), two tanks received the ^{14}C-labelled test item from an application solution with a flow-through volume of 250 L/24 hours (i.e. about 3 times the tank volume), delivered via a Hamilton dispenser unit into a mixing flask where the application solution was pre-diluted with tap water. Before placing the fish into the respective tank, the flow-through system was run for 2 - 3 times 24 hours with labelled test item (i.e. about 7 - 10 tank volumes) to ensure a stabilised concentration of the test item at the onset of the uptake phase.

Test Conditions:
Surrounding Type: controlled environment room

Illumination: photo-period of 16 hours light, with intensity at light period of approx. 300-400 Lux-
Light Intensity: 480-860 Lux
Temperature: 13-17 °C

STUDY DESIGN AND METHODS

Dates: Experimental Start: March 2, 2004
Experimental Termination: August 13, 2003

For the treated tanks in the main test the minimal duration of the uptake phase was calculated according to the OECD Guideline 305. Based on the log Pow, the expected depuration rate constant (k2) and the optimal duration of the uptake phase (u) are defined as:

$$\log k2 = -0.414 \log (Pow) + 1.47$$

and

$$u = 3.0/k2$$

Based on the log Pow value of 3.1, about 2 days would be needed to reach 95% of "steady state". To be able to fulfil all validation criteria an accumulation and depuration period of 14 days was run. To initiate depuration, the fish were transferred to a flow-through system with untreated water and the elimination of the radioactivity from the fish was determined.

Dosage Preparation and Dosing

Stability of ^{14}C-IR5885: to prove the storage stability of the test item in the stock solutions, an aliquot of the low dose stock solution after 31 days storage at –20 °C was analysed by HPLC (95.5% parent). Compared to the initial radiochemical purity of 14C-IR5885 in the stock solution 1 of 97.1% as determined by HPLC the test item proved to be sufficiently stable in dimethylformamid (DMF).
In a pre-test, the radiochemical purity of ^{14}C-IR5885 in the application solution after stirring at room temperature during 7 days was determined by HPLC and TLC analyses. Compared to the initial radiochemical purity of 87.9% (HPLC) and 98.5% (TLC), the radiochemical purity of IR5885 after 7 days stirring at room temperature amounted to 86.1% (HPLC) and 99.1% (TLC).
These results prove that the test item was sufficiently stable in the application solution (DMF) at test conditions.

Test Concentration and application of the test item: taking into account available Rainbow Trout toxicity data dose levels of 1000 and 100 µg/L were selected. Taking into account a daily flow-through volume of 250 L and a final concentration of organic solvent in the water not exceeding 0.01%, application solutions for both dose levels were prepared from the corresponding stock solutions by dilution in DMF to a target daily amount of 250 mg/25 mL (high dose of 1000 µg/L) and 25 mg/25 mL application solution (low dose of 100 µg/L).
Therefore, each dosing day an aliquoted portion of the respective stock solution was diluted about 1:1 (v/v) in DMF:

Table 4.2.1.1-01 Test concentration

	High Dose		**Low Dose**	
Application solution for x days	6	4	6	4
Actual concentration dpm/µL (LSC)*	2'293-2'483	2'172	2'152-2'516	2'527
Actual concentration mg/25 mL	263-284	249	22-26	26
n=	2	1	2	1

* Liquid scintillation counting (specific radioactivity: 218 and 2462 dpm/µg for the high and low dose, respectively)

Throughout the entire test, the system was equilibrated with tap water at a flow-through volume of 250 L/day, to which ^{14}C-IR5885 was added from the application solutions at a rate of 25 mL/24 h (52 µL/3 min) to achieve final target concentrations of 1000 µg/L (or 2180 dpm/10mL) at 218 dpm/µg and 100 µg/L (or 2462 dpm/10 mL) at 2462 dpm/µg for the high and low dose level respectively in the treated tanks.

Monitoring:

In addition to sampling of fish and water at selected time intervals (see section 2.4.1) and dosing of ^{14}C-IR5885 during the uptake phase, the following activities were performed throughout the study:

- Determination of natural particle content and TOC in untreated tap water.
- On each working day monitoring of flow rate and as far as appropriate adjustment to present values.
- On each working day monitoring of temperature.
- Monitoring of pH and oxygen concentration once a week during the uptake period and the depuration period in all tanks.
- Monitoring of TOC in all tanks 48 and 24 hours before initiation of the uptake phase and weekly during uptake and depuration phase.
- Monitoring of total hardness once during the test in the control and low dose level tank.
- Feeding after taking water/fish samples.
- Siphoning of uneaten food and fish excreta from the tanks about 1 hour after feeding.
- Monitoring of fish health status including mortality.
- During the uptake phase as far as necessary every 3 - 4 days exchange of the mixture chamber and connection tube. As far as appropriate additional cleaning of cooling system and tank walls.
- Between accumulation and depuration, the test apparatus was cleaned thoroughly. Lack of contamination was proven by the determination of radioactivity in samples obtained after wiping discrete areas (about 100 cm² of cover, various wall positions and drain-pipe) of the respective treated tanks with ethanol (1-2 dpm/cm²).

Sampling and analysis

Fish and water sampling schedule: For the sampling schedule, five sampling points during the uptake phase and four depuration sampling points were spaced throughout the duration of the experiment, assuming a total period of maximally 28 days. The time schedule of fish and water sampling is summarised in Table 4.2.1.1-02:

Table 4.2.1.1-02 Fish and water sampling schedule

Fish sampling		Number of fish samples from each treated tank		Control fish samples		H_2O*** samples	Sample point (days)	Action
		TR	Analyses	TR	Lipid			
						2	-2	Start test
						2	-1	
						2	0	Fish added **
A	1st	6	--	6	10	2 (+2)*	4	
	2nd	6	--	--	--	2 (+2)*	7	
	3rd	6	8	--	--	2 (+2)*	10	
	4th	6	--	--	--	2 (+2)*	12	
	5th	6	8	6	10	2 (+2)*	14	End of uptake period/ onset of depuration
Subtotal		30	16	12	20			
B	6th	6	--	--	--	2	17	
	7th	6	--	--	--	2	19	
	8th	6	--	--	--	2	25	
	9th	6	--	6	--	2	28	
Subtotal		24	0	18	20			
Reserve fish		10	--	7				
Total		64	16	45				
Total number of fish sampled		80		45				

A: Uptake phase of the test
B: Depuration phase of the test
TR: Total radioactivity
2: Two samples before feeding
(+2)*: Additional sampling of water for analytics.
**: After at least 7-10 tank volumes run through the tanks during the pre-equilibrium period of 2 days.
***: Water samples from the tanks were taken on working days during accumulation and at all depuration time points. Based on the results additional water samples were taken from the mixing chambers to evaluate constant exposure concentrations.

Water: Samples were taken from the central area of the respective test tank before feeding and immediately before fish sampling. Additionally, to exclude the influence of excreta, as far as appropriate water samples (for determination of radioactivity by LSC) were also drawn from the corresponding mixture chamber.

Treated tanks: daily during the uptake phase and at selected time points during the depuration phase, duplicate samples of 10 mL were removed from the treated tanks and as far as appropriate from the corresponding mixture chambers; radioactivity was directly determined.
Additionally at selected time intervals, duplicate samples of approximately 500 ml were taken, worked up and stored at –20°C until analyses of one sample/time point/dose level (in total 10 samples were analysed).

Control tank: duplicate background samples were taken at the beginning, in the middle and at the end of the uptake phase and at the end of depuration phase from the control tank and directly analysed by liquid scintillation counting (LSC).

Fish: Treated tanks: on each sampling occasion, six fish were collected randomly from each exposure tank, rinsed with water, sacrificed in 1.5 % (v/v) 2-phenoxy-ethanol in purified water and blotted dry. The fish were

analysed immediately following sacrifice. At two time intervals (e.g. after 10 and 14 days of accumulation) 8 fish were sampled and stored at about -20 °C for additional analyses.

Control tank: at the beginning and at the end of the accumulation period and at the end of the depuration period, six control fish were taken and analysed for total radioactivity.
At two selected time points during the uptake phase ten fish were additionally sampled for lipid determination on both edible and non edible parts.

Sample preparation:

Water: duplicate samples of 10 mL water were directly analysed for total radioactivity by LSC.
The duplicate water samples of 500 mL on all 5 uptake time points were partitioned twice with about 200 mL ethylacetate. The first ethylacetate fraction containing significant amounts of radioactivity was dried and stored at -20 °C until further analyses.

Fish: the six fish sampled from each tank (exposure tanks and control tank) were directly separated into edible (fillet and skin) and non-edible parts of fish (head, fins, viscera and skeleton). The fish parts of each fish were weighed and solubilised (100 mg/mL solubiliser). Thereafter, duplicate solubilised subsamples (corresponding to 100 mg) were measured by LSC.

On day 4 and 14 ten fish were sampled from the control tank and separated into edible and non-edible parts. The individual parts of fish were pooled, weighed and stored at –20 °C until determination of fish lipid/wet weight ratio on edible and non-edible parts by means of chloroform/methanol .

For lipid determination the edible and non-edible fish pools were thawed and homogenised in the presence of dry-ice. Thereafter the homogenates (in total 4) were alternatively extracted once with methanol/dichloromethane (1:1; v/v). The ratio fish against $MeOH/CH_2Cl_2$ was 1:8 (w/v). The first extraction step was performed by gentle shaking (220 rpm) overnight at room temperature. A subsequent extraction step was performed with dichloromethane (fish:CH_2Cl_2; 1:2, w/v) by shaking for about 30 min (220 rpm) at room temperature. The two extracts were combined in a separation funnel and after adding of 10% purified water, the dichloromethane phases could be separated. After drying the dichloromethane phases with sodium sulphate they were concentrated to dryness under reduced pressure. The fish lipid residue was determined after drying at 105°C and subsequent cooling in an exicator to constant weight.

The additional 8 fish sampled per exposure tank on day 10 and 14 were directly separated into edible and non-edible parts. The individual parts of fish were pooled, weighed and stored at –20 °C. Because the average BCF value was below 1000, the pooled fish parts were not needed for additional analyses and therefore remained stored at –20°C.

Analytical method

Measurement of radioactivity: the radioactivity was determined on Packard liquid scintillation counters equipped with DPM and luminescence options. All measurements were performed after scintillation background correction and all samples were determined at least in duplicate.

Measurement of Total Organic Carbon (TOC): for TOC analysis 10-50 mL analyses were performed with a TOC analyser consisting of an autosampler.

HPLC was used as primary method to determine the radiochemical purity of the test item in the stock solution and to determine the metabolite pattern in the exposure water. Analyses were performed with a HPLC-system consisting of a gradient pump, an autosampler, an UV-detector, a radiodetector and data processing system.

TLC (Thin-layer chromatography) was used as secondary method to determine the radiochemical purity/stability of the test item and to determine the metabolite pattern in the exposure-water

RESULTS AND DISCUSSION

Observations and optimisation: the following observations were made and optimisation measures were performed during the course of the study.

1) No mortality or other adverse effects in both control and treated fish occurred.

2) To promote level equilibration at day -2, the levels in the treated tanks were adjusted once by adding an appropriate volume of the respective stock solution to each tank.

3) To check the efficacy of the flow-though system and to evaluate possible non-specific interactions of the test item with fish and fish excreta, additional water samples from the mixing chambers were taken at day 0 and 11. The values were 10 - 18% above the levels in the respective fish tanks, proving some reduction in the test item concentration in the presence of fish and fish excreta.

pH, temperature, oxygen, hardness and TOC concentration in the water

Temperature, pH and oxygen concentration were monitored during the exposure days 0 to 14 and amounted in the presence of fish to 13.3-14.8 °C, 8.0-8.2 and 8.1-9.5 mg/L, respectively. During depuration (day 15-28) the respective values were 13.7-15.0 °C, 7.7-7.9 and 7.9-8.9 mg/L .

Water total hardness measured once during the test (day 5 of exposure) was 10°d (17.8°f) or 1.8 mmol/L for both control tank and low dose tank.

The TOC value of untreated tap water amounted to 1.0 mg/L. TOC values during pre-equilibrium and accumulation period ranged from 44.6–49.3 mg/L for the two ^{14}C-tanks. The corresponding control tank values were 42.3–47.1 mg/L. The DMF (accounting for 46.6 mg C/L) was responsible for the higher TOC values. With respect to the total amount organic carbon in the exposure water, the test item (on average 894 µg/L for the high dose) did not attribute significantly to the TOC values. After correction, TOC values during pre-equilibrium and accumulation period were below 3 mg/L.
In the first week of depuration TOC values had decreased to 0–3.6 mg/L and in the second week to 1.8-2.7 mg/L

Levels of radioactivity in water during pre-equilibrium, exposure and depuration

During the pre-equilibrium phase of 2 days, total radioactivity in the high dose tank ranged on average from 906-935 µg-eq/L and in the low dose tank from 92-97 µg-eq/L.

During the accumulation period (day 0 – 14) in both exposure tanks, total radioactivity levels remained sufficiently constant showing equilibrium.
Total radioactivity levels amounted on average to 893.5 ± 29.1 µg-eq/L (± 3.3%) and 93.5 ± 3.0 µg-eq/L (± 3.2%) in the high dose tank and low dose tank, respectively.

To check the efficacy of the flow through system radioactivity levels in the mixing chambers of both dose levels were determined on day 0 and 11. Total radioactivity amounted on average to 1054.1 ± 81.1 µg-eq/L (± 7.7%) and 102.6 ± 12.8 µg-eq/L (± 12.4%) in the high dose and low dose mixing chamber respectively.
During depuration, radioactivity levels in both exposure tanks rapidly decreased within the first day below 0.3% of the respective average exposure level and below background (0.23 and 0.02 µg-eq/L for high and low dose, respectively) on day 11.

In conclusion, sufficient radioactivity was delivered via the Hamilton dispenser system to obtain constant dose levels in the tanks reflecting on average 89.4% (high dose tank) and 93.5% (low dose tank) of the respective target concentration (i.e. 1000 µg/L and 100 µg/L for high dose and low dose, respectively).

Level of radioactivity in fish during exposure (bioconcentration) and depuration

Average residual radioactivity in edibles, non-edibles and whole fish during exposure to ^{14}C-labelled IR5885 at two dose levels during 14 days and during following depuration for 14 days are presented in Tables 8.2.6-03 and 8.2.6-04. Background levels are presented in Table 8.2.6-05.
The residues were expressed in µg parent equivalents/kg according to the total radioactivity found in edibles, non-edibles and whole fish and their fresh weights.

Tank 2, ^{14}C-high dose :

The residual radioactivity during exposure in edibles, non-edibles and whole fish (calculated) amounted on average to 1160 ± 355 (31%), 2685 ± 325 (12%) and 1940 ± 367 (19%) µg-eq/kg, respectively. The values in edible part of fish were in general below the detection limit (Table 4.2.1.1-05).

The residual radioactivity during depuration in edibles, non-edibles and whole fish (calculated) ranged from 428-486, 474-788 and 451-602 µg-eq/kg, respectively. After 14 days of depuration 18-37% of the respective average amount of radioactivity during exposure were found in whole fish and fish parts. All values determined in fish during depuration were below the detection limit (Table 4.2.1.1-03).

Table 4.2.1.1-03 Concentration of radioactivity in edible and non-edible parts of fish and in the whole fish during the test at the average high dose level of 893.5 µg/L (Cw) in tank 2.

Phase	Time interval		R_6	Concentration of radioactivity in fish (Cf)					
	I	II		edibles		non-edibles		whole fish*	
				µg-eq/kg	%**	µg-eq/kg	%**	µg-eq/kg	%**
A	4	---	1.4	1097 L	---	2645	---	2000	---
A	7	---	1.1	994 L	---	2676	---	1875	---
A	10	---	1.1	1755	---	3219	---	2522	---
A	12	---	0.9	814 L	---	2343	---	1539	---
A	14	---	0.8	1139 L	---	2542	---	1763	---
Mean				1160 L	100	2685	100	1940	100
± SD				355 (31%)	---	325 (12%)	---	367 (19%)	---
D	17	3	0.9	428 L	---	596 L	---	508 L	---
D	19	5	0.9	486 L	---	608 L	---	543 L	---
D	25	11	0.9	436 L	---	788 L	---	602 L	---
D	28	14	1.0	428 L	37	474 L	18	451 L	23

* Calculated value based on non-edibles/edibles fresh weight ratio R_6
L: below or at limit of detection in fish, see Table 8.2.3-05
R_6: Non-edibles/edibles fresh weight ratio
**: Residual radioactivity in fish in percentage of the average concentration in fish during exposure.

Tank 3, ^{14}C-low dose:

The residual radioactivity during exposure in edibles, non-edibles and whole fish (calculated) amounted on average to 142 ± 36 (25%), 283 ± 53 (19%) and 215 ± 46 (21%) µg-eq/kg, respectively.

The residual radioactivity during depuration in edibles, non-edibles and whole fish (calculated) ranged from 48-72, 60-111 and 52-90 µg-eq/kg, respectively. After 14 days of depuration 21-34% of the respective average amount of radioactivity during exposure were found in whole fish and fish parts. In general all values determined in fish during depuration were below the detection limit (Table 4.2.1.1-05).

Table 4.2.1.1-04 Concentration of radioactivity in edible and non-edible parts of fish and in the whole fish during the test at the average low dose level of 93.5 µg/L (Cw) in tank 3.

Phase	Time interval		R_6	Concentration of radioactivity in fish (Cf)					
	I	II		edibles		non-edibles		whole fish*	
				µg-eq/kg	%**	µg-eq/kg	%**	µg-eq/kg	%**
A	4	---	1.4	164	---	258	---	219	---
A	7	---	1.2	129	---	299	---	222	---
A	10	---	1.1	195	---	368	---	285	---
A	12	---	0.9	110	---	247	---	175	---
A	14	---	0.9	114	---	242	---	175	---
Mean				142	100	283	100	215	100
± SD				36 (25%)	---	53 (19%)	---	46 (21%)	---
D	17	3	0.9	72 L	---	111	---	90 L	---
D	19	5	0.9	60 L	---	93 L	---	76 L	---
D	25	11	1.0	43 L	---	62 L	---	52 L	---
D	28	14	1.0	48 L	34	60 L	21	54 L	25

*: Calculated value based on non-edibles/edibles fresh weight ratio R_6
L: below or at limit of detection in fish, see Table 8.2.3-05
R_6: Non-edibles/edibles fresh weight ratio
**: Residual radioactivity in fish in percentage of the average concentration in fish during exposure.

Tank 1, untreated :

Taking into account the specific radioactivity of 3.6 kBq/mg for the high dose level, the limit of detection amounted to 1228, 1170 and 1188 µg-eq/kg for edibles, non-edibles and whole fish (calculated), respectively. For the low dose level, at a specific radioactivity of 41.0 kBq/mg the limit of detection amounted to 109, 104 and 105 µg-eq/kg for edibles, non-edibles and whole fish, respectively.

Table 4.2.1.1-05 Background levels in edible and non-edible parts of untreated fish and in the whole untreated fish during incubation

(values given as dpm per 100 mg fish and expressed in parent equivalents on a fresh weight basis, i.e. mg/kg, taking into account the specific radioactivity of 218 dpm/µg and 2462 dpm/µg for high and low dose, respectively).

Time interval (days)	Edibles			Non-edibles			Whole fish *		
	dpm per 100 mg	µg/kg		dpm per 100 mg	µg/kg		R_6	µg/kg	
		HD	LD		HD	LD		HD	LD
4	17	783	69	16	723	64	1.3	749	66
14	8	348	31	6	283	25	0.8	319	28
28	9	390	35	7	329	29	0.9	361	32
Mean (BG)	11	507	45	10	445	39	---	476	42
SD	---	240	21	---	242	21	---	237	21
LD	---	1228	109	---	1170	104	---	1188	105

*: Calculated value based on non-edibles/edibles fresh weight ratio R6
LD: Limit of detection: Mean + 3x SD
BG: Background
SD: Standard deviation
R6: Non-edibles/edibles fresh weight ratio

In conclusion, the amount of radioactivity found in rainbow trout after high dose as well as after low dose exposure was about 2 fold higher than the exposure concentration in the respective tanks. Due to the extremely low accumulation of the test item, no plateau level could be determined and no half-life calculations and accumulation/depuration kinetics could be performed.

Bioconcentration factor

The bioconcentration parameters are summarised in Tables 4.2.1.1-06, 4.2.1.1-07 and 4.2.1.1-08.

Due to the extremely low accumulation of the test item for both dose levels, no plateau level could be determined for edibles, non-edibles and whole fish. Therefore, the average amount of residual radioactivity in fish during the whole exposure period was used for BCF calculations.

Based on the average radioactivity levels of 1160 and 142 µg-eq/kg in fish edibles after exposure to ^{14}C-labelled IR5885 at the high and low dose, respectively and the total radioactivity present in the exposure water (893.5 and 93.5 µg-eq/L, respectively) the calculated bioconcentration factor (BCF) amounted to 1.3 and 1.5, respectively.

Based on the average radioactivity levels of 2685 and 283 µg-eq/kg in fish non-edibles after exposure to ^{14}C-labelled IR5885 at the high and low dose, respectively and the total radioactivity present in the exposure water (893.5 and 93.5 µg-eq/L, respectively) the calculated BCF amounted to 3.0 for both dose levels.
Based on the average, calculated radioactivity levels of 1940 and 215 µg-eq/kg in whole fish after exposure to ^{14}C-labelled IR5885 at the high and low dose, respectively and the total radioactivity present in the exposure water (893.5 and 93.5 µg-eq/L, respectively) the calculated BCF amounted to 2.2 and 2.3, respectively.

In conclusion, an extremely low accumulation of IR5885 in rainbow trout (edibles, non-edibles and whole fish) was detected, resulting in BCF values below 4.
The lipid content in edible parts of fish amounted on day 4 to 28.6 mg/g and on day 14 to 46.8 mg/g. In non-edible parts of fish the lipid content on day 4 and day 14 was similar and amounted to 56.3 – 58.9 mg/g. Based on the decreasing ratio of non-edible parts to edible parts in fish during the study, the lipid content in whole fish was calculated to be similar on day 4 and day 14 (47.2-51.5 mg/g). Taking into account the respective Cf levels, average lipid-BCF values of both days in edibles, non-edibles and whole fish ranged from 35-50 and 43-46 as calculated for high and low dose, respectively.

Table 4.2.1.1-06 Bioconcentration factors in edible and non-edible parts of fish and in the whole fish during the test at the average high dose level of 893.5 µg/L (Cw) in tank 2.

Phase	Time interval		BCF**		
	I	II	edibles	non-edibles	whole fish
A	4	---	1.2	3.0	2.2
A	7	---	1.1	3.0	2.1
A	10	---	2.0	3.6	2.8
A	12	---	0.9	2.6	1.7
A	14	---	1.3	2.8	2.0
Mean			1.3	3.0	2.2
± SD			0.4 (31%)	0.4 (12%)	0.4 (19%)

**: Cf / Cw
I: Days after the onset of accumulation
II: Days after the onset of depuration
A: Accumulation
D: Depuration
SD: Standard deviation

Table 4.2.1.1-07 Bioconcentration factors in edible and non-edible parts of fish and in the whole fish during the test at the average lowdose level of 93.5 µg/L (Cw) in tank 3.

Phase	Time interval		BCF**		
	I	II	edibles	non-edibles	whole fish
A	4	---	1.8	2.8	2.3
A	7	---	1.4	3.2	2.4
A	10	---	2.1	3.9	3.1
A	12	---	1.2	2.6	1.9
A	14	---	1.2	2.6	1.9
Mean			1.5	3.0	2.3
± SD			0.4 (25 %)	0.6 (19 %)	0.5 (21 %)

**: Cf / Cw
I: Days after the onset of accumulation
II: Days after the onset of depuration
A: Accumulation
D: Depuration
SD: Standard deviation

Table 4.2.1.1-08 Bioconcentration factors calculated for the high dose and the low dose level based on lipid content in control fish as determined on day 4 and day 14

High Dose

	Lipid/g fish (mg)	Cf (µg-eq./kg fish)	Cf (µg-eq./kg lipid)	Cw (µg/L)	BCF** (lipid)
day 4, edibles	28.6	1097	38'328	893.5	43
day 14, edibles	46.8	1139	24'318	893.5	27
Mean BCF					35
day 4, non-edibles	58.9	2645	44'896	893.5	50
day 14, non-edibles	56.3	2542	45'113	893.5	50
Mean BCF					50
day 4, whole fish	47.2*	2000	42'381	893.5	47
day 14, whole fish	51.5*	1763	34'202	893.5	38
Mean BCF					43

Low Dose

	Lipid/g fish (mg)	Cf (µg-eq./kg fish)	Cf (µg-eq./kg lipid)	Cw (µg/L)	BCF** (lipid)
day 4, edibles	28.6	164	5'736	93.5	61
day 14, edibles	46.8	114	2'436	93.5	26
Mean BCF					44
day 4, non-edibles	58.9	258	4'377	93.5	47
day 14, non-edibles	56.3	242	4'289	93.5	46
Mean BCF					46
day 4, whole fish	47.2*	219	4'638	93.5	50
day 14, whole fish	51.5*	175	3'386	93.5	36
Mean BCF					43

* Calculated value based on the non-edibles/edibles fresh weight ratio (R10) of 1.6 at day 4 and 1.0 at day 14
** Cf / Cw

Chromatographic analysis of exposure water

The partitioned radioactivity from exposure water was characterised by TLC and HPLC analysis. Due to the low amount of radioactivity the aqueous phases were not analysed further.

Partitioning of exposure water: at both dose levels and at all time intervals, radioactivity found in tank water partitioned largely into the organic phase. For comparison between various time points, all values were normalised.
At the high dose radioactivity partitioned into ethylacetate ranged from 98.7 to 99.7% during 14 days exposure period. Accordingly, low amounts of radioactivity (0.3-1.3%) remained in the aqueous phase.
At the low dose, radioactivity partitioned into the organic phase ranged from 98.9 to 99.7%. Only low amounts of radioactivity (0.3-1.1%) remained in the aqueous phase.

Pattern of radioactivity in exposure water: At the high dose level, in the organic phase, four radioactive fractions were detected (W0, W1, W2 and W3/4).
After HPLC analysis, besides the parent item two radioactive fractions were detected (W2 and W3/4).
Radioactive fraction W1 (parent item) was found in an average amount of 96.2 ± 0.3% of the radioactivity recovered and radioactive fraction W3/4 in an average amount of 1.9 ± 0.6% of the radioactivity recovered. Radioactive fraction W2 was only detected on day 10 (0.7%) and day 12 (0.5%).

After TLC analyses besides the parent item one radioactive fraction was detected (W0). Radioactive fraction W1 (parent item) was found in an average amount of 97.2 ± 1.6% of the radioactivity recovered. Radioactive fraction W0 was only detected on day 10 and 12 (2.0% for both days).

At the low dose level, in the organic phase in total three radioactive fractions were detected (W0, W1 and W3/4).
After HPLC analyses besides the parent item only the radioactive fraction W3/4 was detected.
Radioactive fraction W1 (parent item) was found in an average amount of 96.6 ± 0.6% of the radioactivity recovered and radioactive fraction W3/4 in an average amount of 1.9 ± 0.3% of the radioactivity recovered.

After TLC analyses besides the parent item one radioactive fraction was detected (W0). Radioactive fraction W1 (parent item) was found in an average amount of 98.0 ± 1.1% of the radioactivity recovered. Radioactive fraction W0 was only detected on day 10 (2.4%).

In conclusion for both dose levels during the whole uptake period, besides the main radioactive fraction W1 (parent item), three minor radioactive fractions (W0,W2,W3/4) were detected in amounts below 3%.

Characterisation of radioactivity in exposure water

Radioactive fraction W1 (^{14}C-IR5885): After HPLC analysis the formation of significant amounts of PCBA in the exposure water was excluded.

After TLC analyses (5 cm x 20 cm plates, without chamber saturation) radioactive fraction W1 (one peak) showed Rf-values of 0.45 - 0.51. The unlabelled parent item showed identical Rf-values of 0.48 - 0.55 (reference spot not shown).

After TLC analyses with chamber saturation the results proved that neither IR5885-acid (code IR5839) nor PCBA occurred in the exposure water.

In conclusion based on TLC and HPLC analyses, radioactive fraction W1 showed the same HPLC and TLC behaviour as IR5885 and proved to be identical to the parent item.

Unknown radioactive fractions W0, W2 and W3/4: All unknown radioactive fractions represented minor metabolites.

The unknown radioactive fraction W0 was only detected by TLC analyses and behaved more apolar than the parent item.
The unknown radioactive fraction W2 was only detected by HPLC at the high dose level.
The unknown radioactive fraction W3/4 was only detected by HPLC analyses.
The HPLC behaviour of W3/4 strongly indicates, that radioactive fraction W3/4 was already present as impurity in the stock solutions in small amounts of 1.8 - 2.4%.

The occurrence of IR5885-acid was excluded.

CONCLUSIONS

The present study on rainbow trout proved lack of bioconcentration of IR5885 at exposure concentrations of 893.5 µg/L (high dose) and 93.5 µg/L (low dose). Therefore, no plateau level could be determined. Based on the average residual radioactivity in fish during exposure, BCF values of IR5885 in edibles, non-edibles and whole fish were extremely low (below 4). Therefore, no half-life calculations as well as accumulation/depuration kinetics could be performed.

For both dose levels, the major radioactive fraction in exposure water represented parent item. Additionally, in total three unknown radioactive fractions occurred in minor amounts (< 3%). IR5885 acid and PCBA were excluded in the exposure water.

4.2.2 Bioaccumulation test with other organisms

Data not available.

4.3 Acute aquatic toxicity

4.3.1 Short-term toxicity to fish

4.3.1.1 Acute toxicity - Rainbow Trout – IR5885

Reference Number:	KII- Section 6, Volume 1, Annex IIA, point 8.2.1.1/01
Report:	See annex conf. 16
Guidelines:	Commission Directive 92/69/EEC, Annex Part C, C.1, 1992 OECD Guideline for Testing of Chemicals, Section 2, No. 203, 1992
Deviations:	None
GLP:	Yes; certified laboratory

Executive Summary:

Groups of 7 young Rainbow trout (*Oncorhynchus mykiss* – length: 4.78 ± 0.48 cm; wet weight: 1.01 ± 0.23 g) were exposed in a static test to aqueous test media containing IR5885. After a pre-test (range finding test) and a pre experiment carried out to find out the range of concentrations to be tested in the main test and the solubility of the IR5885 in the test medium, respectively, the limit concentration of 100 mg/L of the test item suspended in methyl cellulose and two controls holding water and water with methyl cellulose, respectively were tested in order to determine the mortality and symptoms of intoxication over periods of 2.5, 24, 48, 72, 96 hours after start of test. pH, dissolved oxygen concentration and water temperature were recorded daily in each experimental group as well as for the other environmental parameters as light intensity, light regime. Duplicate samples from the freshly prepared test media of the only test concentration and the vehicle control were taken at the start of the test in order to verify the concentration of the test item in the test media. For the determination of the stability of the test item under the test conditions, respectively the maintenance of the test item concentrations during the test period, samples of the test media and vehicle control were taken in duplicate on Day 2 and Day 4 of exposure.
The environmental parameters (pH, dissolved oxygen concentration and water temperature) were into the acceptable range.
The analytically determined concentration of IR5885 was 95 % of the nominal concentration, as average. The active ingredient, was judged to be sufficiently stable during the test period of 96 hours. Thus, all results were related to the nominal concentration of the test item.

In the control and at the test concentration of 100 mg/L no mortality and no symptoms of intoxication were observed. The NOEC of IR5885 in Rainbow Trout resulted to be 100 mg/L, while the LC_{50} and the LOEC were higher than 100 mg/L.

MATERIALS AND METHODS

MATERIALS

Test Material	IR5885
Description	white powder
Lot/Batch #	T 025/02
Purity	99.56% (w/w)
CAS #	283159-90-0

Test Animals:	
Species:	*Oncorhynchus mykiss* (Rainbow trout)
Age:	juveniles
Weights:	the mean body length of the fish (10 fish from the test fish batch were measured during the course of the test) in the test was 4.78 ± 0.48 cm (Mean ± SD), the mean body wet weight was 1.01 ± 0.23 g (Mean ± SD).
Source:	Forellenzuchtbetrieb Tautenhahn, 98646, Trostadt, Germany
Acclimation period:	for 12 days before the start of the test the fish were acclimated to the test temperature
Diet:	during holding until one day before the test start the fish were fed a commercial fish diet for Rainbow Trout.
Test Water:	Reconstituted water
Test Units:	20-litre glass aquaria with 10-litre test medium. Each test unit was uniquely identified with study number, treatment and replicate number.

Test Conditions:	
Surrounding Type:	controlled environment room
Light Regime:	16h light : 8h dark
Light Intensity:	380-420 lux
Aeration of Test Water:	the test media were slightly aerated during the test
pH Values:	pH 7.8 to 8.0
Dissolved Oxygen Concentrations:	at least 8.7 mg/L or higher
Water Temperature:	16-17 °C

STUDY DESIGN AND METHODS

Dates:	Experimental Start:	October 28, 2002
	Experimental Termination:	December 20, 2002

Dosage Preparation and Dosing

Pre-experiments: pre-experiments were carried out to determine the solubility of the test item in test water (not in compliance with GLP regulations).

Range-finding: the test concentrations were based on the results of a range finding test (not in compliance with GLP regulations).

Test concentrations: the only concentration tested was nominal 100 mg test item/L. Thus a limit test was performed in accordance with the Directive 92/69/EEC to demonstrate that the test item has no toxic effects up to this concentration.
The test medium with the nominal test item concentration of 100 mg/L was prepared by dosing 1 g test item and 1 g methyl cellulose into 10 L test water. The test item was mixed into the test water as homogeneously as

possible by ultrasonic treatment for 30 minutes and intense stirring for 72 hours. The test medium was freshly prepared just before the start of the test (introduction of the test fish).
Control: test water was used without addition of the solvent and test item. A solvent control ran in addition to the control. The solvent additive concentration was the same in all test media and the solvent control (100 mg methyl cellulose/L).

Analysis of IR5885 concentration:

Duplicate samples from the freshly prepared test media of the test concentration and the solvent control were taken at the start of the test. For the determination of the stability of the test item under the test conditions, samples were taken in duplicate out of the test media and the solvent control on Day 2 and Day 4 of exposure.
The samples were prepared for chromatography directly after sampling. The samples were stored in the refrigerator until analysis was performed and the remaining part of the samples was stored in refrigerator and protected from light until the study completion.

The concentration of IR5885 was analysed in the duplicate test media from the test concentration of nominal 100 mg/L at all sampling times. Only one of the duplicates was analysed at each sampling time from the solvent control samples.

Experimental Design

At the start of the test 7 fish were randomly introduced into each aquarium. A static test procedure was chosen according to the pre-experiment. No feeding was performed during the 96-hour exposure period.
The test fish were observed at approx. 2.5, 24, 48, 72 and 96 hours test duration for symptoms of intoxication and mortality. The water temperature, pH-values and the dissolved oxygen concentrations were determined daily in the test media of the only test concentration and the controls. The behaviour of the test item in the only test concentration was determined once every day during the test.

RESULTS AND DISCUSSION

Analytical results

During the test period test item concentrations were measured in the range from 95% to 96% of the nominal value at the start of the test. At the end of the test concentrations ranging from 89% to 92% of nominal were found. Under the test conditions IR5885 resulted sufficiently stable during the 96 hours period, therefore all the reported results are related to the nominal concentration of the test item.

Behaviour of test item: in the solvent control and the 100 mg/L test concentration, foam was observed. At 100 mg/L the test medium was coloured and turbid from the test item and a part of test item was swimming at water surface.

Biological results:

Signs of intoxication: in the control and in the test medium of 100 mg/ test item/L all fish survived until the end of the test and no signs of intoxication were observed. In the solvent control one fish was dead after 72 hours. However, this mortality was not related to the solvent since all the other fish were healthy and in a first experiment all fish survived in the solvent control and showed no symptoms of intoxication.

Results after 96 hours: the 96-hour NOEC and the 96-hour LC_0 of IR5885 to Rainbow Trout were determined to be at least 100 mg test item/L. The NOEC and LC_0 might even be higher. The 96-hour LOEC, the 96-hour LC_{50} and the 96-hour LC_{100} are clearly higher than 100 mg test item/L. These values could not be quantified since concentrations in excess of 100 mg/L have not been tested in accordance with the test guideline.

CONCLUSIONS

The NOEC of IR5885 on Rainbow Trout resulted to be 100 mg test item/L, while the LC_{50} and the LOEC were higher than 100 mg test item/L.

4.3.1.2 Acute toxicity to Zebra fish (Brachydanio rerio)

Reference Number:	KII- Section 6, Volume 1, Annex IIA, point 8.2.1.2/01
Report:	See annex conf. 19
Guidelines:	Commission Directive 92/69/EEC, Annex Part C, C.1, 1992 OECD Guideline for Testing of Chemicals, Section 2, No. 203, 1992
Deviations:	Concerning the test conditions, the temperature in the aquaria was determined to be 20°C during the test period, instead of 21–24°C (with a maximum deviation of ± 1 °C) according to the study protocol. This deviation has technical reasons, but there are no effects on the study, since the deviation in only minor.
GLP:	Yes; certified laboratory

Executive Summary:

Groups of 7 juveniles Zebra fish were exposed in a static test to aqueous test media containing IR5885. After a pre test (range finding test) and a pre experiment carried out to find out the range of concentrations to be tested in the main test and the solubility of the IR5885 in the test medium, respectively, one concentration at 100 mg/L of the test item suspended in methyl cellulose and two controls holding water and water with methyl cellulose, respectively were tested in order to determine the mortality and symptoms of intoxication over periods of 2.5, 24, 48, 72, 96 hours after start of test. pH, dissolved oxygen concentration and water temperature were recorded daily in the test media of 100 mg/L and in the controls, as well as for the other environmental parameters as light intensity, light regime. For the analytical dose verification of IR5885 duplicate samples from the freshly prepared test media of the test concentration and the vehicle control were taken at the start of the test. For the determination of the stability of the test item under the test conditions, respectively the maintenance of the test item concentrations during the test period, samples of the test media and vehicle control were taken in duplicate on Day 4 of exposure.
The environmental parameters (pH, dissolved oxygen concentration and water temperature) were into the acceptable range.
The analytically determined concentration of IR5885 in analysed test medium varied in the range from 90% to 95% of nominal. The active ingredient was judged to be sufficiently stable during the test period of 96 hours. Thus, all results were related to the nominal concentration of the test item.

In the control and at the test concentration of 100 mg/L no mortality and no signs of intoxication were observed. The NOEC of IR5885 in Zebra fish resulted to be 100 mg/L, while the LC_{50} and the LOEC were higher than 100 mg/L.

MATERIALS AND METHODS

MATERIALS

Test Material	IR5885
Description	white powder
Lot/Batch #	T 025/02
Purity	99.56% (w/w)

CAS #	283159-90-0
Test Animals:	
Species:	*Brachidanio rerio* (Zebra fish)
Age and Size:	juveniles; the mean body length of the fish (10 fish from the test fish batch were measured during the course of the test) in the test was 3.11 ± 0.18 cm (Mean ± SD), the mean body wet weight was 0.24 ± 0.06 g (Mean ± SD).
Source:	City Zoo, 64572 Büttelborn, Germany
Acclimation period:	for 4 weeks before the start of the test the fish were acclimated to the test temperature
Diet:	during holding until one day before the test start the fish were fed a commercial fish diet.
Test Water:	Reconstituted water
Test Units:	9-litre glass aquaria with 4-litre test medium. Each test unit was uniquely identified with study number, treatment and replicate number.

Test Conditions:	
Surrounding Type:	controlled environment room
Light Regime:	16h light : 8h dark
Light Intensity:	510-750 lux
Aeration of Test Water:	the test media were slightly aerated during the test
pH Values:	7.8 to 8.1
Dissolved Oxygen Concentrations:	at least 8.5 mg/L or higher
Water Temperature:	20 °C

STUDY DESIGN AND METHODS

In life dates:	Experimental Start:	May 27, 2002
	Experimental Termination:	December 13, 2002

Dosage Preparation and Dosing

Pre-experiments: pre-experiments were carried out to determine the solubility of the test item in test water (not in compliance with GLP regulations).

Range-finding: the test concentrations were based on the results of a range finding test (not in compliance with GLP regulations).

Test concentrations: the only concentration tested was nominal 100 mg test item/L. Thus a limit test was performed in accordance with the Directive 92/69/EEC to demonstrate that the test item has no toxic effects up to this concentration.
The test medium with the nominal test item concentration of 100 mg/L was prepared by dosing 400 mg test item and 400 mg methyl cellulose into 4 L test water. The test item was mixed into the test water as homogeneously as possible by ultrasonic treatment for 30 minutes and intense stirring for 72 hours. The test medium was freshly prepared just before the start of the test (introduction of the test fish).

Control: in the control test water was used without addition of the solvent and test item. A solvent control ran in addition to the control. The solvent additive concentration was the same in all test media and the solvent control (100 mg methyl cellulose/L).

Analysis of IR5885 concentration:

Duplicate samples from the freshly prepared test media of the test concentration and the solvent control were taken at the start of the test. For the determination of the stability of the test item under the test conditions, samples were taken in duplicate out of the test media and the solvent control on Day 4 of exposure.
All samples were analysed directly after sampling.
The concentration of IR5885 was analysed in the duplicate test media samples from the test concentration of nominal 100 mg/L from all sampling times. From the solvent control samples only one of the duplicate was analysed from each of all sampling times.

Experimental Design

At the start of the test 7 fish were introduced into each aquarium in random order. No feeding was performed and the exposure time was of 96 hours.
The test fish were observed after approx. 2.5, 24, 48, 72 and 96 hours test duration for symptoms of intoxication and mortality. The water temperature, pH-values and the dissolved oxygen concentrations were determined daily in the test media of the only test concentration and the controls. The behaviour of the test item in the only test concentration was determined once every day during the test.

RESULTS AND DISCUSSION

Analytical results

During the test period test item concentrations were measured in the range from 90% to 95% of the nominal value at the start of the test. Under the test conditions IR5885 resulted sufficiently stable during the 96 hours period, therefore all the reported results are related to the nominal concentration of the test items.

Behaviour of test item: in the solvent control and the 100 mg/L test concentration, foam was observed. At 100 mg/L the test medium was coloured by the test item and a part of test item was swimming at water surface and was lying at the bottom of the aquarium.

Biological results:

Signs of intoxication: in the control and in the test medium of 100 mg test item/L all fish survived until the end of the test and no signs of intoxication were observed.

Results after 96 hours: the 96-hour NOEC and the 96-hour LC_0 of IR5885 to Zebra fish were determined to be at least 100 mg test item/L. The NOEC and LC_0 might even be higher. The 96-hour LOEC, the 96-hour LC_{50} and the 96-hour LC_{100} are clearly higher than 100 mg test item/L.

CONCLUSIONS

The NOEC of IR5885 on Zebra fish resulted to be 100 mg test item/L, while the LC_{50} and the LOEC were higher than 100 mg test item/L.

4.3.1.3 Acute toxicity to Sheepshead Minnow (Cyprinodon variegatus)

Reference Number: KII - **Post submission 02**, Section 6, Volume 2, Annex IIA, point 8.2.1/03

Report:	**See annex conf. 29**

Guidelines: U.S. EPA OPPTS NUMBER 850.1075

Deviations: none

GLP: Yes; certified laboratory

Executive Summary:

The objective of this study was to determine the toxicity of IR5885 to the sheepshead minnow, *Cyprinodon variegatus*, during a 96-hour exposure period under static test conditions.
Sheepshead minnows were exposed to a geometric series of five test concentrations, a negative control (filtered saltwater), and a solvent control (dimethyl formamide). Two replicate test chambers were maintained in each treatment and control group, with 10 sheepshead minnows in each test chamber, for a total of 20 fish per test concentration.
Nominal test concentrations were selected in consultation with the Sponsor, and were based upon the results of an exploratory range finding toxicity test. Nominal test concentrations selected were 1.9, 3.8, 7.5, 15 and 30 mg IR5885/L. When measured concentrations of the samples collected throughout the test were averaged, the mean measured test concentrations for this study were 1.7, 3.2, 6.7, 12 and 15 mg/L, representing 89, 84, 89, 80 and 50% of nominal concentrations, respectively. The results of the study were based on the mean measured concentrations.
All organisms were observed periodically to determine the number of mortalities in each treatment group. The numbers of individuals exhibiting signs of toxicity or abnormal behavior also were evaluated. Observations were made approximately 5, 24, 48, 72 and 96 hours after test initiation.
There were no treatmentrelated effects noted at any concentration tested. The 96-hour LC_{50} value was >15 mg/L, the highest concentration tested. The no-mortality concentration and the NOEC were 15 mg/L.

MATERIALS AND METHODS

MATERIALS

Test Material IR5885
Description white powder
Lot/Batch # T 025/02
Purity 99.63% (w/w)
CAS # 283159-90-0

Test Animals:
Species: Sheepshead Minnow (*Cyprinodon variegatus*)
Age and Size: juveniles; the average total length of 10 negative control fish measured at the end of the test was 2.7 cm, with a range of 2.2 to 3.3 cm. The average wet weight (blotted dry) of 10 negative control fish measured at the end of the test was 0.30 grams, with a range of 0.13 to 0.57 grams.
Source: Aquatic BioSystems, Inc. Of Fort Collins, Colorado.
Acclimation period: at least 14 days prior to the test in water from the same source and at approximately the same temperature as used during the test
Diet: Daily during the holding period, the fish were fed a commercially- repared diet supplied by Zeigler Brothers, Inc., Gardners, Pennsylvania, and *Artemia*

	nauplii supplied by Brine Shrimp Direct, Ogden, Utah. The fish were not fed for at least two days prior to the test or during the test.
Test Water:	natural seawater collected at Indian River Inlet, Delaware that was filtered and diluted to a salinity of approximately 20‰ with well water.
Test Units:	Test chambers were 25-L stainless steel aquaria containing 20 L of test solution. The depth of the test water in a representative test chamber was 23.5 cm. Test chambers were indiscriminately positioned in an environmental chamber designed to maintain the desired test temperature throughout the test period.

Test Conditions:	
Light Regime:	16 hours of light and 8 hours of darkness
Light Intensity:	91 lux
pH Values:	8.0 to 8.5
Dissolved Oxygen Concentrations:	6.7 to 7.4 mg/L (= 86% of saturation)
Water Temperature:	22 ± 2ºC

STUDY DESIGN AND METHODS

1.	**In life dates:**	Experimental Start:	July 31, 2005
		Experimental Termination:	August 5, 2005

Dosage Preparation and Dosing

A primary stock solution was prepared by dissolving the test substance in dimethyl formamide (DMF) at a concentration of 300 mg/mL. The stock was sonicated for10 minutes and inverted to mix, and appeared clear and very light yellow after mixing. Aliquots of the primary stock solution were serially diluted with DMF to prepare secondary stock solutions at concentrations of 19, 38, 75 and 150 mg/mL. The secondary stocks were mixed by inversion and ranged in appearance from clear and colorless to clear and very slightly yellow. Aliquots of the appropriate primary or secondary stock solution were diluted in 20 L of filtered saltwater to prepare two replicates of each test solution at nominal concentrations of 1.9, 3.8, 7.5, 15 and 30 mg/L. The test solutions were mixed for approximately 1 – 2 minutes using top-down electric mixers. The solvent control solution was prepared by mixing 2.0 mL of DMF into 20 L of dilution water. The solvent concentration in the solvent control and all test solutions was 0.1 mL/L. The 1.9, 3.8 and 7.5 mg/L test solutions appeared clear and colorless at test initiation and termination. The 15 and 30 mg/L test solutions appeared clear, with a white precipitate present on the surface at test initiation and on the surface and bottom of the test chambers at test termination.

Analysis of IR5885 concentration:

The method used for the analysis of IR5885 in saltwater samples consisted of diluting the samples in saltwater, as necessary, and analyzing by direct injection high performance liquid chromatography (HPLC) with UV detection at 220 nm. Concentrations of IR5885 in the samples were determined by HPLC using an Agilent Series 1100 High Performance Liquid Chromatograph (HPLC) equipped with an Agilent Series 1100 Variable Wavelength Detector. Chromatographic separations were achieved using a YMC-Pack ODS AM column (150 mm x 4.6 mm, 3-µm particle size).
Calibration standards of IR5885, ranging in concentration from 1.00 to 10.0 mg/L, were prepared in saltwater using a stock solution of IR5885 in acetonitrile. Fresh standards were prepared and analyzed with each sample set. Linear regression equations were generated using the sum of the peak area responses versus the respective concentrations of the calibration standards. The concentration of IR5885 in the samples was determined by substituting the sum of the peak area responses of the samples into the applicable linear regression equation.

The method limit of quantitation (LOQ) for these analyses was defined as 1.00 mg/L, calculated as the product of the concentration of the lowest calibration standard (1.00 mg/L) and the dilution factor of the matrix blank samples (1.00). Three matrix blank samples were analyzed to determine possible interferences.
No interferences were observed at or above the LOQ during the sample analyses.
Samples of saltwater were fortified at 1.80, 7.00 and 30.0 mg/L using a stock solution of IR5885 in acetonitrile, and were analyzed concurrently with the test samples. The measured concentrations for the matrix fortification samples ranged from 92.6 to 102% of nominal concentrations.

Experimental Design

All organisms were observed periodically to determine the number of mortalities in each treatment group. The numbers of individuals exhibiting signs of toxicity or abnormal behavior also were evaluated. Observations were made approximately 5, 24, 48, 72 and 96 hours after test initiation.
Dissolved oxygen and pH were measured in each test chamber at the beginning and end of the test and at approximately 24-hour intervals during the test. Temperature was measured in each test chamber at the beginning and end of the test and at approximately 24-hour intervals during the test. Temperature also was measured continuously during the test in one negative control test chamber.

RESULTS AND DISCUSSION

Analytical results

Nominal concentrations selected for use in this study were 1.9, 3.8, 7.5, 15 and 30 mg/L. Samples collected during the test from the 1.9, 3.8 and 7.5 mg/L solutions had measured concentrations that ranged from 80 to 96% of nominal concentrations. Samples collected during the test from the 15 and 30 mg/L solutions in which visible precipitates were present had measured concentrations that ranged from 27 to 89% of nominal concentrations. The lower analytical results and the presence of precipitate at 15 and 30 mg/L confirmed that the test was conducted to the limit of solubility. When measured concentrations of the samples collected throughout the test were averaged, the mean measured test concentrations for this study were 1.7, 3.2, 6.7, 12 and 15 mg/L, representing 89, 84, 89, 80 and 50% of nominal concentrations, respectively. The results of the study were based on the mean measured concentrations.

Biological results:

All sheepshead minnows in the negative and solvent control groups appeared healthy and normal throughout the test. All sheepshead minnows in the 1.7, 3.2, 6.7, 12 and 15 mg/L treatment groups also appeared normal throughout the test, with no mortalities or overt signs of toxicity observed.
The no mortality concentration and the NOEC were 15 mg/L. LC_{50} values at 24, 48, 72 and 96 hours were estimated to be greater than 15 mg/L, the highest concentration tested.

CONCLUSIONS

Sheepshead minnows, *Cyprinodon variegatus*, were exposed for 96 hours under static conditions to five mean measured concentrations of IR5885 ranging from 1.7 to 15 mg/L. There were no treatmentrelated effects noted at any concentration tested. The 96-hour LC_{50} value was >15 mg/L, the highest concentration tested. The no-mortality concentration and the NOEC were 15 mg/L.

4.3.1.4 Acute toxicity to Bluegill sunfish (Lepomis macrochirus)

Report:	See annex conf 65

Guidelines: OPPTS 850.1075

Deviations: None

GLP: Yes

Executive Summary
In a 96-hour acute toxicity study, Bluegill sunfish (*Lepomis macrochirus*) were exposed to valifenalate technical at nominal concentrations 0 (control), 0 (vehicle control), and 40 mg a.i./L under static conditions in accordance with the OPPTS 850.1075 guideline. The control treatment met the acceptability criteria for survival set by the study protocol. The 24-, 48-, 72-, and 96-hour LC_{50} values, based on nominal concentrations, were estimated to be >40 mg a.i./L, the highest concentration tested. The 96-hour NOEC was 40 mg a.i./L, based on less than 10% mortality and a lack of observed sublethal effects at this, the highest nominal test substance concentration.

Materials and Methods:

Experimental Test Dates: 30 July 2014 to 03 August 2014

Test Material:

Name:	Valifenalate Technical
Batch no.:	PL14-0060 (Manufacturing Lot No.: P/13/019 (Isagro))
Analysed content:	98.9%
Expiry:	03 March 2015

Test Species:

Species:	Bluegill sunfish (*Lepomis macrochirus*)
Source:	Osage Catfisheries, Osage Beach, Missouri

Test Conditions:

Temperature:	21.2 to 22.8°C
Dissolved Oxygen:	5.7 to 8.5 mg/L (67 to 101% sat.)
pH:	8.0 to 8.4
Alkalinity:	152 mg CaCO3/L
Hardness:	150 mg CaCO3/L
Conductivity:	341 µS/cm
Photoperiod:	16-hr light:8-hr dark
Light Intensity:	699 lux

Test Design: Based on the functional solubility of the test substance in freshwater, nominal concentrations of 0 (control), 0 (vehicle control, 0.50 mL dimethylformamide (DMF)/L), and 40 mg a.i./L were selected for the definitive test. The test chambers were 21-L glass jars, each containing approximately 20 L of control or test solution in the definitive test. Each glass jar was covered with a clear glass lid. The jars were labelled with study number, treatment, and replicate. For the treatment solution, 10 mL of primary standard

was stirred into a 21 L glass jar containing a volume of 20 L blended freshwater (FW-blended). Vehicle control solutions were prepared at a concentration of 0.50 mL DMF/L by stirring the appropriate volume of DMF into dilution water. The control consisted of dilution water only. Test fish were impartially assigned to treatments by adding one fish per chamber proceeding from the control to vehicle control then test substance treatment, and repeating steps as necessary until ten fish were present in each replicate test chamber. The treatments were replicated three times for a total of 30 fish per treatment, with individual replicates within each treatment labelled A, B, or C respectively. Observations for mortality and sublethal responses were made at approximately 24, 48, 72, and 96 hours.

Test solutions were analyzed for the concentrations of valifenalate technical using a high performance liquid chromatography with ultraviolet detection (HPLC-UV) system. Analysis was accomplished based on a method provided by the Sponsor and validated at ABC Laboratories, Inc. The concentration of valifenalate technical was measured in test solution (freshwater) samples collected at initiation (0 hour), 48, and 96 hours of the definitive test. Control and valifenalate technical-fortified samples were also prepared for analysis at each sample period.

Since mortality was less than 10% in the control and test substance treatment, no statistical analyses were performed.

Validity Criteria:
The following criteria must be found in the control replicates:

- <10% mortality in control

Results and Discussion:
Validity Criteria: The results of the control and vehicle control met the required guideline validity criteria.
***Analytical results*:** Measured concentrations of valifenalate technical in the replicate test substance treatment solutions at initiation (0 hour) were 36.8, 38.2, and 37.5 mg a.i./L, which represented recoveries of 92 to 96% of the nominal concentrations. Measured concentrations in the replicate test solutions at 48 hours were 35.8, 37.5, and 37.4 mg a.i./L, or 90 to 94% of the nominal concentrations. Measured concentrations in the replicate test solutions at 96 hours were 36.4, 36.3, and 36.0 mg a.i./L, or 90 to 91% of the nominal concentrations. No residues of valifenalate technical were detected in the control or vehicle control solutions above the MQL for this analyte during the exposure period. The mean measured valifenalate technical concentrations in the replicate test substance treatment solutions were 36.3, 37.3, and 37.0 mg a.i./L, which represented recoveries of 91 to 93% of the nominal concentrations.

Biological Results: After 96 hours of exposure, mortality was 0% in the 0 (control), 0 vehicle control, and 40 mg a.i./L treatment. The 24-, 48-, 72-, and 96-hour LC_{50} values, based on nominal concentrations, were estimated to be >40 mg a.i./L, the highest concentration tested. No sublethal effects were observed. The 96-hour NOEC was 40 mg a.i./L, based on less than 10% mortality and a lack of observed sublethal effects at this, the highest nominal test substance concentration.

Table 1: Effect of valifenalate technical on mortality (bluegill sunfish, *Lepomis macrochirus*)

Nominal concentration (mg a.i./L)	Initial fish count	Cumulative Mortality (% Mortality)			
		24 Hours	48 Hours	72 Hours	96 Hours
Negative control	10	0 (0)	0 (0)	0 (0)	0 (0)
Solvent control	10	0 (0)	0 (0)	0 (0)	0 (0)
40	10	0 (0)	0 (0)	0 (0)	0 (0)

Conclusions: Acute toxicity to Bluegill Sunfish was tested with Valifenalate technical. The control treatment met the acceptability criteria for survival set by the study protocol. The 24-, 48-, 72-, and 96-hour LC_{50} values, based on nominal concentrations, were estimated to be >40 mg a.i./L, the highest concentration tested. The 96-hour NOEC was 40 mg a.i./L, based on less than 10% mortality and a lack of observed sublethal effects at this, the highest nominal test substance concentration.

4.3.2 Short-term toxicity to aquatic invertebrates

4.3.2.1 Acute toxicity to aquatic invertebrates - Daphnia magna

Reference Number: KII- Section 6, Volume 2, Annex IIA, point 8.3.1.1/01

Report:	See annex conf. 13

Guidelines: Commission Directive 92/69/EEC, Annex Part C, C.2 "Acute Toxicity for *Daphnia*", Official Journal of the European Communities No. L383, 29 December, 1992

OECD Guideline for testing of Chemicals, Section 2, No. 202, April 04 1984

Deviations: None

GLP: Yes; certified laboratory

Executive Summary:

The aim of this study was to determine the influence of the test item IR5885 on the mobility and survival of *Daphnia magna*. A pre test (range finding test) was carried out to find out the range of concentrations to be tested in the main test. In progress of that one, groups of 20 young Daphnia were exposed, in static conditions, to the test item IR5885 suspended in methylcellulose for a period of 48 hours. The concentrations tested were: 4.6, 10, 21, 46 and 100 mg/L. Two control groups were tested in parallel; one holding water and the other holding water with 100 mg methyl cellulose/L. Besides, a parallel test (analytical test) was carried out to verify that the concentration of test item was constantly above 80 % of initial concentration over all the period of the test. As regards to the analytical phase, under test condition the active ingredient was stable during the test period (48 h) with mean of recoveries in the test samples of 93%. Concerning the biological results no significant immobility or mortality up to the highest concentration tested was observed. Thus the EC50 and the NOEC were determined to be higher than 100 mg/L and 100 mg/L, respectively.

MATERIALS AND METHODS

MATERIALS

Test Material	IR5885
Description	white powder
Lot/Batch #	T 025/02
Purity	99.56% (w/w)
CAS #	283159-90-0
Test Animals:	
Species:	*Daphnia magna* (Straus), clone 5
Age:	7-22 hours old
Source:	supplied 1997 by the Umweltbundesamt, Institut fur Wasser-Boden und Lufthygiene, Berlin, Germany
Acclimation period:	for 7 hours under test conditions
Breeding conditions:	*Daphnia* were bred at IBACON Lab. under similar temperature and light conditions as in the test and in reconstituted water of similar pH, ions components and total hardness as the test water. The test organisms were not first brood progeny.
Test Water:	Reconstituted water

Test Units: glass beakers of 250 mL with 150 mL test medium. Each test unit was uniquely identified with study number, treatment and replicate number.

Test Conditions:
Surrounding Type: controlled environment room
Water temperature: 21-22°C
Light Regime: 16h light / 8h dark
Light Intensity: 420 lux
pH Values: pH 7.7 to 8.1
Dissolved Oxygen Concentrations: 8.2 mg/L or higher
Water Temperature: 21-22 °C

Toxic Standard: for the evaluation of the quality of the *Daphnia* clone and the experimental conditions the substance potassium dichromate p.A. is tested at least twice a year in order to demonstrate satisfactory test conditions.

STUDY DESIGN AND METHODS

Dates: Experimental Start: May 27, 2002
Experimental Termination: May 29, 2002

Dosage Preparation and Dosing

Pre-experiments: pre-experiments were carried out to determine the solubility of the test item in test water (not in compliance with GLP regulations).

Range-finding: the test concentrations were based on the results of a range finding test (not in compliance with GLP regulations).

Test concentrations: 4.6, 10, 21, 46 and 100 mg test item/L. Additionally, a control and a solvent control (100 mg methyl cellulose/L) were tested in parallel.

Dosage of the test Item: before the test start, the test medium of the highest test concentration of nominal 100 mg/L was prepared by dosing 100 mg test item and 100 mg methyl cellulose in 1 litre test water. The test item was mixed into test water as homogeneously as possible by intense stirring for 75 hours and short ultrasonic treatment for 15 minutes.
Adequate volumes of this test concentration were diluted with test water to prepare the desired concentrations. The solvent additive concentrations were the same in all test media and the solvent control.

Analysis of IR5885 concentration:

One sample from the freshly prepared stock solution and duplicate samples from the freshly prepared test media of the test concentration and the solvent control were taken at the start of the test. For the determination of the stability of the test item under the test conditions, duplicate samples from the test media of all test concentrations and the solvent control were collected at the end of the test (48 hours).
All samples were analysed directly after sampling.
The concentrations of IR5885 were analysed in the duplicate test media samples from all the test concentrations and both sampling times (0 and 48 hours). From the solvent control samples only one of the duplicate was analysed from each of all sampling times.

Experimental Design:

At the start of the test 20 Daphnia per control and test concentrations divided into two groups of ten animals, each group in 150 mL test medium, were introduced into the beakers. The exposure time was of 48 hours.
The immobility or mortality of the *Daphnia* was determined by visual controls after 24 and 48 hours. Those animals not able to swim within 15 seconds after gentle agitation of the test beaker were considered to be immobile.

At the beginning of the test and at the end the pH values and the dissolved oxygen concentrations were determined in the test media of all test concentrations and the controls. At the same time the water temperature was determined in the test medium of one control beaker.

The behaviour of the test item in test water was determined at the start of the test, after 24 and 48 hours test duration in all test concentrations.

RESULTS AND DISCUSSION

Analytical results:

During the test period test item concentrations were measured in the range from 83 to 103 of the nominal values measured. Under the test conditions IR5885 resulted sufficiently stable during the 48 hours period, therefore all the reported results are related to the nominal concentration of the test items.

Behaviour of test item: in the freshly prepared test media foam was observed in all test concentrations and the solvent control. At the test concentrations of 21 to 100 mg/L the test media were turbid for the test item and at 100 mg/L also a part of the test item was swimming on the water surface.
After 24 and 48 hours at the concentrations from 4.6 to 21 mg/L and the solvent control no remarkable observations were made. At 46 and 100 mg/L the test media were turbid and a part of the test item was swimming on the surface. However, no influence on the outcome of the study came from such a behaviour since at the two highest test concentrations the analysis gave values between 86% and 96% of the nominal concentrations.

Biological results:

Signs of intoxication after 24 and 48 hours: after 24 and 48 hours in the controls and in the test concentrations up to 100 mg test item/L no mortality or immobilization of the test animals were observed. At the two highest test concentrations of 46 and 100 mg/L the bodies of the Daphnia were swollen, however this had no influence on the mobility of the test animals.

CONCLUSIONS

The 24-hour and 48-hour NOEC and the 24-hour and 48-hour EC_0 of IR5885 to *Daphnia magna* were determined to be at least 100 mg test item/L were not tested. The NOEC and EC_0 might even be higher not having tested concentrations above 100 mg/L. The 48-hour LOEC and the 48-hour EC_{50} were clearly higher than 100 mg test item/L. These values could not be quantified due to the absence of toxicity of IR5885 up to the highest concentration.

4.3.2.2 Acute toxicity test with the saltwater mysid (*Americamysis bahia*)

Reference Number: KII - **Post submission 02**, Section 6, Volume 2, Annex IIA, point 8.2.4/02

Report:	**See annex conf. 31**

Guidelines: U.S. EPA OPPTS NUMBER 850.1035

Deviations: None

GLP: Yes; certified laboratory

Executive Summary:

The objective of this study was to determine the toxicity of IR5885 to the saltwater mysid, *Americamysis bahia*, during a 96-hour exposure period under static test conditions. Saltwater mysids were exposed to a geometric series of five test concentrations, a negative control (filtered saltwater), and a solvent control (dimethyl formamide). Two replicate test chambers were maintained in each treatment and control group, with 10 mysids in each test chamber, for a total of 20 mysids per test concentration. Nominal test concentrations were selected in consultation with the Sponsor, and were based upon the results of exploratory range finding toxicity tests and the results of the initial trial. Nominal test concentrations selected were 0.50, 1.0, 2.0, 4.0 and 8.0 mg IR5885/L. Mean measured test concentrations were determined from samples of test water collected from each treatment and control group at test initiation, at the approximate mid-point of the test and at test termination. The mean measured concentrations of IR5885 ranged from 0.50 to 7.5 mg/L.

Saltwater mysids were impartially assigned to exposure chambers at test initiation. Observations of mortality and other signs of toxicity were made approximately 5, 24, 48, 72 and 96 hours after test initiation. The 96-hour LC50 value was 2.8 mg/L. The no-mortality concentration and the NOEC were both 1.9 mg/L.

MATERIALS AND METHODS

MATERIALS

Test Material	IR5885
Description	white powder
Lot/Batch #	T 025/02
Purity	99.63% (w/w)
CAS #	283159-90-0
Test Animals:	
Species:	saltwater mysid (*Americamysis bahia*)
Age:	Juveniles (<24 hours old)
Source:	Wildlife International, Ltd. Easton, Maryland 21601
Acclimation period:	14-day
Diet:	Mysids in the cultures were fed live brine shrimp *Artemia nauplii* (Brine Shrimp Direct, Ogden, Utah) daily, occasionally enriched with ALGAMAC-2000 (Bio-Marine, Inc., Hawthorn, California), to prevent cannibalism. During the test, the juvenile mysids were fed live brine shrimp *Artemia nauplii* daily.
Test Water:	The water used for culturing and testing was natural seawater collected at Indian River Inlet, Delaware that was filtered and diluted to a salinity of approximately 20‰ with well water.

Test Units:	Test chambers were 2-L glass beakers containing approximately 1.5 L of test solution. The depth of the test water in a representative test chamber was 12.5 cm. Test chambers were indiscriminately positioned an environmental chamber designed to maintain the desired test temperature throughout the test period.
Test Conditions:	
Light Regime:	16 hours of light and 8 hours of darkness
Light Intensity:	135 lux
pH Values:	8.0 to 8.3
Dissolved Oxygen Concentrations:	6.2 mg/L (= 85% of saturation)
Water Temperature:	25 ± 2ºC

STUDY DESIGN AND METHODS

In life dates:	Experimental Start:	June 6, 2005
	Experimental Termination:	July 29, 2005

Dosage Preparation and Dosing

A primary stock solution was prepared by dissolving the test substance in dimethyl formamide (DMF) at a concentration of 300 mg/mL. The stock was sonicated for10 minutes and inverted to mix, and appeared clear and very light yellow after mixing. Aliquots of the primary stock solution were serially diluted with DMF to prepare secondary stock solutions at concentrations of 19, 38, 75 and 150 mg/mL. The secondary stocks were mixed by inversion and ranged in appearance from clear and colorless to clear and very slightly yellow. Aliquots of the appropriate primary or secondary stock solution were diluted in 20 L of filtered saltwater to prepare two replicates of each test solution at nominal concentrations of 1.9, 3.8, 7.5, 15 and 30 mg/L. The test solutions were mixed for approximately 1 – 2 minutes using top-down electric mixers. The solvent control solution was prepared by mixing 2.0 mL of DMF into 20 L of dilution water. The solvent concentration in the solvent control and all test solutions was 0.1 mL/L. The 1.9, 3.8 and 7.5 mg/L test solutions appeared clear and colorless at test initiation and termination. The 15 and 30 mg/L test solutions appeared clear, with a white precipitate present on the surface at test initiation and on the surface and bottom of the test chambers at test termination.

Analysis of IR5885 concentration:

The method used for the analysis of IR5885 in saltwater samples was based upon methodology developed by Wildlife International, Ltd. The analytical method consisted of diluting the samples in saltwater, as necessary, and analyzing by direct injection high performance liquid chromatography (HPLC) with UV detection at 220 nm. Concentrations of IR5885 in the samples were determined by HPLC using an Agilent Series 1100 High Performance Liquid Chromatograph (HPLC) equipped with an Agilent Series 1100 Variable Wavelength Detector. Chromatographic separations were achieved using a YMC-Pack ODS AM column (150 mm x 4.6 mm, 3-µm particle size). Calibration standards of IR5885, ranging in concentration from 0.250 to 2.50 mg/L, were prepared using a stock solution of IR5885 in acetonitrile. Fresh standards were prepared and analyzed with each sample set. Linear regression equations were generated using the sum of the peak area responses versus the respective concentrations of the calibration standards. The concentration of IR5885 in the samples was determined by substituting the sum of the peak area responses of the samples into the applicable linear regression equation. The method limit of quantitation (LOQ) for these analyses was defined as 0.250 mg/L, calculated as the product of the concentration of the lowest calibration standard (0.250 mg/L) and the dilution factor of the matrix blank samples (1.00). Three matrix blank samples were analyzed to determine possible interferences. No interferences were observed at or above the LOQ during the sample analyses. Samples of saltwater were fortified at 0.400, 1.00 and 8.00 mg/L using stock solutions of IR5885 in acetonitrile, and were analyzed concurrently with the test samples. The measured concentrations for the matrix fortification samples ranged from 92.5 to 104% of nominal concentrations.

Experimental Design

All organisms were observed periodically to determine the number of mortalities in each treatment group. The numbers of individuals exhibiting signs of toxicity or abnormal behavior also were evaluated. Observations were made approximately 5, 24, 48, 72 and 96 hours after test initiation.
Dissolved oxygen, pH and temperature were measured in each test chamber at the beginning and end of the test and at approximately 24-hour intervals during the test. Temperature also was measured continuously during the test in one negative control test chamber.

RESULTS AND DISCUSSION

Analytical results

Nominal concentrations selected for use in this study were 0.50, 1.0, 2.0, 4.0 and 8.0 mg/L. Samples collected at test initiation had measured concentrations that ranged from 96 to 113% of nominal concentrations. Samples collected at 48-hours had measured concentrations that ranged from 89 to 98% of nominal concentrations. Samples collected at test termination had measured concentrations that ranged from 83 to 92% of nominal concentrations. When measured concentrations of the samples collected throughout the test were averaged, the mean measured test concentrations for this study were 0.50, 0.95, 1.9, 3.6 and 7.5 mg/L, representing 100, 95, 95, 90 and 94% of nominal concentrations, respectively. The results of the study were based on the mean measured concentrations.

Biological results:

All mysids in the negative and solvent control groups appeared healthy and normal throughout the test. All mysids in the 0.50, 0.95 and 1.9 mg/L treatment groups also appeared normal throughout the test, with no mortalities or overt signs of toxicity observed. Percent mortality in the 3.6 and 7.5 mg/L treatment groups at test termination was 90 and 100%, respectively. The two surviving mysids in the 3.6 mg/L treatment group were observed to be on the bottom of the test chamber and exhibiting erratic swimming behavior at test termination. The no-mortality concentration and the NOEC were 1.9 mg/L. LC_{50} values at 24, 48, 72 and 96 hours were determined from the mortality.

CONCLUSIONS

Saltwater mysids, *Americamysis bahia*, were exposed for 96 hours under static conditions to five mean measured concentrations of IR5885 ranging from 0.50 to 7.5 mg/L. The 96-hour LC_{50} value was 2.8 mg/L, with a 95% confidence interval of 1.9 to 3.6 mg/L. The no-mortality concentration and the NOEC were both 1.9 mg/L.

4.3.2.3 96-hour shell deposition test in Eastern Oyster (*Crassostrea virginica*) – IR5885

Reference Number: KII - **Post submission 02**, Section 6, Volume 2, Annex IIA, point 8.2.4/03

Report:	**See annex conf. 32**

Guidelines: U.S. EPA OPPTS NUMBER 850.1025

Deviations: none

GLP: Yes; certified laboratory

Executive Summary:

The objective of this study was to determine the effects of IR5885 on the shell deposition of the eastern oyster, *Crassostrea virginica*, during a 96-hour exposure period under flow-through test conditions.
Eastern oysters were exposed to a geometric series of five test concentrations, a negative control (filtered saltwater), and a solvent control (dimethyl formamide). One test chamber was maintained in each treatment and control group with 20 eastern oysters in each test chamber. Nominal test concentrations were selected in consultation with the Sponsor, and were based upon the results of an exploratory range finding toxicity test and the results of the initial trial. Nominal test concentrations selected were 0.38, 0.75, 1.5, 3.0 and 6.0 mg IR5885/L. When measured concentrations of the samples collected during the test were averaged, the mean measured test concentrations for this study were 0.38, 0.76, 1.5, 3.0 and 4.3 mg/L, representing 100, 101, 100, 100 and 72% of nominal concentrations, respectively. The results of the study were based on the mean measured test concentrations.
Observations of mortality and other clinical signs were made approximately 6, 24, 48, 72 and 96 hours after test initiation. Measurements of shell deposition for each oyster were made at 96 hours, and were used to determine the EC_{50} value and the no-observed-effect-concentration (NOEC). The EC_{50} is the concentration of test substance in water that is calculated to induce a 50% reduction in shell deposition, relative to the control.
There were no mortalities or clinical signs of toxicity observed at any concentration tested. Based on inhibition of shell deposition, the 96-hour EC_{50} value was 3.1 mg/L. The NOEC was determined to be 1.5 mg/L, based on the statistically significant inhibition of shell growth observed at 3.0 and 4.3 mg/L.

MATERIALS AND METHODS

MATERIALS

Test Material	IR5885
Description	white powder
Lot/Batch #	T 025/02
Purity	99.63% (w/w)
CAS #	283159-90-0
Test Animals:	
Species:	Eastern oyster (*Crassostrea virginica*)
Age and Size:	The average length of the 20 representative oysters was 36.9 ± 2.5 mm, with a range of 32.3 to 42.9 mm.
Source:	Taylor Shellfish Farms, Shelton, Washington..
Acclimation period:	The oysters were received 10 days prior to test initiation and were held in filtered saltwater from the same source as used during the test.
Diet:	a suspension of marine microalgae (Reed Mariculture, Inc., San Jose, California). This suspension was provided continuously during holding and testing

Test Water:	The water used for culturing and testing was natural seawater collected at Indian River Inlet, Delaware, that was filtered and diluted to a salinity of approximately 20‰ with well water.
Test Units:	Test chambers were 54-L glass aquaria filled with approximately 27 L of test water. The depth of the test water in a representative chamber was 17.5 cm. Test chambers were indiscriminately positioned in a temperature-controlled environmental chamber set to maintain the desired test temperature.
Test Conditions:	
Light Regime:	16 hours of light and 8 hours of darkness
Light Intensity:	397 lux
pH Values:	7.7 to 8.4
Dissolved Oxygen Concentrations:	7.0 to7.6 mg/L (=88% of saturation)
Water Temperature:	20 ± 2ºC

STUDY DESIGN AND METHODS

In life dates:	Experimental Start:	July 14, 2005
	Experimental Termination:	September 23, 2005

Dosage Preparation and Dosing

One stock solution was prepared for each of the five concentrations tested. A primary stock solution was prepared by dissolving the test substance in DMF at a nominal concentration of 60 mg/mL. The stock solution was sonicated and mixed by inversion, and was clear and light amber in appearance. The primary stock solution was proportionally diluted with DMF to prepare four secondary stock solutions at nominal concentrations of 3.8, 7.5, 15 and 30 mg/mL. The secondary stock solutions were mixed by inversion and ranged in appearance from clear and colorless to clear and slightly amber. The stock solutions were injected into the diluter mixing chambers (at a rate of 35.0 µL/min) where they were mixed with filtered saltwater (at a rate of 350 mL/minute) to achieve the desired test concentrations. The solvent concentration in all IR5885 treatment groups and the solvent control was 0.1 mL DMF/L. The negative control was dilution water without solvent or test solution.
At test initiation, the negative and solvent control solutions and the 0.38 mg/L test solution appeared clear and colorless in the test chambers and in the diluter mixing chambers. The 0.75 and 1.5 mg/L test solutions appeared clear and colorless in the test chambers, with a white precipitate present in the diluter mixing chambers. The 3.0 and 6.0 mg/L test solutions appeared clear and colorless, with a precipitate present in the test chambers and in the diluter mixing chambers. At test termination, the test solutions appeared the same as at test initiation, with a green tint to the water in the test chambers due to the algal feed.

Analysis of IR5885 concentration:

The method used for the analysis of IR5885 in saltwater samples consisted of diluting the samples in saltwater, as necessary, and analyzing by direct injection high performance liquid chromatography (HPLC) with UV detection at 220 nm. Concentrations of IR5885 in the samples were determined by HPLC using an Agilent Series 1100 High Performance Liquid Chromatograph (HPLC) equipped with an Agilent Series 1100 Variable Wavelength Detector. Chromatographic separations were achieved using a YMC-Pack ODS AM column (150 mm x 4.6 mm, 3-µm particle size).
Calibration standards of IR5885, ranging in concentration from 0.200 to 2.00 mg/L, were prepared in saltwater using a stock solution of IR5885 in acetonitrile. Fresh standards were prepared and analyzed with each sample set. Linear regression equations were generated using the sum of the peak area responses versus the respective concentrations of the calibration standards. The concentration of IR5885 in the samples was determined by substituting the sum of the peak area responses of the samples into the applicable linear regression equation.
The method limit of quantitation (LOQ) for these analyses was defined as 0.200 mg/L, calculated as the product of the concentration of the lowest calibration standard (0.200 mg/L) and the dilution factor of the

matrix blank samples (1.00). Three matrix blank samples were analyzed to determine possible interferences. No interferences were observed at or above the LOQ during the sample analyses.
Samples of saltwater were fortified at 0.350, 1.00 and 6.00 mg/L using a stock solution of IR5885 in acetonitrile, and were analyzed concurrently with the test samples. The measured concentrations for the matrix fortification samples ranged from 84.0 to 103% of nominal concentrations.

Experimental Design

Observations were made periodically to determine the numbers of mortalities. The numbers of individuals exhibiting clinical signs of toxicity or abnormal behavior also were evaluated. Observations were made approximately 6, 24, 48, 72 and 96 hours after test initiation. Measurements of shell deposition for each oyster were made at 96 hours, and were used to determine the EC_{50} value and the no-observed-effect-concentration (NOEC). At the end of the test, the longest finger of new shell growth on each oyster was measured to the nearest 0.1 mm using calipers.

RESULTS AND DISCUSSION

Analytical results

Nominal concentrations selected for use in this study were 0.38, 0.75, 1.5, 3.0 and 6.0 mg/L. Samples collected at test initiation, at 48 hours, and at test termination from the 0.38, 0.75, 1.5, and 3.0 mg/L solutions had measured concentrations that ranged from approximately 98 to 102% of nominal concentrations. Samples collected at test initiation, at 48 hours, and at test termination from the 6.0 mg/L solution had measured concentrations that declined over time from approximately 89% of nominal at test initiation to approximately 46% of nominal at termination. The lower measured concentrations in the 6.0 mg/L solutions, as well as the observations of a precipitate in the 3.0 and 6.0 mg/L test chambers, confirm that the test was conducted to the limit of solubility in the test system. When measured concentrations of the samples collected during the test were averaged, the mean measured test concentrations for this study were 0.38, 0.76, 1.5, 3.0 and 4.3 mg/L, representing 100, 101, 100, 100 and 72% of nominal concentrations, respectively. The results of the study were based on the mean measured test concentrations.

Biological results:

There were no mortalities among oysters in any treatment or control group during the test.
All oysters appeared normal throughout the 96-hour exposure period. After 96-hours, the mean shell deposition in the negative and solvent control groups was 2.34 and 2.12 mm, respectively. When the shell deposition data for the negative control was compared with the solvent control, no statistically significant differences were found at the 95% level of confidence. Therefore, the control groups were pooled for comparison with the treatment groups. Mean shell deposition in the 0.38, 0.76, 1.5, 3.0 and 4.3 mg/L treatment groups was 1.54, 1.61, 1.54, 1.20 and 0.24 mm, respectively.
Percent inhibition of shell growth was calculated relative to the control data. Inhibition of shell growth in the 0.38, 0.76, 1.5, 3.0 and 4.3 mg/L treatment groups was 31, 28, 31, 46 and 89%, respectively. The 96-hour EC_{50} was calculated to be 3.1 mg/L, with a 95% confidence interval of 1.8 to 3.4 mg/L. The percent inhibition at 0.38, 0.76 and 1.5 mg/L was not concentration-responsive and mean shell growth in those treatment groups was not significantly different from the pooled controls. Consequently, the NOEC was determined to be 1.5 mg/L.

CONCLUSIONS

Eastern oysters, *Crassostrea virginica*, were exposed for 96 hours under flow-through test conditions to five concentrations of IR5885 ranging from 0.38 to 4.3 mg/L. There were no mortalities or clinical signs of toxicity observed at any concentration tested. Based on inhibition of shell deposition, the 96-hour EC_{50} value was 3.1 mg/L, with a 95% confidence interval of 1.8 to 3.4 mg/L. The NOEC was determined to be 1.5 mg/L, based on the statistically significant inhibition of shell growth observed at 3.0 and 4.3 mg/L.

This study is specifically performed for the registration in non EU Countries and it is not considered representative for the EU. Therefore it is not included in the risk assessment.

4.3.2.4 Whote sediment acute toxicity to a Marine Amphipod *(Leptocheirus plumulosus*)

Report:	**Aufderheide, J., (2015). Valifenalate Technical: Whole Sediment Acute Toxicity to a Marine Amphipod *(Leptocheirus plumulosus*). ABC Laboratories. FMC Corporation, FMC Tracking No.: 2014ETX-VAL1348. Report date**

Guidelines: OCSPP 850.1740

Deviations: none

GLP: Yes

Executive Summary

In a 10-day acute toxicity study, the marine amphipod, *Leptocheirus plumulosus*, was exposed to valifenalate technical at nominal concentrations of 0 (control) and 201 mg a.i./kg dry sediment in accordance with the OCSPP 850.1740 guideline. The NOEC value based on mean measured concentrations in sediment was 109 mg a.i./kg for survival. The LOEC value based on mean measured concentrations in sediment was >109 mg a.i./kg for survival. The LC_{50} value based on mean calculated concentrations in sediment was >109 mg a.i./kg for survival. There were no abnormalities noted in any of the test substance treatments during the 10-day test. This toxicity study is classified as acceptable and satisfies the guideline requirements for the marine amphipod, *Leptocheirus plumulosus* acute toxicity study.

Table 4.3.2.4-1: Summary of endpoints for Valifenalate Technical after 10-day exposure

Statistical Endpoint	Survival
NOEC (mg a.i./kg)	**109**
LOEC (mg a.i./kg)	**>109**
LC_{50} (95% Confidence Limit)	**>109 (not calculated)**

MATERIALS AND METHODS:

Experimental Test Dates:

Test Material:

Name: Valifenalate Technical
Batch no.: PL14-0060 (Manufacturing Lot No.: P/13/019 (Isagro))
Analyzed content: 98.9%
Expiry: 03 March 2016
Name: Valifenalate Technical (IR5885 analytical reference substance)
Batch no.: 20071/77
Analyzed content: 99.27%
Expiry: November 2017

Test Species:

Species: Marine amphipod, *Leptocheirus plumulosus*

Source: Chesapeake Cultures, Hayes, Virginia

Test Conditions:

Test medium:	The sediment was natural marine sediment collected by personnel at Environ, Port Gable, Washington. Sediment used in the definitive test was collected on 18 February 2015, and received at ABC Laboratories on 23 February 2015. The sediment was assigned lot number MSED 230215.	
Dissolved Oxygen:	5.7 to 6.9 mg/L (80 to 96% saturation)	
pH:	7.9 to 8.3	
Salinity:	20.3 to 21.9‰	
Un-ionized Ammonia:	Pore water Day-0:	0.133 to 0.157 mg/L
	Overlying water Day-0:	0.0503 to 0.0898 mg/L
	Overlying water Day-10:	0.164 to 0.252 mg/L

Temperature & photoperiod:

Temperature:	24.7 to 25.0°C
Photoperiod:	16-hr light: 8-hr dark
Light intensity:	509 lux

Test Design: The in-life definitive toxicity test was performed from 12 to 22 March 2015. The testing was performed at nominal sediment concentrations of 0 (control) and 201 mg a.i./kg dry sediment. A 0.3552 g amount of valifenalate technical (IR5885) (0.353 g corrected for 99.27% purity) was directly added to 5,400 g of marine sediment (oven dry equivalent of 1,656.4 g) and 100.0 g of clean sand, and was mixed by hand for approximately five minutes to produce a dosed sediment with a nominal concentration of 201 mg a.i./kg dry sediment. The control sediment consisted of 5,400 g of marine sediment (oven dry equivalent of 1,656.4 g) and 100.0 g of clean sand without test substance.

The test chambers were glass jars approximately 17 cm in height by 8.5 cm in diameter. Approximately 250 g (approximately 5 cm sediment depth) of dosed sediment was added to each replicate test chamber. Approximately 600 mL of saltwater was carefully added to the test chambers using a deflector to minimize the disturbance of the sediments. Eight replicate test chambers were prepared for the biological parameters,i.e., survival and behavior observations. A total of four replicate chambers were prepared for analytical confirmation of valifenalate technical and acid concentrations within samples collected of the overlying water, pore water, and sediments. An additional replicate was prepared ammonia analysis of the overlying and pore water performed at initiation. The test chambers were prepared one day prior to test initiation (addition of the test organisms). The test chambers were placed in a temperature-controlled water bath in a randomized block arrangement.

Temperature was set at a target range of 25 ± 1°C. A 16-hour light:8-hour dark photoperiod with 30-minute dawn and dusk periods was maintained. Aeration was provided at an initial rate of 60-100 bubbles per minute to each test chamber through a glass pipet. The pipet was inserted such that its tip was 2–3 cm from the sediment surface. Aeration was discontinued during the addition of the animals and resumed approximately 24 hours after addition was completed. The aeration was adjusted as necessary to maintain the dissolved oxygen concentrations at a target level ≥60% saturation within each test chamber.

Twenty amphipods were impartially added to a set of labeled containers with each container representing one treatment replicate. Each container was randomly assigned to one treatment replicate. The amphipods in each container were then released into the corresponding test chamber. All test chambers received 20 amphipods, except for the additional test replicates used for test initiation analytical confirmation and ammonia analyses.

Measurements of temperature, dissolved oxygen concentration, pH, and salinity of the overlying water were measured with a WTW Multi 3500i multi meter in all biological replicates at test initiation and termination. These same parameters were measured daily in one replicate test chamber in each treatment. Water bath temperature was continuously measured and recorded with an electronic data logger. On days 0 and 10, overlying water samples were taken from each biological test chamber, approximately 1-2 cm above the

sediment surface, and composited by treatment for measurement of overlying water ammonia concentrations. Pore water ammonia samples were collected at test initiation after the sediments from the additional replicate test chambers were centrifuged for approximately 20 minutes, which separated the pore water from the sediments. Ammonia was measured using an Accumet Model 25 pH meter equipped with an ammonium specific probe. Light intensity, measured at the test solution level on day 0 with a LI-COR Model LI-189 light meter equipped with a photometric sensor, was 509 lux. At test termination (day 10), the entire contents of biological replicate test chambers from each treatment level were removed and the live and dead amphipods were enumerated. The amphipods were carefully separated from the sediment by sieving the sediment through a stainless steel sieve (500 µm) and observing for movement. Observations of general health and behavior of the amphipods were also noted (normal, lethargic, etc.). Any of the amphipods that were not accounted for at test termination (not found) were considered dead.

Based on the environmental fate profile of valifenalate, its hydrolysis product valifenalate acid was included in analytical verification of the test substance in different compartments of the test system: overlying water, interstitial (pore) water and sediment. Overlying water, interstitial (pore) water, and sediment were analyzed for valifenalate and its hydrolysis product valifenalate acid using a liquid chromatographic with tandem mass spectrometry (LC-MS/MS) system. Analysis of water samples (i.e., overlying and pore water) and sediment samples was accomplished based on a method provided by the Sponsor and validated as part of ABC study 81308.

Statistical analysis of the concentration versus effect data was performed using SAS (version 9.3 for Windows). The no-observable effects (NOEC) and lowest-observable effects (LOEC) values for survival were determined by using a one-way analysis of variance (ANOVA), followed by a Dunnett's test for determination of significance and a Fisher's one-tailed exact test. The Dunnett's test was performed with the alternate hypothesis being that response in the treatment had been reduced in comparison to the control ($p<0.05$). Prior to the ANOVA and Dunnett's tests, a Shapiro-Wilk's test and a Levene's test were conducted to test for normality and homogeneity of variance, respectively. The survival data was not normally distributed and the variances were not homogeneous, therefore an ANOVA was performed on the ranks of the data. Since the mortality or other adverse effects observed were less than 10% within the test substance treatment group, the LC_{50} value was reported as being greater than the highest treatment level being tested.

Validity Criteria:

The following criteria must be found in the control replicates:

- Mean survival ≥90% for control.

RESULTS AND DISCUSSION:

Validity Criteria: The control animals met the acceptability criteria for mean survival (i.e., >90%) as specified by the study protocol and the OPPTS 850.1740 testing guideline.

***Analytical results*:**

Sediment: The treatment mean measured concentration of valifenalate technical at day 0 was 190 mg a.i./kg (95% of nominal) for the 201 mg a.i./kg nominal treatment. The treatment mean measured concentration of valifenalate technical at day 10 was 28.6 mg a.i./kg (14% of nominal) for the 201 mg a.i./kg nominal treatment. The overall mean and geometric measured concentrations of valifenalate technical was 109 and 72.6 mg a.i./kg (54 and 36% of nominal), respectively, for the 201 mg a.i./kg nominal treatment. No residues of valifenalate technical were detected in the control samples at or above the MQL of 4.00 mg a.i./kg. Quality control fortification recoveries from the day 0 sediment sample set were 140 and 148% of the nominal fortification concentrations. The 10-day sediment fortification recoveries were 91 and 97% of the nominal concentrations. It's possible that there was a matrix enhancement that was picked up in the day 0 QC samples that was not measured in the day 10 samples even though the same spiking solutions and fortification preparations were used for both data sets. The biological results were reported based on the overall mean measured valifenalate technical sediment concentrations.

Based on the environmental fate in soil/sediment, the parent substance Valifenalate exhibits rapid degradation to valifenalate acid, p-chlorobenzoic acid (PCBA) and ultimately mineralizes to CO_2. The DT-50s of

valifenalate, valifenalate acid, and PCBA were 5 hours, 15 hours and 3 days, respectively, indicating a very rapid degradation in soil/sediment systems. Because the duration of the present study was longer (10 days) than the degradation times established in e-fate studies, the loss of test substance at the end of the study was expected. In addition to the valifenalate technical analysis, only valifenalate acid was measured as part of this study. Therefore the measured analytes in the sediment and waters were not able to fully account for the total valifenalate concentrations within the sediment at the time the sediments were dosed.

The measured concentrations of valifenalate acid at day 0 ranged from 22.1 to 30.9 mg/kg for the test substance treatment, with a mean of 26.5 mg/kg. The measured concentrations of valifenalate acid at day 10 ranged from 1.22 to 1.49 mg/kg for the test substance treatment, with a mean of 1.36 mg/kg. No residues of valifenalate acid were detected in the control samples at or above the MQL of 4.00 mg/kg. The losses of the valifenalate technical over time in the treatment sediments were not unanticipated.

Overlying Water: The measured concentrations of valifenalate technical at day 0 ranged from 0.316 to 0.345 mg a.i./L for the test substance treatment, with a mean of 0.331 mg a.i./L. The measured concentrations of valifenalate technical at day 10 ranged from 0.686 to 0.696 mg a.i./L for the test substance treatment, with a mean of 0.691 mg a.i./L. No residues of valifenalate technical were detected in the control samples at or above the MQL of 0.00200 mg a.i./L. The mean and geometric mean concentrations over the duration of the exposure were 0.511 and 0.478 mg a.i./L, respectively. Similar to the sediment QC fortifications, the recoveries from the day 0 sample set were 170 and 193% of the nominal fortification concentrations. The 10-day overlying fortification recoveries were 99 and 104% of the nominal concentrations. It's possible that there was a matrix enhancement that was picked up in the day 0 QC samples that was not measured in the day 10 samples even though the same spiking solutions and fortification preparations were used for both data sets.

The measured concentrations of valifenalate acid at day 0 ranged from 0.0749 to 0.0797 mg/L for the test substance treatment, with a mean of 0.0773 mg/L. The measured concentrations of valifenalate acid at day 10 ranged from 0.0298 to 0.0313 mg/L for the test substance treatment, with a mean of 0.0306 mg/L. No residues of valifenalate acid were detected in the control samples at or above the MQL of 0.00200 mg/L. The mean and geometric mean concentrations over the duration of the exposure were 0.0539 and 0.0486 mg/L, respectively. The day 0 high QC fortification for the valifenalate acid overlying water analysis had a recovery of 231% of nominal. All other valifenalate acid overlying water QC fortification recoveries ranged from 89 to 114% of the nominal concentration.

Pore (Interstitial) Water: The measured concentrations of valifenalate technical at day 0 ranged from 6.94 to 7.70 mg a.i./L for the test substance treatment, with a mean of 7.32 mg a.i./L. The measured concentrations of valifenalate technical at day 10 ranged from 1.70 to 2.54 mg a.i./L for the test substance treatment, with a mean of 2.12 mg a.i./L. No residues of valifenalate technical were detected in the control samples at or above the MQL of 0.00200 mg a.i./L. The mean and geometric mean concentrations over the duration of the exposure were 4.72 and 3.90 mg a.i./L, respectively. The day 0 and day 10 valifenalate pore water QC fortification recoveries ranged from 153 to 156% and 94 to 96% of the nominal concentration, respectively.

The measured concentrations of valifenalate acid for day 0 ranged from 2.65 to 2.67 mg/L for the test substance treatment, with a mean of 2.66 mg/L. The measured concentrations of valifenalate acid at day 10 ranged from 10.7 to 11.3 mg/L for the test substance treatment, with a mean of 11.0 mg/L. No residues of valifenalate acid were detected in the control samples at or above the MQL of 0.00200 mg/L. The mean and geometric mean concentrations over the duration of the exposure were 6.83 and 5.41 mg/L, respectively. Quality control fortifications ranged from 86 to 111% of the nominal concentrations, respectively.

Biological Results: After 10 days of exposure, survival was 100 and 99% in the control and 109 mg a.i./kg treatments, respectively. The mean survival of the control treatment was greater than the 90% survival acceptability criterion set by the study protocol. All surviving animals were normal in appearance and behavior. Based on mean measured sediment concentrations, the 10-day LC_{50} was estimated to be >109 mg a.i./kg , the highest concentration tested. The 10-day NOEC and LOEC values for survival were 109 and >109 mg a.i./kg respectively, the highest concentration tested. The results are summarized in Table 4.3.2.4-2.

Table 4.3.2.4-2: Survival Observations after 10-Day Exposure of *Leptocheirus plumulosus* to Valifenalate Technical

Mean Calculated Sediment Concentration (mg/kg dry Sediment)	**REP**	**Day 10 Survival [a] (% survival)**	**REP**	**Day 10 Survival [a] (% survival)**	**Treatment Percent Survival**
0 (Control)	A	20 (100)	E	20 (100)	100
	B	20 (100)	F	20 (100)	
	C	20 (100)	G	20 (100)	
	D	20 (100)	H	20 (100)	
108	A	19 (95)	E	20 (100)	99
	B	19 (95)	F	20 (100)	
	C	20 (100)	G	20 (100)	
	D	20 (100)	H	20 (100)	

[a] Animals that were not found were treated as dead.

Note; There were not statistically significant (Dunnett's' test, Fisher's Exact test p = 0.05) reduction in animal survival as compared to the control. The achieved NOEC, LOEC, and LC_{50} values are summarized in Table 4.3.2.4-3.

Table 4.3.2.4-3: Calculated Effects Concentrations for Survival of the Marine Amphipod, *Leptocheirus plumulosus*, Exposed to Valifenalate in Whole Sediment

Based on Mean Calculated Valifenalate Concentrations in Sediment:

Biological Parameter	NOEC	LOEC	LC_{50} (95% Confidence Limit)
10-day Survival	109 mg/kg	>109 mg/kg	>109 mg/kg (not calculated)

NOEC = No-observable-effect concentration and LOEC = lowest-observable-effect concentration.

CONCLUSION

In a 10-day acute toxicity test, marine amphipod *Leptocheirus plumulosus* was exposed to Valifenalate technical. The control animals met the acceptability criteria for mean survival (i.e., >90%) as specified by the study protocol and the OPPTS 850.1740 testing guideline.

Results Based on Mean Calculated Concentrations in Sediment:

Biological Parameter	NOEC	LOEC	LC_{50} (95% Confidence Limit)
10-Day Survival	109 mg/kg	>109 mg/kg	>109 mg/kg (not calculated)

4.3.3 Algal growth inhibition tests

4.3.3.1 Effects on green algae (*Scenedesmus subspicatus)*

Reference Number: KII- Section 6, Volume 2, Annex IIA, point 8.4/01

Report: See annex conf. 14

Guidelines: Commission Directive 92/69/EEC, Annex Part C, C.3: "Algal Inhibition Test"
OECD Guideline for Testing of Chemicals, Section 2, No.201: "Alga, Growth Inhibition Test", 7 June 1984

Deviations: None

GLP: Yes; certified laboratory

Executive Summary:

The effect of IR5885 was determined, by a limit test, on the unicellular green algae *Scenedesmus subspicatus*. Exponentially growing cultures of this green algal species were exposed in a static test to aqueous test media containing IR5885 suspended in methyl cellulose at a concentration of 100 mg/L under defined conditions. Two controls groups holding respectively water and water with methyl cellulose, were tested in parallel. Previously a pre test and a pre experiment were carried out to find out respectively the range of concentrations to be tested in the main test and the solubility of the test item in the test water. Besides, an analytical test was carried out over all the period of the test to verify that the concentration of test item was constantly above 80 % of initial concentration.
The inhibition of growth in relation to control cultures, measured as growth rate and biomass, was determined over test periods of 24, 48 and 72 hours and thus over several algal generations.
At the end of test both the biomass and the growth rate were not significantly different from control.
Thus the NOEC and the EC_{50} values were determined to be respectively at least 100 mg/L and higher than 100 mg/L.

MATERIALS AND METHODS

MATERIALS

Test Material IR5885
Description white powder
Lot/Batch # T 025/02
Purity 99.56% (w/w)
CAS # 283159-90-0

Test Animals:
Species: *Scenedemus subspicatus* CHODAT, Strain No. 86.81 SAG
Source: supplied by the "Sammlung von Algenkulturen, Pflanzenphysiologisches Institut der Universität Gottingen", 37073 Gottingen, Germany.
Breeding conditions: The algae were cultivated under standardised conditions according to the test Guidelines.
Test Units: Erlenmeyer flasks of 50 mL volume with 50 mL test medium. Each test unit was uniquely identified with the study number, treatment and replicate number.
Test Conditions:
Light Regime: continuous illumination

Light Intensity:	7597 lux (mean value), achieved with fluorescent tubes.
pH Value:	7.8 – 8.0 at test start 8.2 – 8.0 at test end
Water temperature:	23°C
Toxic Standard:	for the evaluation of the quality of the Algae and the experimental conditions the substance potassium dichromate p.A. is tested at least twice a year in order to demonstrate satisfactory test conditions.

STUDY DESIGN AND METHODS

Dates:	Experimental Start:	May 28, 2002
	Experimental Termination:	June 20, 2002

Dosage Preparation and Dosing

Pre-experiments: pre-experiments were carried out to determine the solubility of the test item in test water (not in compliance with GLP regulations).

Range-finding: the test concentrations were based on the results of a range finding test (not in compliance with GLP regulations).

Test concentrations: the only tested concentration was 100 mg/L. Additionally, a control and a solvent control (100 mg methyl cellulose/L) were tested in parallel.

Dosage of the test Item: before the test start the test medium of nominal 100 mg/L was prepared by dosing 60 mg test item and 60 mg methyl cellulose into 600 mL test water. The test item was mixed into the test water as homogeneously as possible (stirring and ultrasonic treatment). The test medium was freshly prepared just before the introduction of algae.

Analysis of IR5885 concentration:

Duplicate samples from the freshly prepared test medium (without algae) and from the solvent control were taken at the start of the test. For the determination of the stability of the test item under the test conditions respectively the maintenance of the test item concentrations during the test, sufficient volumes of the freshly prepared test medium of nominal 100 mg/L and the solvent control were incubated under the same conditions as in the test and were sampled in duplicate at the end of the same (after 72 hours test).
All samples were analysed directly after sampling.
The concentrations of IR5885 were analysed in the duplicate test media samples from the test concentration of nominal 100 mg/L from both sampling times (0 and 72 hours). From the solvent control samples only one of the duplicate was analysed from both sampling times.

Experimental Design:

Introduction of Algae: the test started (0 hours) by inoculation of a biomass of 10000 algal cells per mL test medium. These cells were taken from an exponentially growing pre-culture (set-up 3 days prior to the test start at same test condition).

Replicates: the test was performed with three replicates per test concentration and six replicates in the control. The Erlenmeyer flasks (50 mL volume) were continuously stirred and were covered with glass dishes. The flasks were incubated in a water bath.

Blanks: additionally, one replicate per test concentration was prepared without algae to provide as “blank” for the spectrophotometrical measurements.

Exposure time: 72 hours.

Determination of the Algal growth Inhibition: defined volumes of the algae suspensions from all replicates and from the blanks were sampled after 24, 48 and 72 hours of exposure, and were not replaced. The cell densities were determined by spectrophotometrical measurements. For the determination of algal cell densities the absorption of the blanks were subtracted from the absorption of the samples with algae.
The cell density in one control was counted by microscope after 72 hours test. Based on the counted cell densities and based on the determined absorption of the control and five dilutions of the control, a linear regression was performed for the calculation of the cell densities in all other samples measured spectrophotometrically during the test.

The inhibition of algae growth was determined from:

- area under growth curves (biomass)
- specific growth rates r for exponentially growing cultures

Microscopic examination: from the 100 mg/L test concentration a sample after 72 hours exposure was taken. The shape of the treated algal cells compared to controls was examined.

Measurement of pH, Water Temperature and Test Item behaviour: the pH values of the test media were measured in samples from the test concentration and the controls at the start and at the end of the test. During the experiment the test media temperatures were measured daily in a control flask with water incubated at the same conditions of the test.
The behaviour of the test item in test water was determined daily.

RESULTS AND DISCUSSION

Analytical results:

During the test period test item concentrations were measured in the range from 99 to 103 of the nominal values. Under the test conditions IR5885 resulted sufficiently stable during the 72 hours period, therefore all the reported results are related to the nominal concentration of the test items.
Behaviour of the test item: the test medium was turbid by the test item.

Biological results:

Limit test: in the test concentration of nominal 100 mg/L no significant reduction of growth of algal cells was determined during the test period of 72 hours compared to the control and to the solvent control.

Microscopic examination: at the examination of the shape of the algal cells after 72 hours, no difference was observed between the algae growing in the test concentration of 100 mg/L and the algae growing in the control.

Cell density in control: in the control the cell density has increased from nominal N = 1 x 10^4 cells/mL at the start to N = 43.2 x 10^4 cells/mL (mean value) after 72 hours, with a factor of approx. 43. In the solvent control this factor was 42.82. Yhus the algal growth in the controls were sufficiently high under the test conditions.

CONCLUSIONS

The 72-hour NOEC in unicellular green algae, *Scenedesmus subspicatus*. was determined to be at least 100 mg test item/L The 72-hour LOEC and the 72-hour EC_{50} were clearly higher than 100 mg test item/L.

4.3.3.2 96-hour growth inhibition test in Marine Diatom, *Skeletonema costatum*) –

Report:	Hicks S (2015b). Valifenalate Technical: Growth Inhibition Test with the Marine Diatom, *Skeletonema costatum* . ABC Laboratories, FMC Corporation, FMC Tracking No.: 2014ETX-VAL1346

Guidelines: OCSPP 850.4500

Deviations: none

GLP: Yes

Executive Summary

In a 96-hour acute toxicity study, the cultures of marine diatom, *Skeletonema costatum* were exposed to valifenalate technical at nominal concentrations of 0 (control), 0 (vehicle control; 50 µL DMF/L), 0.040, 0.12, 0.37, 1.1, 3.3, and 10 mg a.i./L under static conditions in accordance with the OCSPP 850.4500 guideline. The NOEC values based on area under the growth curve, growth rate, and mean yield were all 0.106 mg a.i./L, respectively. The 96-hour IC_{10}, IC_{20}, and IC_{50} values based on area under the growth curve were 0.183, 1.47, and >9.48 mg a.i./L, respectively. The percent area under the growth curve inhibition in the treated algal culture as compared to the control ranged from -1 to 32%. The 96-hour IC_{10}, IC_{20}, and IC_{50} values based on growth rate were >9.48 mg a.i./L. The percent growth rate inhibition in the treated algal culture as compared to the control ranged from 0 to 3%. The 96-hour IC_{20} and IC_{50} values based on mean yield were >9.48 mg a.i./L, respectively. The 96-hour yield data did allow for calculation of the IC_{10}; therefore, the value was estimated to be 0.976 mg a.i./L based on the treatment mean percent inhibition. The percent yield inhibition in the treated algal culture as compared to the control ranged from -2 to 12%. There were no abnormalities noted in any of the test substance treatments during the 96-hour test. This toxicity study is classified as acceptable and satisfies the guideline requirements for marine diatom, *Skeletonema costatum* static acute toxicity study.

Table 4.3.3.2-1: Summary of endpoints for Valifenalate Technical after 96-hour exposure

Statistical Endpoint	Area Under the Growth Curve	Growth rate	Yield
NOEC (mg a.i./L)	0.106	0.106	0.106
IC_{10} (mg a.i./L) (95% C.I.)	0.183 (0.0407 to 0.326)	>9.48 (Could not be determined)	Estimated - 0.976 (Could not be determined)
IC_{20} (mg a.i./L) (95% C.I.)	1.47 (0.947 to 2.00)	>9.48 (Could not be determined)	>9.48 (Could not be determined)
IC_{50} (mg a.i./L) (95% C.I.)	>9.48 (Not statistically sound)	>9.48 (Could not be determined)	>9.48 (Could not be determined)
Reference chemical, if used	NA	NA	NA

MATERIALS AND METHODS:

Experimental Test Dates: 02 to 06 February 2015

Test Material:

Name: Valifenalate Technical
Batch no.: PL14-0060 (Manufacturing Lot No.: P/13/019 (Isagro))
Analysed content: 98.9%

Expiry:	03 March 2015
Test Species:	
Species:	Marine diatom, *Skeletonema costatum*
Source:	Department of Botany, Culture Collection of Algae, University of Texas at Austin
Test Conditions:	
Growth medium:	Saltwater algal nutrient medium (SWAM) as recommended by OCSPP 850.4500
pH (initiation):	7.9 to 8.0
pH (termination):	8.1 to 8.2
Salinity:	30‰
Temperature & photoperiod:	
Temperature:	20.9 to 21.9°C
Photoperiod:	14 hours light, 10 hours dark
Light intensity:	4,700 to 4,761 lux by cool-white fluorescent bulbs

Test Design: Based on the a range-finding test and the functional solubility of the test substance in SWAM, nominal concentrations of 0 (control), 0 (vehicle control; 50 µL DMF/L), 0.040, 0.12, 0.37, 1.1, 3.3, and 10 mg a.i./L were selected for the definitive test. The exposure flasks were 250-mL Erlenmeyer flasks with foam stoppers and labeled with study number, treatment, replicate, and grid position. The control, vehicle control, and each test substance treatment were replicated four times (replicates A, B, C, and D). Each replicate contained 50 mL of the appropriate parent solution. An additional replicate ("81306, level 1, abiotic") of the 0.040 mg a.i./L test substance treatment, containing 100 mL of the parent solution, was also prepared and used to evaluate the potential for incorporation of the test substance into the algal biomass. At test initiation, each A, B, C, and D replicate of the control, vehicle control, and each test substance treatment were inoculated with 1.0 mL of an algal concentrate containing approximately 5.2×10^5 cells/mL, resulting in a final target density of approximately 1.0×10^4 cells/mL for each flask. At 24, 48, 72, and 96 hours (±1 hour), cell density was measured in replicates A, B, C, and D of the control, vehicle control, and each test substance treatment by direct microscopic counting with a hemacytometer. The "81306, level 1, abiotic" replicate of the 0.040 mg a.i./L test substance treatment was not inoculated with algae.

Test solutions were analyzed for the concentration of valifenalate technical using a liquid chromatography with tandem mass spectrometry (LC-MS/MS) system. Analysis was accomplished based on a method provided by FMC and validated at ABC Laboratories as part of this study. The concentration of valifenalate technical was measured in test solution samples collected at 0 hour (initiation) and 96 hours (termination) of the definitive test. Valifenalate technical-fortified QC samples were also prepared for analysis at each sample period.

All statistical analyses were performed with SAS software (Version 9.3 for windows). Prior to the IC and NOEC calculations, the control and vehicle control groups were evaluated for statistical differences by comparing the means of cell density yield at the 72- and 96-hour time points, and the area under the growth curve and specific growth rate over 0-72 and 0-96 hours. The planned comparison, or Least Square Means test, was performed by inspecting the *p* value for the t-test between control means and showed no statistically significant difference between the control groups. All further statistical analyses were conducted using the blank control. The NOEC values, based on area under the growth curve, growth rate, and yield, were estimated using a one-way analysis of variance (ANOVA) procedure and a one-tailed Dunnett's test ($p = 0.05$) where the alternate hypothesis was the mean for the growth parameter was reduced in comparison to the control. The I_bC_{50}, I_rC_{50}, and I_yC_{50} estimates were calculated using a 4-parameter logistic (sigmoid-shaped) model fit to the data with percent inhibition as the dependent variable and concentration as the independent variable.

Validity Criteria:

The following criteria must be found in the control replicates:

- Logarithmic phase growth after 96 hours.
- The coefficients of variation for yield and average specific growth rates at termination do not exceed 15%.

RESULTS AND DISCUSSION:

Validity Criteria: The results of the control and vehicle control met the required guideline validity criteria.

***Analytical results*:** Measured concentrations of valifenalate technical in the test substance treatment solutions at initiation were 0.0390, 0.118, 0.350, 1.08, 3.44, and 10.1 mg a.i./L, which represented recoveries of 95 to 104% of the nominal concentrations. Measured concentrations of valifenalate technical in the test substance treatment solutions at 96 hours (termination) were 0.0386, 0.0947, 0.329, 0.882, 2.62, and 8.89 mg a.i./L, representing recoveries of 79 to 97% of the nominal concentrations. No residues of valifenalate technical were detected in the control or vehicle control solutions above the MQL of 0.00100 mg a.i./L. The measured concentration in the 0.040 mg a.i./L (nominal) abiotic sample at 96 hours was 0.0423 mg a.i./L, indicating that the test substance was stable during the test exposure. The geometric mean measured concentrations in the test substance treatment solutions were 0.0388, 0.106, 0.339, 0.976, 3.00, and 9.48 mg a.i./L, representing recoveries of 88 to 97% of the nominal concentrations.

Biological Results: The mean cell density in the valifenalate technical treatments at 96 hours ranged from a low of 89.2×10^4 cells/mL at the concentration of 3.00 mg a.i./L to a high of 103×10^4 cells/mL at the concentration of 0.0388 mg a.i./L. Percent inhibition in area under the growth curve at 96 hours, as compared to the control, ranged from -1% at 0.0388 mg a.i./L to 32% at 9.48 mg a.i./L. Percent inhibition in growth rate at 96 hours, as compared to the control, ranged from 0% at 0.0388 and 0.106 mg a.i./L to 3% at 3.00 mg a.i./L. The percent inhibition in yield at 96 hours, as compared to the control, ranged from -2% at 0.0388 mg a.i./L to 12% at 3.00 mg a.i./L. There were no abnormalities noted in any of the test substance treatments during the 96-hour test. The results for cell density, area under the growth curve, growth rate and yield during the 96-hour exposure are summarized in Tables 4.3.3.2-2, 3, 4, and 5, respectively.

Table 4.3.3.2- 2: Effect of valifenalate technical on cell density (marine diatom, *Skeletonema costatum*)

Treatment geometric mean measured concentration (mg a.i./L)	Initial cell density	Cell density at (cells/mL × 10^4)				
		24 Hours	48 Hours	72 Hours	96 Hours	
					cell count	% inhibition
Negative control	1.0×10^4	5.03	17.3	50.7	101	NA
Solvent control	1.0×10^4	5.34	17.4	50.6	103	NA
0.0388	1.0×10^4	5.20	17.3	51.5	103	NA
0.106	1.0×10^4	5.39	15.7	51.4	99.4	NA
0.339	1.0×10^4	5.14	14.8	42.5	97.2	NA
0.976	1.0×10^4	4.72	7.97	38.4	90.5	NA
3.00	1.0×10^4	3.64	7.14	35.8	89.2	NA
9.48	1.0×10^4	3.75	7.45	27.9	91.5	NA

Table 4.3.3.2-3: Effect of valifenalate technical on area under the growth curve (marine diatom, *Skeletonema costatum*)

Treatment geometric mean measured concentration (mg a.i./L)	Area Under the Growth Curve				
	0-24 Hours	0-48 Hours	0-72 Hours	0-96 Hours	
				Area Under the Growth Curve	% inhibition
Negative control	48.4	293	1,080	2,880	---
Solvent control	52.0	301	1,090	2,910	NA
0.0388	50.3	296	1,100	2,920	-1
0.106	52.7	282	1,060	2,850	1
0.339	49.7	265 *	929 *	2,580 *	10
0.976	44.7	173 *	705 *	2,230 *	23
3.00	31.7 *	137 *	629 *	2,100 *	27
9.48	33.0 *	143 *	543 *	1,950 *	32

* Significant reduction in area as compared to the negative control (Dunnett's test, p = 0.05).

Table 4.3.3.2-4: Effect of valifenalate technical on growth rate (marine diatom, *Skeletonema costatum*)

Treatment geometric mean measured concentration (mg a.i./L)	Mean Growth Rate				
	0-24 Hours	0-48 Hours	0-72 Hours	0-96 Hours	
				Mean Growth Rate	% inhibition
Negative control	0.0672	0.0594	0.0545	0.0481	---
Solvent control	0.0697	0.0595	0.0545	0.0483	NA
0.0388	0.0684	0.0593	0.0547	0.0482	0
0.106	0.0699	0.0573	0.0547	0.0479	0
0.339	0.0681	0.0561 *	0.0520 *	0.0477 *	1
0.976	0.0646	0.0432 *	0.0507 *	0.0469 *	2
3.00	0.0537 *	0.0409 *	0.0497 *	0.0468 *	3
9.48	0.0549 *	0.0418 *	0.0462 *	0.0470 *	2

* Significant reduction in growth rate as compared to the negative control (Dunnett's test, p = 0.05).

Table 4.3.3.2-5: Effect of valifenalate technical on yield (marine diatom, *Skeletonema costatum*)

Treatment geometric mean measured concentration (mg a.i./L)	Mean Yield (cells/mL × 10^4) [a]				
	24 Hours	48 Hours	0-72 Hours	96 Hours	
				Mean Growth Rate	% inhibition
Negative control	4.03	16.3	49.7	100	---
Solvent control	4.34	16.4	49.6	102	NA
0.0388	4.20	16.3	50.5	102	-2
0.106	4.39	14.7	50.4	98.4	2
0.339	4.14	13.8 *	41.5 *	96.2 *	4
0.976	3.72	6.97 *	37.4 *	89.5 *	11
3.00	2.64 *	6.14 *	34.8 *	88.2 *	12
9.48	2.75 *	6.45 *	26.9 *	90.5 *	10

[a] Yield calculated as the biomass at the end of the exposure period minus the starting biomass (i.e., target inoculation cell density of 1.0 × 10^4 cells/mL).

* Significant reduction in growth rate as compared to the negative control (Dunnett's test, p = 0.05).

The achieved NOEC and IC values are summarized in Table **4.3.3.2-**6.

Table 4.3.3.2-6: Effects of Valifenalate Technical on *Skeletonema costatum* growth and yield after 96-hour exposure.

Statistical Endpoint	Area Under the Growth Curve	Growth rate	Yield
NOEC (mg a.i./L)	0.106	0.106	0.106
IC_{10} (mg a.i./L) (95% C.I.)	0.183 (0.0407 to 0.326)	>9.48 (Could not be determined)	Estimated - 0.976 (Could not be determined)
IC_{20} (mg a.i./L) (95% C.I.)	1.47 (0.947 to 2.00)	>9.48 (Could not be determined)	>9.48 (Could not be determined)
IC_{50} (mg a.i./L) (95% C.I.)	>9.48 (Not statistically sound)	>9.48 (Could not be determined)	>9.48 (Could not be determined)

CONCLUSION

A 96-hour acute toxicity study was conducted to determine the effect of valifenalate technical on growth and yield of a marine diatom, *Skeletonema costatum*. The NOEC values based on area under the growth curve, growth rate, and mean yield were all 0.106 mg a.i/L. The 96-hour IC_{50} values based on area under the growth curve, growth rate and mean yield were >9.48 mg a.i./L.

4.3.3.3 Effects on freshwater diatom (*Navicula pelliculosa)*

Report:	Bergfield, A., (2015a): Valifenalate Technical: Growth Inhibition Test with the Freshwater Diatom, *Navicula pelliculosa* . ABC Laboratories, FMC Corporation, FMC Tracking No.: 2014ETX-VAL1344; Report date 2015 dates of experimental work:

Guidelines: OCSPP 850.4500
Deviations: None
GLP: Yes; certified laboratory

Executive Summary

In a 96-hour acute toxicity study, the cultures of freshwater diatom, *Navicula pelliculosa* were exposed to valifenalate technical at nominal concentrations of 0 (control), 0 (vehicle control; DMF 50 µL/L), 0.38, 0.75, 1.5, 3.0, and 6.0 mg a.i./L under static conditions in accordance with the OCSPP 850.4500 guideline. The NOEC values based on geometric mean measured concentration for area under the growth curve, growth rate, and mean yield were all 5.45 mg a.i./L. The 96-hour IC_{10}, IC_{20}, and IC_{50} values based on geometric mean measured concentration for area under the growth curve and were >5.45 mg a.i./L,. The 96-hour percent area under the growth curve inhibition in the treated algal culture as compared to the control ranged from -4 to 4%. The 96-hour IC_{10}, IC_{20}, and IC_{50} values based on geometric mean measured concentration for growth rate were >5.45 mg a.i./L. The 96-hour percent growth rate inhibition in the treated algal culture as compared to the control was 0%. The 96-hour IC_{10}, IC_{20} and IC_{50} values based on geometric mean measured concentration mean yield were >5.45 mg a.i./L. The 96-Hour percent yield inhibition in the treated algal culture as compared to the control ranged from -1 to 3%. There were no abnormalities noted in any of the test substance treatments during the 96-hour test. This toxicity study is classified as acceptable and satisfies the guideline requirements for freshwater diatom, *Navicula pelliculosa* static acute toxicity study.

Table 4.3.3.3-1: Summary of endpoints for Valifenalate Technical after 96-hour exposure

Statistical Endpoint	Area Under the Growth Curve	Growth rate	Yield
NOEC (mg a.i./L)	5.45	5.45	5.45
IC_{10} (mg a.i./L) (95% C.I.)	>5.45 (Not statistically sound)	>5.45 (Not statistically sound)	>5.45 (Not statistically sound)
IC_{20} (mg a.i./L) (95% C.I.)	>5.45 (Not statistically sound)	>5.45 (Not statistically sound)	>5.45 (Not statistically sound)
IC_{50} (mg a.i./L) (95% C.I.)	>5.45 (Not statistically sound)	>5.45 (Not statistically sound)	>5.45 (Not statistically sound)
Reference chemical, if used	NA	NA	NA

MATERIALS AND METHODS

Experimental Test Dates: 05 to 09 January 2015

Test Material:
Name: Valifenalate Technical
Batch no.: PL14-0060 (Manufacturing Lot No.: P/13/019 (Isagro))
Analysed content: 98.9%

Expiry: 03 March 2016
Test Species:
Species: Freshwater diatom, *Navicula pelliculosa*
Source: Department of Botany, Culture Collection of Algae, University of Texas at Austin
Test Conditions:
Growth medium: Freshwater algal nutrient medium (FWAM) as recommended by OCSPP 850.4500
pH (initiation): 7.5
pH (termination): 7.9 to 8.0
Salinity: NA
Temperature & photoperiod:
Temperature: 23.1 to 24.7°C
Photoperiod: Continuous
Light intensity: 4,415 to 4,534 lux by cool-white fluorescent bulbs

Test Design: Based on the a range-finding test and the functional solubility of the test substance in FWAM, nominal concentrations of 0 (control), 0 (vehicle control), 0.38, 0.75, 1.5, 3.0, and 6.0 mg a.i./L were selected for the definitive test. The exposure flasks were 250-mL Erlenmeyer flasks with foam stoppers and labeled with study number, treatment, replicate, and grid position. The control, vehicle control, and each test substance treatment were replicated four times (replicates A, B, C, and D). Each replicate contained 100 mL of the appropriate solution. An additional replicate ("81304, Level 1, Abiotic") of the 0.38 mg a.i./L test substance treatment, containing 100 mL of the parent solution, was also prepared and used to evaluate the potential for incorporation of the test substance into the algal biomass. At test initiation, each A, B, C, and D replicate of the control, vehicle control, and each test substance treatment were inoculated with 1.0 mL of an algal concentrate containing approximately 1.0×10^6 cells/mL, resulting in a final target density of approximately 1.0×10^4 cells/mL for each flask. At 24, 48, 72, and 96 hours (±1 hour), cell density was measured in replicates A, B, C, and D of the control, vehicle control, and each test substance treatment by direct microscopic counting with a hemacytometer. The "81304, Level 1, Abiotic" replicate of the 0.38 mg a.i./L test substance treatment was not inoculated with algae.

Test solutions were analyzed for the concentration of valifenalate technical using high performance liquid chromatography with ultraviolet detection (HPLC-UV) system. Analysis was accomplished based on a method provided by FMC and validated at ABC Laboratories as part of ABC Study No.: 81300; FMC Study 2014ETX-VAL1340. The concentration of valifenalate technical was measured in test solution samples collected at 0 hour (initiation) and 96 hours (termination) of the definitive test. Valifenalate technical-fortified QC samples were also prepared for analysis at each sample period.

All statistical analyses were performed with SAS software (Version 9.3 for windows). All statistical analyses were conducted using the blank control. The NOEC values, based on area under the growth curve, growth rate, and yield, were estimated using a one-way analysis of variance (ANOVA) procedure and a one-tailed Dunnett's test ($p = 0.05$) where the alternate hypothesis was the mean for the growth parameter was reduced in comparison to the control. The I_bC_{50}, I_rC_{50}, and I_yC_{50} estimates were calculated using a 4-parameter logistic (sigmoid-shaped) model fit to the data with percent inhibition as the dependent variable and concentration as the independent variable.

Validity Criteria:
The following criteria must be found in the control replicates:

- Logarithmic phase growth after 96 hours.
- The coefficients of variation for yield and average specific growth rates at termination do not exceed 15%.

RESULTS AND DISCUSSION

Validity Criteria: The results of the control and vehicle control met the required guideline validity criteria.

***Analytical results*:** Measured concentrations of valifenalate technical in the test substance treatment solutions at initiation were 0.368, 0.728, 1.46, 3.12, and 5.69 mg a.i./L, which represented recoveries of 95 to 104% of the target nominal concentrations. Measured concentrations of valifenalate technical in the test substance treatment solutions at 96 hours (termination) were 0.342, 0.696, 1.36, 2.70, and 5.22 mg a.i./L, respectively, which represented recoveries of 87 to 93% of the target nominal concentrations. No residues of valifenalate technical were detected in the control or vehicle control solutions above the MQL of 0.0200 mg a.i./L. The measured concentration in the 0.38 mg a.i./L (nominal) abiotic sample at 96 hours was 0.369 mg a.i./L, indicating that the test substance was stable during the test exposure. The geometric mean measured concentrations in the test substance treatment solutions were 0.355, 0.712, 1.41, 2.90, and 5.45 mg a.i./L, which represented recoveries of 91 to 97% of the target nominal concentrations.

Biological Results: The mean cell density in the valifenalate technical treatments at 96 hours ranged from a low of 53.3 × 10^4 cells/mL at 5.45 mg a.i./L to a high of 54.4 × 10^4 cells/mL at 2.90 mg a.i./L. Percent inhibition in area under the growth curve at 96 hours, as compared to the control, ranged from -4% at 0.712 mg a.i./L to 4% at 5.45 mg a.i./L. Percent inhibition in growth rate at 96 hours, as compared to the control, was 0% at all test substance treatment levels. The percent inhibition in yield at 96 hours, as compared to the control, ranged from -1% at the concentration of 2.90 mg a.i./L to 1% at the concentrations of 0.368 and 5.45 mg a.i./L. There were no abnormalities noted in any of the test substance treatments during the 96-hour test. The results for cell density, area under the growth curve, growth rate and yield during the 96-hour exposure are summarized in Tables 4.3.3.3-2, 3, 4, and 5, respectively.

Table 4.3.3.3-2: Effect of valifenalate technical on cell density (freshwater diatom, *Navicula pelliculosa*)

Treatment geometric mean measured concentration (mg a.i./L)	Initial cell density	Cell density at (cells/mL × 10^4)				
		24 Hours	48 Hours	72 Hours	96 Hours	
					cell count	% inhibition
Negative control	1.0 × 10^4	3.06	7.06	19.4	54.0	---
Solvent control	1.0 × 10^4	3.14	7.64	20.6	52.5	3
0.368	1.0 × 10^4	3.33	7.22	20.0	53.5	1
0.712	1.0 × 10^4	3.28	8.06	20.3	53.9	0
1.41	1.0 × 10^4	3.20	7.64	19.9	53.4	1
2.90	1.0 × 10^4	3.14	7.20	20.3	54.4	-1
5.45	1.0 × 10^4	2.03 *	5.47 *	20.4	53.3	1

* Significant reduction in area as compared to the control (Dunnett's test, p = 0.05).

Table 4.3.3.3-3: Effect of valifenalate technical on area under the growth curve (freshwater diatom, *Navicula pelliculosa*)

Treatment geometric mean measured concentration (mg a.i./L)	Area Under the Growth Curve				
	0-24 Hours	0-48 Hours	0-72 Hours	0-96 Hours	
				Area Under the Growth Curve	% inhibition
Negative control	24.7	122	415	1,270	---
Solvent control	25.7	131	446	1,300	-2
0.368	28.0	131	433	1,290	-1
0.712	27.3	139	455	1,320	-4
1.41	26.3	132	439	1,290	-2
2.90	25.7	126	432	1,300	-3
5.45	12.3 *	78.3 *	365 *	1,220	4

* Significant reduction in area as compared to the control (Dunnett's test, p = 0.05).

Table 4.3.3.3-4: Effect of valifenalate technical on growth rate (freshwater diatom, *Navicula pelliculosa*)

Treatment geometric mean measured concentration (mg a.i./L)	Mean Growth Rate				
	0-24 Hours	0-48 Hours	0-72 Hours	0-96 Hours	
				Mean Growth Rate	% inhibition
Negative control	0.0463	0.0407	0.0412	0.0415	---
Solvent control	0.0476	0.0423	0.0420	0.0413	0
0.368	0.0501	0.0411	0.0416	0.0414	0
0.712	0.0493	0.0435	0.0418	0.0415	0
1.41	0.0482	0.0423	0.0416	0.0414	0
2.90	0.0473	0.0411	0.0418	0.0416	0
5.45	0.0291 *	0.0353 *	0.0419	0.0414	0

* Significant reduction in growth rate as compared to the control (Dunnett's test, p = 0.05).

Table 4.3.3.3-5: Effect of valifenalate technical on yield (freshwater diatom, *Navicula pelliculosa*)

Treatment geometric mean measured concentration (mg a.i./L)	Mean Yield (cells/mL × 10^4) [a]				
	24 Hours	48 Hours	0-72 Hours	96 Hours	
				Mean Growth Rate	% inhibition
Negative control	2.06	6.06	18.4	53.0	---
Solvent control	2.14	6.64	19.6	51.5	3
0.368	2.33	6.22	19.0	52.5	1
0.712	2.28	7.06	19.3	52.9	0
1.41	2.20	6.64	18.9	52.4	1
2.90	2.14	6.20	19.3	53.4	-1
5.45	1.03 *	4.47 *	19.4	52.3	1

[a] Yield calculated as the biomass at the end of the exposure period minus the starting biomass (i.e., target inoculation cell density of 1.0 × 10^4 cells/mL).

* Significant reduction in growth rate as compared to the control (Dunnett's test, p = 0.05).

The achieved NOEC and IC values are summarized in Table 4.3.3.3-6.

Table 4.3.3.3-6: Effects of Valifenalate Technical on *Navicula pelliculosa* growth and yield after 96-hour exposure.

Statistical Endpoint	Area Under the Growth Curve	Growth rate	Yield
NOEC (mg a.i./L)	5.45	5.45	5.45
IC_{10} (mg a.i./L) (95% C.I.)	>5.45 (Not statistically sound)	>5.45 (Not statistically sound)	>5.45 (Not statistically sound)
IC_{20} (mg a.i./L) (95% C.I.)	>5.45 (Not statistically sound)	>5.45 (Not statistically sound)	>5.45 (Not statistically sound)
IC_{50} (mg a.i./L) (95% C.I.)	>5.45 (Not statistically sound)	>5.45 (Not statistically sound)	>5.45 (Not statistically sound)

CONCLUSION

A 96-hour acute toxicity study was conducted to determine the effect of valifenalate technical on growth and yield of a freshwater diatom, *Navicula pelliculosa*. The NOEC values based on area under the growth curve, growth rate, and mean yield were all 5.45 mg a.i/L. The 96-hour IC_{50} values based on area under the growth curve, growth rate and mean yield were >5.45 mg a.i./L.

4.3.3.4 Effects on Cyanobacteria (*Anabaena flos-aquae)*

Report:	Aufderheide, J., (2015), Valifenalate Technical: Growth Inhibition Test with the Cyanobacterium, *Anabaena flos-aquae*. ABC Laboratories, FMC Corporation, FMC Tracking No.: 2014ETX-VAL1345; Report date 2015 dates of experimental work:

Guidelines: OCSPP 850.4550
Deviations: None
GLP: Yes

Executive Summary

In a 96-hour acute toxicity study, the cultures of cyanobacterium, *Anabaena flos-aquae* were exposed to valifenalate technical at nominal concentrations of 0 (control), 0 (vehicle control; DMF 50 µL/L), 0.38, 0.75, 1.5, 3.0, and 6.0 mg a.i./L under static conditions in accordance with the OCSPP 850.4550 guideline. The functional solubility of valifenalate technical in test medium, as determined as part of this study, was approximately 6 mg/L. The NOEC values based on area under the growth curve, growth rate, and mean yield were 2.15, 4.13, and 4.13 mg a.i./L geometric mean measured concentration, respectively. The 96-hour IC_{10}, IC_{20}, and IC_{50} values based on area under the growth curve and geometric mean measured concentration were >4.13 mg a.i./L, respectively. The percent area under the growth curve inhibition in the treated algal culture as compared to the control ranged from -9 to 9%. The 96-hour IC_{10}, IC_{20}, and IC_{50} values based on growth rate and geometric mean measured concentration were >4.13 mg a.i./L. The percent growth rate inhibition in the treated algal culture as compared to the control ranged from -4 to 1%. The 96-hour IC_{10}, IC_{20} and IC_{50} values based on mean yield and geometric mean measured concentration were >4.13 mg a.i./L. The percent yield inhibition in the treated algal culture as compared to the control ranged from -16 to 5%. There were no abnormalities noted in any of the test substance treatments during the 96-hour test. This toxicity study is classified as acceptable and satisfies the guideline requirements for cyanobacterium, *Anabaena flos-aquae* static acute toxicity study.

Table 4.3.3.4-01: Summary of endpoints for Valifenalate Technical after 96-hour exposure

Statistical Endpoint	Area Under the Growth Curve	Growth rate	Yield
NOEC (mg a.i./L)	2.15	4.13	4.13
IC_{10} (mg a.i./L) (95% C.I.)	>4.13 (Not statistically sound)	>4.13 (Could not be calculated)[a]	>4.13 (Could not be calculated)[a]
IC_{20} (mg a.i./L) (95% C.I.)	>4.13 (Not statistically sound)	>4.13 (Could not be calculated)[a]	>4.13 (Could not be calculated)[a]
IC_{50} (mg a.i./L) (95% C.I.)	>4.13 (Not statistically sound)	>4.13 (Could not be calculated)[a]	>4.13 (Could not be calculated)[a]
Reference chemical, if used	NA	NA	NA

[a] Data did not allow for calculation of the IC value or 95% Confidence Limits due to a lack of inhibitory response in the highest concentration.

MATERIALS AND METHODS

Experimental Test Dates: 08 to 12 December 2014

Test Material:
Name: Valifenalate Technical
Batch no.: PL14-0060 (Manufacturing Lot No.: P/13/019 (Isagro))
Analysed content: 98.9%
Expiry: 03 March 2015

Test Species:
Species: Cyanobacterium, *Anabaena flos-aquae*
Source: Department of Botany, Culture Collection of Algae, University of Texas at Austin
Test Conditions:
Growth medium: Freshwater algal nutrient medium (FWAM) as recommended by OCSPP 850.4550
pH (initiation): 7.4 to 7.5
pH (termination): 9.3 to 9.5
Temperature & photoperiod:
Temperature: 24.2 to 24.6°C
Photoperiod: Continuous
Light intensity: 2,158 to 2,244 lux by cool-white fluorescent bulbs

Test Design: Based on the a range-finding test and the functional solubility of the test substance in FWAM, nominal concentrations of 0 (control), 0 (vehicle control; 50 µL/L), 0.38, 0.75, 1.5, 3.0, and 6.0 mg a.i./L were selected for the definitive test. The exposure flasks were 250-mL Erlenmeyer flasks with foam stoppers and labeled with study number, treatment, replicate, and grid position. The control, vehicle control, and each test substance treatment were replicated four times. Each replicate contained 100 mL of the appropriate parent solution. An additional replicate (abiotic replicate) of the 0.38 mg a.i./L test substance treatment, containing 100 mL of the parent solution, was also prepared and used to evaluate the potential for incorporation of the test substance into the algal biomass. At test initiation, each biological replicate of the control, vehicle control, and each test substance treatment were inoculated with 1.0 mL of an algal concentrate containing approximately 1.95×10^6 cells/mL, resulting in a final target density of approximately 1.0×10^4 cells/mL for each flask. At 24, 48, 72, and 96 hours (±1 hour), cell density was measured in the biological replicates of the control, vehicle control, and each test substance treatment by direct microscopic counting with a hemacytometer. The abiotic replicate of the 0.38 mg a.i./L test substance treatment was not inoculated with algae.

Test solutions were centrifuged to remove any algae or undissolved test substance, thus the analysis were indicative of the test substance in solution. Each test solution was analyzed for the concentration of valifenalate technical using high performance liquid chromatography with ultraviolet detection (HPLC-UV) system. Analysis was accomplished based on a method provided by FMC and validated at ABC Laboratories as part of ABC Study No.: 81300; FMC Study 2014ETX-VAL1340. The concentration of valifenalate technical was measured in test solution samples collected at 0 hour (initiation) and 96 hours (termination) of the definitive test. Valifenalate technical-fortified QC samples were also prepared for analysis at each sample period.

All statistical analyses were performed with SAS software (Version 9.3 for windows). All statistical analyses were conducted using the blank control. The no-observable effects (NOEC) values, based on area under the growth curve, growth rate, and yield, were estimated using a one-way analysis of variance (ANOVA) procedure and a one-tailed Dunnett's test ($p = 0.05$) where the alternate hypothesis was the mean for the growth parameter was reduced in comparison to the control. The I_bC_{50}, I_rC_{50}, and I_yC_{50} estimates were calculated using a 4-parameter logistic (sigmoid-shaped) model fit to the data with percent inhibition as the dependent variable and concentration as the independent variable.

Validity Criteria:

The following criteria must be found in the control replicates:

- Logarithmic phase growth after 96 hours.
- The control coefficient of variation for yield did not exceed 20% and the control coefficient of variation for average specific growth rates did not exceed 12% at termination.

RESULTS AND DISCUSSION

Validity Criteria: The results of the control and vehicle control met the required guideline validity criteria.

***Analytical results*:** Measured concentrations of valifenalate technical in the test substance treatment solutions at initiation were 0.368, 0.712, 1.46, 2.76, and 5.76 mg a.i./L, which represented recoveries of 92 to 97% of the target nominal concentrations. Measured concentrations of valifenalate technical in the test substance treatment solutions at 96 hours (termination) were 0.217, 0.432, 0.861, 1.67, and 2.96 mg a.i./L, which represented recoveries of 49 to 58% of the target nominal concentrations. No residues of valifenalate technical were detected in the control or vehicle control solutions above the MQL of 0.0200 mg a.i./L. The measured concentration in the 0.38 mg a.i./L (nominal) abiotic sample at 96 hours was 0.358 mg a.i./L, indicating that the test substance was stable during the test exposure. The geometric mean measured concentrations in the test substance treatment solutions were 0.283, 0.555, 1.12, 2.15, and 4.13 mg a.i./L, which represented recoveries of 69 to 75% of the target nominal concentrations.

Biological Results: After 96 hours of exposure, mean cell density in the control was 65.9 × 10^4 cells/mL, or 66 times the initial nominal cell density, which verified logarithmic growth meeting the acceptability criterion for this growth parameter. The mean cell density in the valifenalate technical treatments at 96 hours ranged from a low of 63.0 × 10^4 cells/mL at 4.13 mg a.i./L to a high of 76.6 × 10^4 cells/mL at 0.283 mg a.i./L. Percent inhibition in area under the growth curve at 96 hours, as compared to the control, ranged from -9% at 0.283 mg a.i./L to 9% at 4.13 mg a.i./L. The coefficient of variation of average specific growth rates after 96 hours in control replicates was 1%, which met the acceptability criterion of a %CV value <12% for this growth parameter. Percent inhibition in growth rate at 96 hours, as compared to the control, -4% at 0.283 mg a.i./L to 1% at 4.13 mg a.i./L. The coefficient of variation of cell density yield in control replicates was 4% after 96 hours, which met the acceptability criterion of a %CV value <20% for this growth parameter. The percent inhibition in yield at 96 hours, as compared to the control, ranged from -16% at 0.283 and 0.555 mg a.i./L to 5% at 4.13 mg a.i./L. There were no abnormalities noted in any of the test substance treatments during the 96-hour test. The results for cell density, area under the growth curve, growth rate and yield during the 96-hour exposure are summarized in Tables 4.3.3.4-02, 03, 04, and 05, respectively.

Table 4.3.3.4-02: Effect of valifenalate technical on cell density (cyanobacterium, *Anabaena flos-aquae*)

Treatment geometric mean measured concentration (mg a.i./L)	Initial cell density	Cell density at (cells/mL × 10^4)				
		24 Hours	48 Hours	72 Hours	96 Hours	
					cell count	% inhibition
Negative control	1.0 × 10^4	2.67	7.95	33.4	65.9	---
Solvent control	1.0 × 10^4	3.75	8.28	35.5	63.6	---
0.283	1.0 × 10^4	3.31	7.70	34.7	76.6	-16
0.555	1.0 × 10^4	3.53	7.67	33.3	76.5	-16
1.12	1.0 × 10^4	3.75	6.89 *	33.9	64.7	2
2.15	1.0 × 10^4	4.17	7.53	34.9	70.8	-7
-4.13	1.0 × 10^4	2.61	5.58 *	30.7 *	63.0	4

* Significant reduction in area as compared to the negative control (Dunnett's test, p = 0.05).

Table 4.3.3.4-03: Effect of valifenalate technical on area under the growth curve (cyanobacterium, *Anabaena flos-aquae*)

Treatment geometric mean measured concentration (mg a.i./L)	Area Under the Growth Curve				
	0-24 Hours	0-48 Hours	0-72 Hours	0-96 Hours	
				Area Under the Growth Curve	% inhibition
Negative control	20.0	123	596	1,760	---
Solvent control	33.0	153	654	1,820	---
0.283	27.7	136	620	1,930	-9
0.555	30.3	141	608	1,900	-8
1.12	33.0	137	602	1,760	0
2.15	38.0	154	639	1,880	-7
4.13	19.4	93.7 *	504 *	1,610 *	9

* Significant reduction in area as compared to the negative control (Dunnett's test, p = 0.05).

Table 4.3.3.4-04: Effect of valifenalate technical on growth rate (cyanobacterium, *Anabaena flos-aquae*)

Treatment geometric mean measured concentration (mg a.i./L)	Mean Growth Rate (cells/mL/hour)				
	0-24 Hours	0-48 Hours	0-72 Hours	0-96 Hours	
				Mean Growth Rate	% inhibition
Negative control	0.0408	0.0432	0.0487	0.0436	---
Solvent control	0.0551	0.0440	0.0495	0.0432	---
0.283	0.0498	0.0425	0.0492	0.0452	-4
0.555	0.0524	0.0424	0.0487	0.0452	-3
1.12	0.0550	0.0402 *	0.0489	0.0434	0
2.15	0.0594	0.0420	0.0493	0.0444	-2
4.13	0.0399	0.0357 *	0.0475 *	0.0432	1

* Significant reduction in growth rate as compared to the negative control (Dunnett's test, p = 0.05).

Table 4.3.3.4-05: Effect of valifenalate technical on yield (cyanobacterium, *Anabaena flos-aquae*)

Treatment geometric mean measured concentration (mg a.i./L)	Mean Yield (cells/mL × 10^4) [a]				
	24 Hours	48 Hours	0-72 Hours	96 Hours	
				Mean Growth Rate	% inhibition
Negative control	1.67	6.95	32.4	64.9	---
Solvent control	2.75	7.28	34.5	62.6	---
0.283	2.31	6.70	33.7	75.6	-16
0.555	2.53	6.67	32.3	75.5	-16
1.12	2.75	5.89 *	32.9	63.7	2
2.15	3.17	6.53	33.9	69.8	-7
4.13	1.61	4.58 *	29.7 *	62.0	5

[a] Yield calculated as the biomass at the end of the exposure period minus the starting biomass (i.e., target inoculation cell density of 1.0 × 10^4 cells/mL).

* Significant reduction in growth rate as compared to the negative control (Dunnett's test, p = 0.05).

The achieved NOEC and IC values are summarized in Table 4.3.3.4-06.

Table4.3.3.4-06: Effects of Valifenalate Technical on *Anabaena flos-aquae* growth and yield after 96-hour exposure.

Statistical Endpoint	Area Under the Growth Curve	Growth rate	Yield
NOEC (mg a.i./L)	2.15	4.13	4.13
IC_{10} (mg a.i./L) (95% C.I.)	>4.13 (Not statistically sound)	>4.13 (Could not be calculated)[a]	>4.13 (Could not be calculated)[a]
IC_{20} (mg a.i./L) (95% C.I.)	>4.13 (Not statistically sound)	>4.13 (Could not be calculated)[a]	>4.13 (Could not be calculated)[a]
IC_{50} (mg a.i./L) (95% C.I.)	>4.13 (Not statistically sound)	>4.13 (Could not be calculated)[a]	>4.13 (Could not be calculated)[a]

[a] Data did not allow for calculation of the IC value or 95% Confidence Limits due to a lack of inhibitory response in the highest concentration.

CONCLUSION

A 96-hour acute toxicity study was conducted to determine the effect of valifenalate technical on growth and yield of a cyanobacterium, *Anabaena flos-aquae*. The NOEC values based on area under the growth curve, growth rate, and mean yield were 2.15, 4.13, and 4.13 mg a.i/L, respectively. The 96-hour IC_{50} values based on area under the growth curve, growth rate and mean yield were >4.13 mg a.i./L.

4.3.3.5 Effects on Duckweed - *Lemna gibba*

Report:	Bergfield, A., (2015), Valifenalate Technical: Growth Inhibition Test with with the Freshwater Aquatic Plant, Duckweed, *Lemna gibba*. ABC Laboratories, FMC Corporation, FMC Tracking No.: 2014ETX-VAL1343; Report date 2015 dates of experimental work:

Guidelines: OCSPP 850.4400
Deviations: None
GLP: Yes
Executive Summary

In a 7-day acute toxicity study, the cultures of the freshwater aquatic plant, duckweed, *Lemna gibba* were exposed to valifenalate technical at nominal concentrations of 0 (control), 0 (vehicle control; DMF 50 µL/L), 0.38, 0.75, 1.5, 3.0, and 6.0 mg a.i./L under static-renewal conditions in accordance with the OCSPP 850.4400 guideline. The NOEC values based on geometric mean measured concentration for frond average specific growth rate, frond yield, biomass yield as dry weight, and biomass average specific growth rate as dry weight were all 5.02 mg a.i./L. The 7-day EC_{10}, EC_{20}, and EC_{50} values based on geometric mean measured concentration for frond average specific growth rate were >5.02 mg a.i./L. The percent average specific growth rate inhibition in the treated duckweed culture as compared to the control ranged from 0 to 1%. The 7-day EC_{10}, EC_{20}, and EC_{50} values based on geometric mean measured concentration for frond yield were >5.02 mg a.i./L. The percent frond yield inhibition in the treated duckweed culture as compared to the control ranged from -1 to 4%. The 7-day EC_{10}, EC_{20}, and EC_{50} values based on biomass as dry weight and geometric mean measured concentration were >5.02 mg a.i./L. The percent biomass as dry weight inhibition in the treated duckweed culture as compared to the control ranged from -13 to -1%. The 7-day EC_{10}, EC_{20}, and EC_{50} values based on geometric mean measured concentration for biomass average specific growth rate were

>5.02 mg a.i./L. The percent biomass average specific growth rate inhibition in the treated duckweed culture as compared to the control ranged from -4 to -1%. This toxicity study is classified as acceptable and satisfies the guideline requirements for *Lemna gibba* static-renewal acute toxicity study.

Table 4.3.3.5-1: Summary of endpoints for Valifenalate Technical after 7-day exposure

Statistical Endpoint	Frond Average Specific Growth Rate	Frond Yield	Biomass Yield as Dry Weight	Biomass Average Specific Growth Rate as Dry Weight
NOEC (mg a.i./L)	5.02	5.02	5.02	5.02
LOEC (mg a.i./L)	>5.02	>5.02	>5.02	>5.02
EC_{10} (mg a.i./L) (95% C.I.)	>5.02 (Not statistically sound)	>5.02 (Not statistically sound)	Could not be calculated [a]	Could not be calculated [a]
EC_{20} (mg a.i./L) (95% C.I.)	>5.02 (Not statistically sound)	>5.02 (Not statistically sound)	Could not be calculated [a]	Could not be calculated [a]
EC_{50} (mg a.i./L) (95% C.I.)	>5.02 (Not statistically sound)	>5.02 (Not statistically sound)	Could not be calculated [a]	Could not be calculated [a]
Reference chemical, if used	NA	NA	NA	NA

[a] Values could not be calculated due to lack of convergence. EC_{50} was estimated to be greater than the highest treatment level due to less than 50% inhibition at that treatment.

MATERIALS AND METHODS

Experimental Test Dates: 04 to 11 February 2015

Test Material:
Name: Valifenalate Technical
Batch no.: PL14-0060 (Manufacturing Lot No.: P/13/019 (Isagro))
Analysed content: 98.9%
Expiry: 03 March 2016
Test Species:
Species: Duckweed, *Lemna gibba* G3 clone
Source: USDA/ARS Beltsville Agricultural Research Center, Beltsville, Maryland

Test Conditions:
Growth medium: 20X Freshwater algal nutrient medium (20X FWAM) as recommended by OCSPP 850.4400
pH (fresh solutions): 7.5 to 7.7
pH (spent solutions): 8.6 to 9.1
Salinity: NA
Temperature & photoperiod:
Temperature (fresh solutions): 24.4 to 25.8°C
Temperature(spent solutions): 23.7 to 24.2°C
Photoperiod: Continuous
Light intensity: 5,760 to 5,844 lux by cool-white fluorescent bulbs

Test Design: Based on the a range-finding test and the functional solubility of the test substance in FWAM, nominal concentrations of 0 (control), 0 (vehicle control; 50 µL/L), 0.38, 0.75, 1.5, 3.0, and 6.0 mg a.i./L were selected for the definitive test. The exposure flasks were 500-mL Erlenmeyer flasks with foam stoppers and

labeled with study number, treatment, replicate, and grid position. The control, vehicle control, and each test substance treatment were replicated four times (replicates A, B, C, and D). Each replicate contained 200 mL of the appropriate parent solution. Each test flask received three plants, for a total of 12 fronds, at test initiation. Aseptic addition of *Lemna gibba* was initiated and completed within one hour of test solution preparation. Subsequently, at each renewal of test media, the plants were aseptically transferred, without harming roots or fronds, from spent test solutions to fresh test solutions. Plant growth was monitored in each flask. Growth was measured by determining the change in the number of total fronds during the exposure period. Every frond that visibly projected beyond the edge of the parent frond was counted as a separate frond. Any change in plant development, frond size, appearance, necrosis or chlorosis was noted if observed. Frond observations and counts were performed on days 2, 5, and 7 for all replicates of the control, vehicle control, and each test substance treatment. Biomass (dry weight) measurements of each control, vehicle control, and test substance treatment replicate were performed on day 7 (test termination), after frond observations had been conducted, as well as on four representative samples at test initiation. Each representative sample at test initiation was comprised of three plants, for a total of 12 fronds per sample. The biomass of each replicate was determined by transferring all plants (fronds and roots) from each replicate to individually labeled and pre-weighed aluminum pans. All appropriately labeled pans containing *Lemna gibba* were allowed to dry until a constant weight was reached at a range of 65 to 71ºC for six days. The aluminum pans were reweighed after drying to measure total biomass.

Test solutions were analyzed for the concentration of valifenalate technical using high performance liquid chromatography with ultraviolet detection (HPLC-UV) system. Analysis was accomplished based on a method provided by FMC and validated at ABC Laboratories as part of ABC Study No.: 81300; FMC Study 2014ETX-VAL1340. The concentration of valifenalate technical was measured in test solution samples collected from the control, vehicle control, and each test substance treatment. Samples from freshly-prepared parent solutions were collected at day 0 (initiation), day 2 and day 5. Composite samples, after combining replicate solutions by treatment, were collected on day 2, day 5 and day 7 (termination). Valifenalate technical-fortified QC samples were also prepared for analysis at each sample period.

All statistical analyses were performed with SAS software (Version 9.3 for windows). All statistical analyses were conducted using the blank control. The NOEC values, based on total frond, frond growth rate, frond yield, dry weight yield, and average specific grow rate dry weight, were estimated using a one-way analysis of variance (ANOVA) procedure and a one-tailed Dunnett's test ($p = 0.05$) where the alternate hypothesis was the mean for the growth parameter was reduced in comparison to the control. The E_rC_{50} and E_yC_{50} estimates were calculated using a 4-parameter logistic (sigmoid-shaped) model fit to the data with percent inhibition as the dependent variable and concentration as the independent variable.

Validity Criteria:

The following criteria must be found in the control replicates:

- Frond doubling time <2.5 days.
- Greater than a seven-fold increase in the number of fronds.

RESULTS AND DISCUSSION

Validity Criteria: The results of the control met the required guideline validity criteria.

***Analytical results*:** Measured concentrations of valifenalate technical in the test substance treatment solutions at initiation were 0.338, 0.690, 1.42, 2.78, and 5.54 mg a.i./L, which represented recoveries of 89 to 95% of the nominal concentrations. Measured concentrations of valifenalate technical in the test substance treatment fresh solutions at Day 2 were 0.304, 0.702, 1.30, 2.49, and 5.11 mg a.i./L and Day 5 were 0.345, 0.693, 1.43, 2.86, and 5.81 mg a.i./L, which ranged from 80 to 97% of the nominal concentrations. Measured concentrations of valifenalate technical in spent test substance treatment solutions were 0.293, 0.612, 1.27, 2.52, and 5.25 mg a.i./L on day 2; 0.235, 0.548, 1.01, 1.86, and 4.23 mg a.i./L on day 5; and 0.240, 0.481, 0.991, 2.08 and 4.36 mg a.i./L on day 7, which ranged from 62 to 88% of the nominal concentrations. No residues of valifenalate technical were detected in the control or vehicle control solutions above the MQL of 0.0200 mg a.i./L. The mean measured concentrations in the test substance treatment solutions during the 7-day exposure were 0.293, 0.621, 1.24, 2.43, and 5.05 mg a.i./L, which represented recoveries of 77 to 84%

of the nominal concentrations. The geometric mean measured concentrations in the test substance treatment solutions during the 7-day exposure were 0.289, 0.615, 1.22, 2.40, and 5.02 mg a.i./L, which represented recoveries of 76 to 84% of the nominal concentrations.

Biological Results: The doubling time of total frond number in the control was 1.4 days, corresponding to greater than 7-fold increase in seven days, which met the doubling time requirement established by the OECD 221 test guideline (i.e., <2.5 days, corresponding to approximately a seven-fold increase in seven days). The control average specific growth rate for total number of fronds on day 0 to 7 was 0.471 day^{-1}, exceeding the 0.275 day^{-1} minimum required by the OECD 221 test guideline. The mean number of total fronds in the valifenalate technical treatments at 7 days ranged from a low of 312 at 2.40 mg a.i./L to a high of 329 at 0.615 mg a.i./L. Percent inhibition in the mean average specific growth rate at 7 days, as compared to the control, ranged from 0% at 0.289, 0.615, and 1.22 mg a.i./L to 1% at 2.4 and 5.02 mg a.i./L. Percent inhibition in mean frond yield at 7 days, as compared to the control, -1% at 0.615 mg a.i./L to 4% at 2.4 mg a.i./L. The percent inhibition in biomass (dry weight) at 7 days, as compared to the control, ranged from -13% at 1.22 mg a.i./L to -3% at 0.289 mg a.i./L. The percent inhibition in biomass average specific growth rate at 7 days, as compared to the control, ranged from -4% at 0.615 and 1.22 mg a.i./L to -1% at 0.289 and 5.02 mg a.i./L. The results for total fronds, average specific growth rate, frond yield, biomass (dry weight), and biomass average specific growth rate during the 7-day exposure are summarized in Tables 4.3.3.5-2, 3, 4, 5, and 6, respectively.

Table 4.3.3.5-2: Effect of valifenalate technical on total fronds (duckweed, *Lemna gibba*)

Treatment geometric mean measured concentration (mg a.i./L)	Initial number of fronds	Mean Number of Total Fronds			
		Day 2	Day 5	Day 7	
				Number of fronds	% inhibition
Negative control	48	29.0	107	325	---
Solvent control	48	26.5	107	322	1
0.289	48	27.5	112	321	1
0.615	48	28.8	114	329	-1
1.22	48	27.3	114	326	0
2.40	48	25.5	106	312	4
5.02	48	28.0	107	320	2

Table 4.3.3.5-3: Effect of valifenalate technical on mean average specific growth rate of fronds (duckweed, *Lemna gibba*)

Treatment geometric mean measured concentration (mg a.i./L)	Mean Average Specific Growth Rate (day $^{-1}$)			
	Day 0-2	Day 0-5	Day 0-7	
			Mean Average Specific Growth Rate	% inhibition
Negative control	0.440	0.437	0.471	---
Solvent control	0.395	0.437	0.470	0
0.289	0.414	0.446	0.470	0
0.615	0.434	0.449	0.473	0
1.22	0.408	0.450	0.472	0
2.40	0.376	0.436	0.465	1
5.02	0.422	0.437	0.469	1

Table 4.3.3.5-4: Effect of valifenalate technical on mean frond yield (duckweed, *Lemna gibba*)

Treatment geometric mean measured concentration (mg a.i./L)	Mean Yield of Total Fronds			
	Day 2	Day 5	Day 7	
			Mean Yield of Total Fronds	% inhibition
Negative control	17.0	94.5	313	---
Solvent control	14.5	94.5	310	1
0.289	15.5	100	309	1
0.615	16.8	102	317	-1
1.22	15.3	102	314	0
2.40	13.5	94.0	300	4
5.02	16.0	94.8	308	2

Table 4.3.3.5-5: Effect of valifenalate technical on biomass (dry weight) (duckweed, *Lemna gibba*)

Treatment geometric mean measured concentration (mg a.i./L)	Biomass (Dry Weight) [a]	
	Day 7	
	Treatment Mean Yield (g)	% inhibition
Negative control	0.0338	---
Solvent control	0.0342	-1
0.289	0.0349	-3
0.615	0.0375	-11
1.22	0.0383	-13
2.40	0.0353	-4
5.02	0.0351	-4

[a] Yield calculated as day 7 biomass – day 0 biomass.

Table 4.3.3.5-6: Effect of valifenalate technical on biomass (dry weight) average specific growth rate (duckweed, *Lemna gibba*)

Treatment geometric mean measured concentration (mg a.i./L)	Biomass (Dry Weight) Average Specific Growth Rate (day $^{-1}$)	
	Day 7	
	Treatment Mean	% inhibition
Negative control	0.387	---
Solvent control	0.389	-1
0.289	0.392	-1
0.615	0.401	-4
1.22	0.404	-4
2.40	0.393	-2
5.02	0.393	-1

The achieved NOEC, LOEC, and estimated EC values are summarized in Table 4.3.3.5-7.

Table 4.3.3.5-7: Effects of Valifenalate Technical on *Lemna gibba* growth and yield after 7-day exposure.

Statistical Endpoint	Frond Average Specific Growth Rate	Frond Yield	Biomass Yield as Dry Weight	Biomass Average Specific Growth Rate as Dry Weight
NOEC (mg a.i./L)	5.02	5.02	5.02	5.02
LOEC (mg a.i./L)	>5.02	>5.02	>5.02	>5.02
EC_{10} (mg a.i./L) (95% C.I.)	>5.02 (Not statistically sound)	>5.02 (Not statistically sound)	Could not be calculated [a]	Could not be calculated [a]
EC_{20} (mg a.i./L) (95% C.I.)	>5.02 (Not statistically sound)	>5.02 (Not statistically sound)	Could not be calculated [a]	Could not be calculated [a]
EC_{50} (mg a.i./L) (95% C.I.)	>5.02 (Not statistically sound)	>5.02 (Not statistically sound)	Could not be calculated [a]	Could not be calculated [a]
Reference chemical, if used	NA	NA	NA	NA

[a] Values could not be calculated due to lack of convergence. EC_{50} was estimated to be greater than the highest treatment level due to less than 50% inhibition at that treatment.

CONCLUSION

A 7-day acute toxicity study was conducted to determine the effect of valifenalate technical on growth and yield of a freshwater aquatic plant, duckweed, *Lemna gibba*. The NOEC values based on geometric mean measured concentration for frond average specific growth rate, frond yield, biomass yield as dry weight, and biomass average specific growth rate as dry weight were all 5.02 mg a.i./L. The 7 day EC_{50} values based on frond average specific growth rate, frond yield, biomass yield as dry weight, and biomass average specific growth rate as dry weight were >5.02 mg a.i./L.

4.3.3.6 Effects on Midge Larvae- (*Chironomus dilutus*)

Report:	Aufderheide, J (2015c), Valifenalate Technical: Whole Sediment Acute Toxicity Test with Midge Larvae (*Chironomus dilutus*). ABC Laboratories, FMC Corporation, FMC Tracking No.: 2014ETX-VAL1347 ; Report date 2015 dates of experimental work:

Guidelines: OCSPP 850.1735
Deviations: None
GLP: Yes

Executive Summary

In a 10-day acute toxicity study, the freshwater midge, *Chironomus dilutus* were exposed to valifenalate technical at nominal concentrations of 0 (control), 13, 25, 50, 100 and 200 mg a.i./ kg dry sediment in accordance with the OCSPP 850.1735 guideline. The NOEC values based on mean calculated concentrations in sediment were 108 mg/kg for survival and 14.1 mg/kg for growth (ash-free dry weights). The LOEC values

based on mean calculated concentrations in sediment were >108 mg/kg for survival and 37.7 mg/kg for growth (ash-free dry weights). The LC_{50} values based on mean calculated concentrations in sediment were >108 mg/kg for survival and were not calculated for growth (ash-free dry weights). There were no abnormalities noted in any of the test substance treatments during the 10-day test. This toxicity study is classified as acceptable and satisfies the guideline requirements for the freshwater midge, *Chironomus dilutus* acute toxicity study.

Table 4.3.3.6-1: Summary of endpoints for Valifenalate Technical after 10-day exposure

Statistical Endpoint	Survival	Growth (ash-free dry weights)
NOEC (mg/kg)	108 mg/kg	14.1 mg/kg
LOEC (mg/kg)	>108 mg/kg	37.7 mg/kg
LC_{50} (95% Confidence Limit)	>108 mg/kg	

MATERIALS AND METHODS

Experimental Test Dates: 25 February 2015 to 07 March 2015

Test Material:
Name: Valifenalate Technical
Batch no.: PL14-0060 (Manufacturing Lot No.: P/13/019 (Isagro))
Analyzed content: 98.9%
Expiry: 03 March 2016

Test Species:
Species: Freshwater midge, *Chironomus dilutus*
Source: Aquatic Bio Systems, Inc., Fort Collins, Colorado

Test Conditions:
Test medium: artificial sediment consisting of 75% sand, 20% clay, and 5% peat and was assigned lot number ASD230215 as recommended by OECD Guideline for Testing of Chemicals, Method 219.
Dissolved Oxygen: 6.4 to 8.6 mg/L (77 to 104% saturation)
pH: 7.4 to 8.5
Un-ionized
Ammonia: 0.0130 to 0.0899 mg/L
Temperature & photoperiod:
Temperature: 22.4 to 23.7°C
Photoperiod: 16-hr light: 8-hr dark
Light intensity: 506 lux

Test Design: The in-life definitive toxicity test was performed from 25 February to 07 March 2015. The testing was performed at nominal sediment concentrations of 0 (control), 13, 25, 50, 100, and 200 mg/kg. Appropriate weights of valifenalate technical (corrected for purity) were directly added to 100 grams of fine silica sand (lot no. SS052505) that was then added to 2,950.5 g of artificial sediment (lot no. ASD230215). The total air-dry equivalent weight of the combined sand and sediment was of 3.0 kg dry sediment. Each of the dosed sediments were thoroughly mixed for approximately 15 minutes using a "V" style stainless steel mixer to produce a series of dosed sediments with nominal concentrations of 13, 25, 50, 100, and 200 mg/kg dry sediment. The control sediment consisted of 3.0 kg of formulated sediment without test substance. An approximately 1,050-mL volume of dilution water was added to each treated sediment and the control, resulting in a hydration level of approximately 35% for each treatment.

The test chambers were glass jars approximately 17 cm in height by 8.5 cm in diameter. Approximately 320 g (approximately 3 cm sediment depth) of the prepared formulated sediment, pre-wetted with dilution water, was added to each replicate test chamber. Approximately 600 mL of dilution water was carefully added to the

test chambers and a plastic deflector was used during the water addition to minimize the disturbance of the sediment. Eight replicate test chambers were prepared for the biological parameters. Two additional replicate chambers were prepared for the various analyses of the overlying water, pore water, and sediment samples. The test chambers were prepared one day prior to study initiation. The test chambers were placed in a temperature-controlled water bath arranged by treatment level. Temperature was set at a target range of 23 ± 1 °C. A 16-hour light:8-hour dark photoperiod with 30-minute dawn and dusk periods was maintained. Aeration was provided at an initial rate of 60-100 bubbles per minute to each test chamber through a glass pipette. The pipette was inserted such that the tip was two to three centimeters from the sediment surface. Aeration was discontinued during the addition of the larvae and resumed within 24 hours.

Ten midge larvae were impartially added to labeled containers. Each container was randomly assigned to a treatment replicate by a computer-generated random number table. The individuals within a container were released into each biological replicate and the termination analytical replicates. The larvae were fed 1.5 mL of a 4.0 g/L flake food suspension daily. At test termination (day 10), the entire contents of each chamber were poured through a stainless steel sieve (500 µm mesh) and the live and dead organisms were enumerated. Observations of general health and behavior of the organisms were also noted (i.e., normal). Any midge that were not accounted for at test termination (i.e., not found) were considered dead. All surviving organisms were also retained for weight measurements. All surviving larvae were pooled by replicate, dried at approximately 70 ºC to a constant weight, allowed to cool to room temperature in a desiccator, and then weighed to the nearest 0.0001 mg. The dried larvae in the pan were then ashed at approximately 550 ºC for approximately 2 hours. The pan with larvae was re-weighed and the tissue mass was determined as the difference between the weight of the dried larvae plus pan and the weight of the ashed larvae plus pan.

Based on the environmental fate profile of valifenalate, its hydrolysis product valifenalate acid was included in analytical verification of the test substance in different compartments of the test system: overlying water, interstitial (pore) water and sediment. Overlying water, interstitial (pore) water, and sediment were analyzed for valifenalate and its hydrolysis product valifenalate acid using a liquid chromatographic with tandem mass spectrometry (LC-MS/MS) system. Analysis of water samples (i.e., overlying and pore water) and sediment samples was accomplished based on a method provided by the Sponsor and validated as part of ABC study 81308. Details of the sample collection, preparation, and method of analysis are described below.

Statistical analysis of the concentration versus effect data was performed using SAS (version 9.3 for Windows). Estimates of the median lethal concentration (LC_{50}) values and their 95% confidence limits were calculated using the probit method and Trimmed Spearman-Karber method. When the *p* value for Goodness of Fit was > 0.05 and there was no other evidence of questionable convergence, the probit method was selected for reporting. When this criterion was not achieved, the Trimmed Spearman-Karber method was selected for reporting.

The no-observable effects (NOEC) and lowest-observable effects (LOEC) values for survival data were analyzed using a one-way analysis of variance (ANOVA) and a one-tailed Dunnett's test and a Fisher's one-tailed exact test ($p \leq 0.05$). Growth data was analyzed using a one-way analysis of variance (ANOVA) and a one-tailed Dunnett's and Williams' test ($p \leq 0.05$). Prior to the Dunnett's test, a Shapiro-Wilk's test and a Levene's test for normality and homogeneity of variance, respectively, of the raw and transformed values over treatments were performed. Where the *p* values for the Shapiro-Wilk's and Levene's test were greater than 0.01, indicating that a parameter met the assumptions of normality and homogeneity, a parametric ANOVA and Dunnett's test on the non-transformed data was performed. Where the *p* values for the Shapiro-Wilk's and Levene's test was less than 0.01, indicated non-normality or heterogeneity of variance, a nonparametric ANOVA and Dunnett's test were performed on the transformed data (i.e., ranks).

Validity Criteria:

The following criteria must be found in the control replicates:

- Mean survival ≥70% for control.
- Control dry weight and ash-free dry weight >0.6 and >0.48 mg, respectively.

RESULTS AND DISCUSSION

Validity Criteria: The control animals met the acceptability criteria (mean survival ≥70% and control dry weight and ash-free dry weight >0.6 and >0.48 mg, respectively) as specified by the study protocol and the OPPTS 850.1735 testing guideline.

***Analytical results*:**

Sediment: The treatment mean measured concentrations of valifenalate technical at day 0 were 4.32, 5.93, 15.2, 39.7, and 154 mg a.i./kg for the 13, 25, 50, 100, and 200 mg a.i./kg test substance treatments, representing 24 to 77% of nominal. The treatment mean measured concentrations of valifenalate technical at day 10 were 7.05, 7.78, 13.1, 35.8, and 62.9 mg a.i./kg for the 13, 25, 50, 100, and 200 mg a.i./kg test substance treatments, representing 26 to 54% of nominal. The overall mean measured concentrations of valifenalate technical were 5.68, 6.86, 14.1, 37.7, and 108 mg a.i./kg (27 to 54% of nominal). The overall geometric mean measured concentrations of valifenalate technical were 5.03, 6.76, 14.0, 37.6, and 97.4 mg a.i./kg (27 to 49% of nominal). No residues of valifenalate technical were detected in the control samples at or above the MQL of 4.00 mg a.i./kg. Quality control fortifications ranged from 83 to 164% of the nominal concentrations. The biological results were reported based on the overall mean calculated valifenalate technical sediment concentrations. The overall mean measured concentrations of valifenalate acid were 2.58, 3.44, 6.85, 13.4, and 20.3 mg a.i./kg.

Based on the environmental fate in soil/sediment, the parent substance valifenalate exhibits rapid degradation to valifenalate acid, p-chlorobenzoic acid (PCBA) and ultimately mineralizes to CO_2. The DT-50s of valifenalate, valifenalate acid, and PCBA were 5 hours, 15 hours and 3 days, respectively, indicating a very rapid degradation in soil/sediment systems. Because the duration of the present study was longer (10 days) than the degradation times established in e-fate studies, the loss of test substance at the end of the study was expected. In addition to the valifenalate technical analysis, only valifenalate acid was measured as part of this study. Therefore the measured analytes in the sediment and waters were not able to fully account for the total valifenalate concentrations within the sediment at the time the sediments were dosed.

The treatment mean measured concentrations of valifenalate acid at day 0 were 2.55, 2.71, 5.18, 8.69, and 15.8 mg a.i./kg for the 13, 25, 50, 100, and 200 mg a.i./kg test substance treatments. The treatment mean measured concentrations of valifenalate acid at day 10 were 2.62, 4.17, 8.51, 18.2, and 24.8 mg a.i./kg for the 13, 25, 50, 100, and 200 mg a.i./kg test substance treatments. The overall geometric mean measured concentrations of valifenalate acid were 2.54, 3.34, 6.62, 12.6, and 19.7 mg a.i./kg. No residues of valifenalate acid were detected in the control samples at or above the MQL of 4.00 mg a.i./kg. Quality control fortifications ranged from 71 to 101% of the nominal concentrations.

Overlying Water: The mean measured concentrations of valifenalate technical at day 0 were 0.0792, 0.144, 0.295, 0.626, and 1.42 mg a.i./L for the 13, 25, 50, 100, and 200 mg a.i./kg test substance treatments. The mean measured concentrations of valifenalate technical at day 10 were 0.201, 0.458, 0.908, 1.58, and 6.24 mg a.i./L for the 13, 25, 50, 100, and 200 mg a.i./kg test substance treatments. No residues of valifenalate technical were detected in the control samples at or above the MQL of 0.00200 mg a.i./L. The mean concentrations over the duration of the exposure were 0.140, 0.301, 0.601, 1.10, and 3.83 mg a.i./L. The geometric mean concentrations over the duration of the exposure were 0.126, 0.257, 0.513, 0.993, and 2.72 mg a.i./L. Quality control fortifications ranged from 106 to 142% of the nominal concentrations.

The mean measured concentrations of valifenalate acid at day 0 were 0.0496, 0.0782, 0.136, 0.202, and 0.285 mg/L for the 13, 25, 50, 100, and 200 mg a.i./kg test substance treatments. The mean measured concentrations of valifenalate acid at day 10 were 0.518, 0.799, 1.75, 3.06, and 7.43 mg/L for the 13, 25, 50, 100, and 200 mg a.i./kg test substance treatments. No residues of valifenalate acid were detected in the control samples at or above the MQL of 0.00200 mg/L. The mean concentrations over the duration of the exposure were 0.284, 0.438, 0.941, 1.63, and 3.86 mg/L. The geometric mean concentrations over the duration of the exposure were 0.160, 0.250, 0.483, 0.783, and 1.38 mg/L. Quality control fortifications ranged from 87 to 119% of the nominal concentrations

Pore (Interstitial) Water: The treatment mean measured concentrations of valifenalate technical at day 0 were 0.662, 0.986, 2.17, 4.39, and 7.66 mg a.i./L for the 13, 25, 50, 100, and 200 mg a.i./kg test substance

treatments. The treatment mean measured concentrations of valifenalate technical at day 10 were 0.768, 1.39, 3.25, 6.47, and 10.3 mg a.i./L for the 13, 25, 50, 100, and 200 mg a.i./kg test substance treatments. No residues of valifenalate technical were detected in the control samples at or above the MQL of 0.00200 mg a.i./L. The mean concentrations over the duration of the exposure were 0.715, 1.19, 2.71, 5.43, and 8.96 mg a.i./L. The geometric mean concentrations over the duration of the exposure were 0.712 1.17, 2.65, 5.33, and 8.86 mg a.i./L. Quality control fortifications ranged from 100 to 139% of the nominal concentrations.

The treatment mean measured concentrations of valifenalate acid at day 0 were 3.27, 4.41, 8.27, 14.8, and 20.2 mg/L for the 13, 25, 50, 100, and 200 mg a.i./kg test substance treatments. The treatment mean measured concentrations of valifenalate acid at day 10 were 5.95, 9.77, 12.4, 23.4, and 43.5 mg/L for the 13, 25, 50, 100, and 200 mg a.i./kg test substance treatments. No residues of valifenalate acid were detected in the control samples at or above the MQL of 0.00200 mg/L. The mean concentrations over the duration of the exposure were 4.61, 7.09, 10.3, 19.1, and 31.8 mg/L. The geometric mean concentrations over the duration of the exposure were 4.40, 6.56, 10.1, 18.6, and 29.6 mg a.i./L. Quality control fortifications ranged from 85 to 117% of the nominal concentrations.

Biological Results: Mean survival in the valifenalate technical treatments was 90, 94, 95, 89, 89, and 86% for the 0 (control), 13, 25, 50, 100, and 200 mg a.i./kg test substance treatments, respectively. Based on mean calculated sediment concentrations, the 10 day LC_{50} was estimated to be >108 mg a.i./kg , the highest concentration tested. The 10-day NOEC and LOEC values for survival were 108 and >108 mg a.i./kg respectively, the highest concentration tested.

The mean individual dry weight for the control was 1.93 mg/animal. Mean dry weights for the treatment animals were 1.87, 1.68, 1.70, 1.59, and 1.54 mg/animal for the 13, 25, 50, 100, and 200 mg a.i./kg test substance treatments, respectively. Since unlike the dry weights data, the ash-free dry weight data were based upon actual tissue weights, this endpoint will be used to determine the overall NOEC and LOEC values for growth. The mean individual ash-free dry weight for the control was 1.30 mg/animal. Mean ash-free dry weights for the treatment animals were 1.30, 1.17, 1.21, 0.957, and 1.07 mg/animal for the 13, 25, 50, 100, and 200 mg a.i./kg test substance treatments, respectively. A statistically significant (p = 0.05) reduction in ash-free dry weights was observed in the 37.7, and 108 mg a.i./kg test substance treatments as compared to the control. The NOEC for dry weight was 14.1 mg a.i./kg and the LOEC was 37.7 mg a.i./kg, based on mean measured sediment concentrations.

The results are summarized in Tables 4.3.3.6-2 and 3, respectively.

Table 4.3.3.6-2 : Survival Observations after 10-Day Exposure of *Chironomus dilutus* to Valifenalate Technical

Mean Calculated Sediment Concentration (mg/kg dry Sediment)	REP	Day 10 Survival [a] (% survival)	REP	Day 10 Survival [a] (% survival)	Treatment Percent Survival
0 (Control)	A	9 (90)	E	6 (60)	90
	B	10 (100)	F	10 (100)	
	C	10 (100)	G	10 (100)	
	D	7 (70)	H	10 (100)	
5.68	A	10 (100)	E	10 (100)	94
	B	9 (90)	F	8 (80)	
	C	9 (90)	G	10 (100)	
	D	9 (90)	H	10 (100)	
6.86	A	10 (100)	E	10 (100)	95
	B	9 (90)	F	9 (90)	
	C	10 (100)	G	10 (100)	
	D	10 (100)	H	8 (80)	
14.1	A	10 (100)	E	8 (80)	89
	B	9 (90)	F	9 (90)	
	C	10 (100)	G	8 (80)	
	D	8 (80)	H	9 (90)	
37.7	A	9 (90)	E	8 (80)	89
	B	8 (80)	F	10 (100)	
	C	8 (80)	G	9 (90)	
	D	10 (100)	H	9 (90)	
108	A	8 (80)	E	9 (90)	86
	B	9 (90)	F	7 (70)	
	C	8 (80)	G	9 (90)	
	D	10 (100)	H	9 (90)	

[a] Animals that were not found were treated as dead.

Note; There were no statistically significant reductions in animal survival as compared to the control (Dunnett's test, Fisher's Exact test p = 0.05).

Table 4.3.3.6-3 : Ash-Free-Dry-Weight After 10-Day Exposure of *Chironomus dilutus* to Valifenalate Technical

Mean Calculated Sediment Concentration (mg/kg dry Sediment)	REP	Mean Tissue Weight/Midge (mg) (AFDW [a])	REP	Mean Tissue Weight/Midge (mg) (AFDW [a])	Treatment Mean Individual Tissue Weight (milligrams)
0 (Control)	A	1.09	E	1.36	1.30
	B	1.27	F	1.56	
	C	1.26	G	1.22	
	D	1.44	H	1.24	
5.68	A	1.20	E	1.40	1.30
	B	1.38	F	1.48	
	C	1.51	G	1.08	
	D	1.38	H	1.00	
6.86	A	0.980	E	1.26	1.17
	B	1.04	F	1.26	
	C	1.33	G	1.14	
	D	1.07	H	1.25	
14.1	A	1.15	E	1.50	1.21
	B	1.37	F	1.23	
	C	0.874	G	1.11	
	D	1.22	H	1.19	
37.7	A	0.457	E	1.21	0.957 *
	B	1.07	F	0.945	
	C	1.18	G	1.08	
	D	0.796	H	0.917	
108	A	1.05	E	1.06	1.07 *
	B	0.967	F	1.03	
	C	1.31	G	0.996	
	D	0.933	H	1.19	

[a] AFDW = Ash-Free-Dry Weight.

* Statistically significant (Dunnett's and Williams' test, p = 0.05) reduction in animal ash-free dry weight as compared to the control (1.30)

The achieved NOEC, LOEC, and LC_{50} values are summarized in Table 4.3.3.6-4.

Table 4.3.3.6-4: Calculated Effects Concentrations for Survival and Growth of the Chironomid, *Chironomus dilutus*, Exposed to Valifenalate in Whole Sediment

Based on Mean Calculated Valifenalate Concentrations in Sediment:

Biological Parameter	NOEC	LOEC	LC_{50} (95% Confidence Limit)
Survival	108 mg/kg	>108 mg/kg	>108 mg/kg
Growth (ash-free dry weights)	14.1 mg/kg	37.7 mg/kg	---

NOEC = No-observable-effect concentration and LOEC = lowest-observable-effect concentration.

CONCLUSION

In a 10 day acute toxicity test, freshwater midge, *Chironomus dilutus* were exposed to valifenalate technical. The control animals met the acceptability criteria (mean survival ≥70% and control dry weight and ash-free dry weight >0.6 and >0.48 mg, respectively) as specified by the study protocol and the OPPTS 850.1735 testing guideline.

Results Based on Mean Calculated Concentrations in Sediment:

Biological Parameter	NOEC	LOEC	LC_{50} (95% Confidence Limit)
Survival	108 mg/kg	>108 mg/kg	>108 mg/kg
Growth (ash-free dry weights)	14.1 mg/kg	37.7 mg/kg	---

4.4 Chronic toxicity

4.4.1 Fish early-life stage (FELS) toxicity test

4.4.1.1 Early life-stage toxicity test with the Fathead Minnow (Pimephales promelas) – IR5885

Reference Number: KII- **Post submission 02**, Section 6, Volume 2, Annex IIA, point 8.2.2.2/01

Report:	**See annex conf. 30**

Guidelines: U.S. EPA OPPTS NUMBER 850.1400
Deviations: None
GLP: Yes; certified laboratory

Executive Summary:

The objective of this study was to determine the effects of IR5885 on the time to hatch, hatching success, survival and growth of fathead minnows *Pimephales promelas*, during early life-stage development. Fathead minnows embryos were exposed to a geometric series of five test concentrations, a negative control (dilution water) and a solvent control (dimethyl formamide) under flow-through conditions. The exposure period included a 5-day embryo hatching period and a 28-day post-hatch juvenile growth period. Nominal test

concentrations were 0.75, 1.5, 3.0, 6.0 and 12.0 mg IR5885/L. The concentrations were selected in consultation with the Sponsor, and were based on exploratory range-finding toxicity data. Mean measured test concentrations were determined from samples of test water collected from each treatment and control group at test initiation, at the weekly interval during the test and at test termination. The mean measured concentrations of IR5885 were 0.74, 1.5, 3.0, 5.4 and 11.0 mg/L (from 99 to 100% of the nominal concentrations).
There were no statistically significant treatment-related effects on hatching success, survival or growth at any concentration tested. Consequently, the NOEC was 11 mg/L, the highest concentration tested, the LOEC was 11 mg/L and the MATC was 11 mg/L.

MATERIALS AND METHODS

Test Material	IR5885
Description	white powder
Lot/Batch #	T 025/02
Purity	99.63% (w/w)
CAS #	283159-90-0

Test Animals:	
Species:	Fathead minnow (*Pimephales promelas*)
Age:	Embryos (<24 hours old)
Source:	Chesapeake Cultures Hayes, Virginia, 23072

- **Acclimation period:** 5-day embryo hatching period
- **Diet:** Newly-hatched larvae were fed live brine shrimp nauplii (*Artemia sp.*) 3 times per day during the first seven days of post-hatch. Thereafter, they were fed live brine shrimp nauplii 3 times per day on weekdays and 2 times per day on weekends until the end of the test. Brine shrimp nauplii were obtained by hatching cysts purchased from Brine Shrimp Direct, Ogden, Utah. Fish were not fed for approximately 48 hours prior to the termination of the test to allow for clearance of the digestive tracts before weight measurements were made.
- **Test Water:** The water used for culturing and testing was freshwater obtained from a well approximately 40 meters deep located on the Wildlife International, Ltd. site. The well water is characterized as moderately-hard water.
- **Test Units:** The test chambers were 9-L glass aquaria filled with approximately 7 L of test solution. The depth of the test water in a representative test chamber was approximately 15 cm.
- **Test Conditions:**
- **Light Regime:** 16 hours of light and 8 hours of darkness
- **Light Intensity:** 126 lux
- **pH Values:** 8.2 to 8.3
- **Dissolved Oxygen**
- **Concentrations:** 71% of saturation (5.8 mg/L)
- **Water Temperature:** 25 ± 1°C

STUDY DESIGN AND METHODS

In life dates:	Experimental Start:	May 16 2005
	Experimental Termination:	June 23, 2005

Dosage Preparation and Dosing

One stock solution was prepared for each of the five concentrations tested. Stock solutions were prepared at approximately two-week intervals during the test. The primary stock was prepared by dissolving the test substance in DMF at a concentration of 120 mg/mL. The stock was sonicated for up to seven minutes and inverted to mix, and appeared clear and light yellow after mixing. Aliquots of the primary stock solution were proportionally diluted with DMF to prepare secondary stocks at concentrations of 7.5, 15, 30 and 60 mg/mL. The secondary stocks were mixed by inversion and appeared clear and colorless after mixing. The five stock solutions and DMF for the solvent control were injected into the diluter mixing chambers (at a rate of 20 µL/minute) where they were mixed with dilution water (at a rate of 200 mL/minute) to achieve the nominal test concentrations of 0.75, 1.5, 3.0, 6.0 and 12 mg/L. All of the test solutions appeared clear and colorless in the test chambers at test initiation and termination. A white precipitate was observed in the diluter mixing chambers for the 6.0 and 12 mg/L test concentrations by test termination.

Two days prior to test initiation, a sample of the test water was collected from each treatment and control group to evaluate diluter performance. The samples were collected after conditioning the diluter for 24 hours. On Days 0, 7, 14, 21, 28 and 33 (test termination), water samples were collected from one alternating replicate test chamber of each treatment and control group to measure concentrations of the test substance. The samples were collected at mid-depth from each test chamber and placed in glass vials for immediate analysis.

Analysis of IR5885 concentration:

The method used for the analysis of IR5885 in freshwater samples consisted of diluting the samples in freshwater, as necessary, and analyzing by direct injection high performance liquid chromatography (HPLC) with UV detection at 220 nm. Concentrations of IR5885 in the samples were determined using an Agilent Model 1100 High Performance Liquid Chromatograph (HPLC) equipped with an Agilent Series 1100 Variable Wavelength Detector. Chromatographic separations were achieved using a YMC-Pack ODS AM column (150 mm x 4.6 mm, 3-µm particle size).

Calibration standards of IR5885, ranging in concentration from 0.400 to 4.00 mg/L, were prepared using a stock solution of IR5885 in acetonitrile. Fresh standards were prepared and analyzed with each sample set. Linear regression equations were generated using the sum of the peak area responses versus the respective concentrations of the calibration standards. The concentration of IR5885 in the samples was determined by substituting the sum of the peak area responses of the samples into the applicable linear regression equation. The method limit of quantitation (LOQ) for these analyses was defined as 0.400 mg/L, calculated as the product of the concentration of the lowest calibration standard (0.400 mg/L) and the dilution factor of the matrix blank samples (1.00). Six matrix blank samples were analyzed to determine possible interferences. No interferences were observed at or above the LOQ during the sample analyses. Samples of freshwater were fortified at 0.700, 3.00 and 12.0 mg/L using a stock solution of IR5885 in acetonitrile, and were analyzed concurrently with the test samples. The measured concentrations for the matrix fortification samples ranged from 89.2 to 101% of nominal concentrations.

Experimental Design

During the first day of exposure, embryos were observed twice for mortality and eggs with fungus. Thereafter, until hatching was complete, observations of embryo mortality and the removal of dead embryos were performed once daily. During the 28-day post-hatch exposure period, the larvae were observed daily to evaluate the number of mortalities and the number of individuals exhibiting clinical signs of toxicity or abnormal behavior.

Temperature was measured in each test chamber at the beginning and end of the test and at weekly intervals during the test using a liquid-in-glass thermometer. Dissolved oxygen concentrations were measured in alternating replicates of each treatment and control group at the beginning and end of the test, daily during the

first seven days of the test, and at weeklyintervals during the test. Measurements of pH were made in alternating replicates of each treatment and control group at the beginning and end of the test and at weekly intervals during the test.

RESULTS AND DISCUSSION

Analytical results

Nominal concentrations used in this study were 0.75, 1.5, 3.0, 6.0 and 12 mg/L. The measured concentrations in the test chambers ranged from approximately 91 to 98% of nominal concentrations. The measured concentrations ranged from approximately 75 to 103% of nominal concentrations. When the measured concentrations of the samples collected during the definitive test were averaged for each treatment group, the mean measured concentrations were 0.74, 1.5, 3.0, 5.4 and 11 mg/L, which represented 99, 100, 100, 90 and 92% of nominal concentrations, respectively. The results of the study were based on the mean measured test concentrations.

Biological results:

Time to Hatch and Hatching Success

Daily observations of the fathead minnow embryos indicated that there were no apparent differences in time to hatch between the control group and any of the IR5885 treatment groups. Hatching success in the negative and solvent control groups was 98 and 90%, respectively. There were no statistically significant differences between the negative and solvent controls ($p > 0.05$). Therefore, the controls were pooled for comparison with the treatment groups. Hatching success in the 0.74, 1.5, 3.0, 5.4 and 11 mg/L treatment groups was 96, 100, 93, 98 and 99%, respectively. Fisher's Exact test indicated that there were no statistically significant differences in hatching success in any IR5885 treatment group in comparison to the pooled controls ($p > 0.05$). Consequently, the NOEC for hatching success was 11 mg/L, the highest concentration tested..

Larval Survival and Clinical Observations

Larval survival in the negative and solvent control groups was 96 and 89%, respectively. There were no statistically significant differences between the negative and solvent controls ($p > 0.05$). Therefore, the controls were pooled for comparison with the treatment groups. Larval survival in the 0.74, 1.5, 3.0, 5.4 and 11 mg/L treatment groups was 90, 93, 93, 92 and 89%, respectively. Fisher's Exact test indicated that there were no statistically significant differences in larval survival in any IR5885 treatment group in comparison to the pooled controls ($p > 0.05$). Consequently, the NOEC for larval survival was 11 mg/L, the highest concentration tested. In general, the majority of the fish appeared normal throughout the test.

Growth

Growth was evaluated at the end of the test by measuring the total length, wet weight and dry weight of each surviving fish. There were no statistically significant differences between the negative and solvent controls for any measured growth parameter ($p > 0.05$). Therefore, the controls were pooled for comparison with the treatment groups. Bonferroni's t-test indicated there were no statistically significant differences in total length, wet weight or dry weight in any of the IR5885 treatment groups in comparison to the pooled controls ($p > 0.05$). Consequently, the NOEC for growth was 11 mg/L, the highest concentration tested.

CONCLUSIONS

Fathead minnows (*Pimephales promelas*) were exposed for 28 days post-hatch to IR5885 at mean measured concentrations of 0.74 to 11 mg/L. There were no statistically significant treatment-related effects on hatching success, survival, or growth at any concentration tested. Consequently, the NOEC was 11 mg/L, the highest concentration tested, the LOEC was >11 mg/L, and the MATC was 11 mg/L.

NOEC, LOEC and MATC values were recalculated by the RMS during review based on mean measured concentrations and are:
NOEC: 12 mg/L
LOEC: >12 mg/L
MATC: 12 mg/L

4.4.2 Fish short-term toxicity test on embryo and sac-fry stages

No data available.

4.4.3 Aquatic Toxicity – Fish, juvenile growth test

4.4.3.1 Chronic Prolonged toxicity test on juvenile fish – Rainbow Trout – IR5885

Reference Number:	KII- Section 6, Volume 1, Annex IIA, point 8.2.3/01
Report:	See annex conf. 17
Guidelines:	OECD Guideline for testing of Chemicals, Section 2, No. 215: "Fish, juvenile growth test", January 2000
Deviations:	1) Concerning test conditions, water temperature According to Guideline: 12.5-16.0 °C Deviation: 17 °C Reason for Deviation: temperature of 17°C was used also in the Acute Toxicity Test Presumed Effect on the Study: none, the temperature range is only a recommended range 2) Concerning test parameters, weighing of test fish According to Protocol and Guideline: the range in individual weights at the start of the test should ideally be kept within ± 10% of arithmetic mean weight and should not exceed 25% Deviation: the individual weights differed up to 27%. Reason for deviation: technical reason Presumed Effect on the Study: none, minor deviation only
GLP:	Yes; certified laboratory

Executive Summary:

Juvenile Rainbow Trout were exposed in a semi static test system to aqueous test media containing the test item. Since in the acute toxicity test with Rainbow Trout no effect was determined up to 100 mg test item/L, in this prolonged study 100 mg test item/L, a control and a solvent control (50 mg methyl cellulose/L) were tested. Mortality and symptoms of intoxication were recorded trought the study and bw of surviving fish were recorded at the start and the end of the test.

During the test period test item concentrations were measured in the range 82-129% of the nominal value and mean measured test item concentration in the test media was found to be 98%. Under the test conditions the test item was sufficiently stable during the test medium renewal period of 48 and 72 hours. Therefore all the results are related to the nominal concentration of the test item.

No mortality or symptoms of intoxication were observed during the test at the test concentration of nominal 100 mg test item/L.
No significant difference was determined comparing the pseudo specific growth rates of the test concentration with the one of control and the solvent control. The 28-day NOEC resulted of at least 100 mg test item/L; the 28-day LOEC and the 28-day Lowest Lethal Concentration (LLC) were higher than 100 mg test item/L.

MATERIALS AND METHODS

MATERIALS

Test Material	IR5885
Description	white powder
Lot/Batch #	T 025/02
Purity	99.56% ± 0.14% (w/w)
CAS #	283159-90-0
Test Animals:	
Species:	Rainbow trout (*Oncorhynchus mykiss*)
Age and Size:	juveniles; the body weight of all test fish were determined at the start and the end of the test. At the start of the test no fish weighting above 1 g as available due to the season. The mean body length of the fish was 4.22 ± 0.3 cm.
Source:	Forellenzucht Tautehahn, 98646 Trostadt, Germany
Acclimation period:	for 3 weeks before the start of the test the fish were acclimated to the test water and test temperature
Diet:	during holding until one day before the test start the fish were fed with a commercial diet for Rainbow trout. The food ration during the test: daily 4% dry weight based on the mean initial fish wet weight in each test vessel. The fish were fed two times per day.
Test Water:	Reconstituted water
Test Units:	20-litre glass aquaria with 15-litre test medium. Each test unit was uniquely identified with study number, treatment and replicate number.
Test Conditions:	
Surrounding Type:	controlled environment room
Light Regime:	16h light / 8h dark
Light Intensity:	480-860 lux
Cleaning:	uneaten food and faecal material were removed when necessary by a vacuum pump
pH Values:	7.8 – 8.1
Dissolved Oxygen Concentrations:	at least 8.8 mg/L or higher
Water Temperature:	17°C

STUDY DESIGN AND METHODS

Dates:	Experimental Start:	July 9, 2003
	Experimental Termination:	August 13, 2003

Dosage Preparation and Dosing

Test Concentration: 100 mg test item/L. Since the test item is not well soluble in test water, a solvent additive (50 mg methyl cellulose/L) was used to suspend the test item as homogeneously as possible in test water. Before the start and before each medium renewal, 1500 mg test item were mixed with 750 mg methyl cellulose. Under intense stirring this mixture was filled up with test water to a volume of 15 litre test medium.

Control: test water was used without addition of the test item. In the solvent control 50 mg methyl cellulose/L were used.

Analysis of test item concentration:

For the analysis of test item concentrations, duplicate samples were taken from the freshly prepared test media of the test concentration and the solvent control just before of the test beginning (day 0) and at weekly intervals (on days 9, 19 and 23). All the samples were approximately taken from the centre of the aquaria without mixing the test media.
The concentrations and the stability of the test item in the old test media were determined on four dates during the test period, corresponding to the renewal periods of 48 and 72 hours. Samples of all test media were taken in duplicate on days 2, 12, 21 and 26.
All samples were stored in a refrigerator.
The concentrations of IR5885 were measured in all duplicate test medium samples from the only test concentration. From the solvent control samples only one of the duplicate samples was analysed from each sampling date.
The quantification of IR5885 was performed using HPLC method.

Experimental Design

At the start of the test 10 fish were randomly introduced into each aquarium. The loading rate was < 1 g fish/litre test medium. During this semi static test the surviving fish were placed in a clean aquarium with freshly prepared test medium of the corresponding treatment. The test media were slightly aerated during the test period. The exposure time was of 28 days.
The water temperature, pH-values and the dissolved oxygen concentrations were determined daily in the test media of the only test concentration and the controls. The behaviour of the test item in the only test concentration was determined once every day during the test.
During the test all the fish were observed at least once each working day for mortality and symptoms of intoxication. Clearly distinguishable differences in appearance or behaviour between treated and control fish were documented each working day. At the start and end of the test, body wet weight of all test fish was determined.

The pseudo specific growth rates were calculated and statistically evaluated on significant differences to the control and solvent control values by the Student-t-test and a pairwise Mann & Whitney-U-Test, after one-way analysis of variance (ANOVA) using Easy Assay, Multiple Testing, Version 4.0. The NOEC, LOEC and LLC were determined directly from the raw data.

RESULTS AND DISCUSSION

Analytical results

During the test period test item concentrations were measured in the range from 82% to 129% of the nominal value. Under the test conditions IR5885 resulted sufficiently stable during the test medium renewal periods of 48 and 72 hours. Therefore all the reported results are related to the nominal concentration of the test items.

Behaviour of test item: in the test media of the only test concentration of 100 mg/L, the test medium was coloured by the test item, a part of the test item was swimming at the surface of the medium and a part was lying at the bottom of the aquarium. In addition (caused by the solvent additive) foam was observed.

Biological results:

Mortality and Signs of intoxication: the mortalities and the symptoms of intoxication are reported in Table 4.4.3.1-01:

Table 4.4.3.1-01 Mortality and symptoms of intoxication, both treatment and solvent control groups consisted in 10 fish each.

Day	Control Dead/symptoms	Solvent Control Dead/symptoms	Treatment 100 mg/L Dead/symptoms
0	0/0	0/0	0/0
1	0/0	0/0	0/0
2	0/0	0/0	0/0
5	0/0	0/0	0/0
6	0/0	0/0	0/0
7	0/0	0/0	0/0
8	0/0	0/0	0/0
9	0/0	0/0	0/0
12	0/0	0/0	0/0
13	0/0	0/0	0/0
14	0/0	0/0	0/0
15	0/0	0/0	0/2 SV
16	1/1	0/0	0/2 SV
19	1/1	0/0	0/0
20	1/1	0/0	0/0
21	1/1	0/0	0/0
22	1/1	0/0	0/0
23	1/1	0/0	0/0
26	1/1	0/0	0/0
27	1/1	0/0	0/0
28	1/1	0/0	0/0

SV: strong ventilation

One fish died during the test in the control group. No mortalities or symptoms of intoxication were observed during the test in the solvent control. In the test concentration of nominal 100 mg test item/L on 2 days two fish showed strong ventilation. This behaviour was not considered as a toxic effect since was not observed any more until the end of the test.

Growth parameters: the mean body wet weight of the control fish increased by a factor of 2.35 (see Table 4.4.3.1-02). The mean body wet weight of the solvent control fish increased by a factor of 2.36. The increasing factor of the mean wet weight of the fish treated at 100 mg/L was not significantly different from the controls with a factor of 2.17. The mean pseudo specific growth rate for the control and solvent control were determined to be 2.971 and of 2.940, respectively. The mean pseudo specific growth rate for the test concentration of 100 mg/L was calculated to be 2.764. According to the results of a Student-t-test and a pairwise Mann & Whitney-U-test no significant difference was determined comparing the test concentration with the control and the solvent control.

Table 4.4.3.1-02 Body wet weight (g) of all test fish at the start and of the surviving fish at the end of the test

Start of the Test

Fish No.	Control	Solvent Control	Treatment 100 mg/L
1	0.53	0.48	0.48
2	0.66	0.45	0.51
3	0.52	0.45	0.69
4	0.54	0.50	0.68
5	0.73	0.63	0.53
6	0.54	0.57	0.67
7	0.54	0.73	0.73
8	0.49	0.48	0.59
9	0.59	0.48	0.54
10	0.54	0.70	0.62
Mean ± SD	0.57 ± 0.07	0.55 ± 0.10	0.60 ± 0.09
Min	0.49	0.45	0.48
Max	0.73	0.73	0.73
n	10	10	10

End of the Test

Fish No.	Control	Solvent Control	Treatment 100 mg/L
1	1.82	0.84	1.83
2	1.03	0.74	1.19
3	0.92	1.48	1.88
4	1.55	1.23	0.98
5	0.98	2.00	0.82
6	1.49	1.10	1.88
7	1.20	1.68	1.12
8	1.49	1.38	0.99
9	1.63	1.46	0.69
10	--	1.13	1.66
Mean ± SD	1.34 ± 0.32	1.30 ± 0.38	1.30 ± 0.46
Min	0.92	0.74	0.69
Max	1.82	2.00	1.88
n	9	10	10
Growth %	235	236	217

-- fish died during the test

CONCLUSIONS

For Rainbow Trout the NOEC of the test item IR5885 after an exposure of 28 days was determined to be at least 100 mg test item/L. The LOEC and the LLC (lowest lethal concentration) were higher then 100 mg test item/L

4.4.4 Chronic toxicity to aquatic invertebrates

4.4.4.1 Chronic toxicity to *Daphnia magna* – IR5885

Reference Number: KII- Section 6, Volume 2, Annex IIA, point 8.3.2.1/01

Report:	See annex conf. 18

Guidelines: OECD Guideline No. 211 "*Daphnia magna* reproduction test" September, 1998
Deviations: None
GLP: Yes; certified laboratory

Executive Summary:

Groups of 10 young *Daphnia* (7.5 – 22.5 hours old) for each control and test concentration, were exposed in semi static conditions to the test item IR5885 for a period of 22 days. The concentrations tested (0.32, 1.0, 3.2, 10, 32 and 100 mg/L) were based on results of the previous acute toxicity test on *Daphnia*, while the two control groups tested held reconstituted water only and water with methylcellulose respectively. The test media of all test concentrations and of the control were renewed on days 2, 5, 7, 9, 12, 14, 16 and 19 of the exposure period. At the mentioned dates the animals were transferred from the old test vessels into the freshly prepared test media of the corresponding concentrations. Observations of adults survival and number of young were carried out daily while pH, dissolved oxygen concentration and water temperature were measured at the start and at the end of each treatment period in the control and all test concentrations.

In order to verify the stability of the test item under the test conditions, a sufficient volume of the freshly prepared test media of the control and of all concentrations were incubated under the same conditions of the test, without animals and food as well for 48 or 72 hours. Samples were collected on days 0-2, 12-14 and 16-19.

At the end of the test period (22 days), the NOEC and the LOEC for reproduction were set at 3.2 mg/L and 10 mg/L respectively. The EC_{50} reproduction rate resulted to be 5.9 mg/L. The NOEC and LOEC for survival were 10 and 32 mg/L, respectively.

MATERIALS AND METHODS

MATERIALS

Test Material IR5885
Description white powder
Lot/Batch # T 025/02
Purity 99.56% (w/w)
CAS # 283159-90-0

Test Animals:
Species: *Daphnia magna* (Straus), clone 5
Age: 7.5-22.5 hours old at the initiation of the test
Source: supplied 1997 by the Umweltbundesamt, Institut fur Wasser-Boden und Lufthygiene, Berlin, Germany
Acclimation period: under test conditions
Breeding conditions: the D*aphnia* were bred in IBACON Lab. Under similar temperature and light conditions as in the test and in reconstituted water of similar pH, ions components and total hardness as the test water. The test organisms were not first brood progeny.

Test Water:	reconstituted water
Test Units:	100 mL glass beakers with 80 mL test medium. Each test unit was uniquely identified with study number, treatment and replicate number.
Test Conditions:	
Surrounding Type:	controlled environment room
Light Regime:	16h light/8h dark
Light Intensity:	450-590 lux
pH Value:	7.6-8.7
Dissolved Oxygen Concentration:	at least 8.3 mg/L
Water temperature:	20-21°C

STUDY DESIGN AND METHODS

Dates:	Biological part:	October 23, 2002 to January 30, 2003
	Analytical part:	January 10 to 30, 2003 (Report 13497221A)

Dosage Preparation and Dosing

Test concentrations: test concentrations were based on the results of the acute toxicity test on *Daphnia* (report No. 13492220, Reference Annex IIA, point 8.3.1.1/01). The following nominal concentrations were tested: 0.32, 1.0, 3.2, 10, 32 and 100 mg test item/L.

Controls: a solvent control (test water with solvent) and a control (test water only) were tested in parallel.

Dosage of the test item: since the test item is not well soluble in test water, a solvent additive (100 mg/L methyl cellulose) was used to prepare the stock solution. The solvent additive concentration was the same in all test media and solvent control.
The test medium of the highest test concentration, nominal 100 mg/L, was prepared by dissolving 150 and 250 mg test item and 150 and 250 mg methyl cellulose in 1.5 and 2.5 litre test water, respectively by short ultrasonic treatment (15 minutes) and intense stirring for at least 24 hours.
Adequate volumes of this medium were diluted with test water to prepare the test media.
Test media were freshly prepared just before each test medium renewal.

Experimental Design

Introduction of individuals: 10 D*aphnia* per control and test concentration, each in 80 mL test medium.

Exposure time: 22 days as was expected from observation at Day 21 that some Daphnia would have being release their offspring from brood pouch in the next hours. For this reason the test was extended for 24 hours until Day 22.

Test medium renewal: in this semi static test, the test media of all test concentrations and of the control were renewed on Days 2, 5, 7, 9, 12, 14, 16 and 19 of the exposure period; a total of 9 treatments were performed.

Feeding of *Daphnia*: the *Daphnia* were fed each working day with green algae (*Desmodesmus subspicatus*) freshly grown in the laboratory. The amount of food was based on the concentration of Total Organic Carbon (TOC) in the food suspension.

Mortality of adults and number of young: the mortality of the test animals and the number of young were recorded each working day. Dead animals and offspring were removed at the same times.

Measurement of pH, dissolved oxygen and water temperature: the pH value and dissolved oxygen concentrations in the control and all the tested concentrations were measured at the beginning and at the end of each treatment period. The water temperature was measured in one control beaker at the beginning and at the end of each treatment period.

Behaviour of the test item in test water: the behaviour of the test item in test water was determined at each test medium renewal period in the freshly prepared and old test media of all test concentrations.

Analysis of IR5885 concentration:

One sample from the freshly prepared stock solutions and duplicate samples from the freshly prepared test media of all test concentrations and the solvent control were taken at the first treatment period (Day 0) and at a treatment period in the second and third week (Days 12 and 16).

For the determination of the stability of the test item under test conditions respectively the maintenance of the test concentrations during the test period at three dates a sufficient volume from the freshly prepared test media of all test concentrations and the solvent control were incubated under the same conditions of the test (without food and *Daphnia*) and were sampled on Days 2, 14 and 19. One of these three stability control treatment last 72 hours, two 48 hours, corresponding to the different test medium renewal periods.

The samples were stored in a refrigerator until analysis. The concentrations of IR5885 were measured in the duplicate test medium samples from all tested concentrations. From the control samples only one of the duplicate samples was analysed from each sampling date.

The accuracy of the analytical method was verified by recoveries which span the concentration range used in the biological test.

RESULTS AND DISCUSSION

Analytical results

During the test period the mean measured test item concentrations of nominal 1.0 to 100 mg/L were determined in the range from 81 to 92% of the nominal values. The lowest test concentration of nominal 0.32 mg/L was below the limit of quantification. All reported results are related to mean measured test item concentrations (Table 4.4.4.1-01).

Table 4.4.4-01

Nominal Concentration (mg/L)	Mean measured test item Concentration (mg/L)
0.32	Not applicable
1.0	0.9
3.2	2.8
10	8.1
32	25.9
100	82.0

Behaviour of test item: at the nominal concentrations of 0.32 to 3.2 mg/L foam was observed. At 10 mg/L a part of the test item was lying at the bottom of the glass beakers. At the two highest concentrations the test media were turbid, a part of the test item was lying at the bottom of the glass beakers or swimming on the water surface and foam was observed in the media.

Biological results:

Survival of adults: in the control all the *Daphnia* survived until the end of the test. In the solvent control the survival rate of adults was 90% after the exposure time of 22 days. No mortality was observed in the three

lowest concentrations of nominal 0.32 to 3.2 mg test item/L. At the nominal concentration of 10 mg/L the survival rate was 90%. At the two highest concentrations of nominal 32 and 100 mg/L all *Daphnia* were dead on Day 16 of observation. Thus the survival rate of *Daphnia magna* after 21 days was significantly reduced at the nominal test concentration of 32 mg/test item/L or above.

Reproduction Rate: it was apparent at the observation of the test animals on Day 21 that some *Daphnia* would release their offspring from brood pouch in the next hours, therefore the test was extended for 24 hours until Day 22. The first young *Daphnia* released from their parent animals were recorded in the control and the nominal test concentrations up to 3.2 mg/L at the observation on Day 8. At the test concentration of 10 mg/L first offspring was observed on Day 9 of exposure. At the two highest concentrations (32 and 100 mg/L) no offspring were reproduced.

In Table 4.4.4.1-02 the reproduction rates calculated for each *Daphnia* survived until the end of the test are given.
The mean reproduction rate of the *Daphnia* in the solvent control was 113.3 ± 17.5 (mean ± SD) alive offspring per adult. In the control the mean reproduction rate was 117.4 ± 13.7 (mean ± SD). According to the results of William test (one sided α=0.05) no significant toxic effect of the test item on the mean reproduction rate was determined up to and including the nominal test concentration of 3.2 mg test item/L, compared to the solvent control.
At the nominal concentration of 10 mg/L the mean of alive offspring reproduced per surviving adult within 22 Days of exposure was significantly lover than in solvent control (19.8% and 113.3 alive offspring *Daphnia*, respectively.

Table 4.4.4.1-02 Number of alive offspring reproduced per surviving adult within 22 days of exposure

	Nominal concentrations of IR5885 (mg/L)							
***Daphnia* No.**	**Solvent Control**	**Control**	**0.32**	**1.0**	**3.2**	**10**	**32**	**100**
1	83	85	115	109	91	13	*	*
2	133	120	105	106	116	34	*	*
3	111	122	107	130	104	*	*	*
4	*	119	102	106	98	27	*	*
5	100	127	110	102	102	32	*	*
6	127	114	111	119	110	23	*	*
7	118	127	97	103	91	14	*	*
8	138	136	102	110	105	3	*	*
9	104	115	98	96	107	12	*	*
10	106	109	121	72	122	20	*	*
Mean	113.3	117.4	106.8	105.3	104.6	19.8	0	0
± SD	17.5	13.7	7.6	15.1	9.9	10.2	-	-
n	9	10	10	10	10	9	0	0
CF%	15.4	11.7	7.1	14.3	9.5	51.7	-	-
Mean %	100	103.6	94.2	92.9	92.3	17.5	-	-
Stat	-	-	n.s.	n.s.	n.s.	s.	-	-

CF% coefficient of variation %
Stat results of Williams-test with the results of the alive offspring (one sided α=0.05)
n.s. mean value not significantly lower than the control
s. mean value significantly lower than the control
* *Daphnia* died during test period

Signs of intoxication: with exception of the reported mortality and the reduced reproduction rate, no particular signs of intoxication were observed during the test.

CONCLUSIONS

Taking into account the reproduction rates of the test animals, the highest concentration of IR5885 tested without toxic effects after the exposure period of 22 days (22-Days NOEC) was 2.8 mg/L (nominal 3.2 mg test item/L). The lowest concentration tested with toxic effects (22-Days LOEC) was determined to be 8.1 mg/L

(nominal 10 mg test item/L) due to the statistically significant reduction in mean reproduction rate of *Daphnia magna* at this concentration.

4.4.5 Chronic toxicity to algae or aquatic plants

Summaries are presented in Section 4.3.3

4.4.5.1 See annex conf. 14

See summary in Section 4.3.3.1

4.4.5.2 Hicks (2015b)

See summary in Section 4.3.3.2.

4.4.5.3 Bergfield (2015a)

See summary in Section 4.3.3.3.

4.4.5.4 Aufderheide (2015b)

See summary in Section 4.3.3.4.

4.4.5.5 Bergfield (2015b)

See summary in Section 4.3.3.5.

4.5 Chronic toxicity to other aquatic organisms

No study available