Bayer CropScience

Document Title

Summary of the toxicological and metabolism studies for Isoxaflutole

Data Requirements

EU Regulation 1107/2009 & EU Regulation 283/2013
Document MCA
Section 5: Toxicological and metabolism studies

According to the guidance document, SANCO 10181/2013, for preparing dossiers for the approval of a chemical active substance

Date

2013-11-15

Author(s)

[redacted].

Bayer CropScience

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Version history

Date	Data points containing amendments or additions[1] and brief description	Document identifier and version number

[1] It is suggested that applicants adopt a similar approach to showing revisions and version history as outlined in SANCO/10180/2013 Chapter 4 How to revise an Assessment Report

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CA 5 TOXICOLOGICAL AND METABOLISM STUDIES ON THE ACTIVE SUBSTANCE

INTRODUCTION

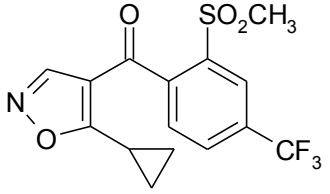
The Acceptable Daily Intake (ADI) established in the first EU review of isoxaflutole was based on the lowest NOAEL observed, in the rat 2-year chronic / oncogenicity study and in the 2-generation reproduction study in the rat. Safety factors of 100 were applied, resulting in an ADI of 0.02 mg/kg bw/day.

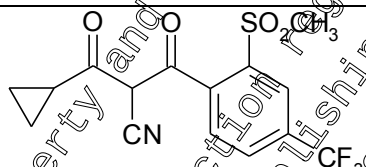
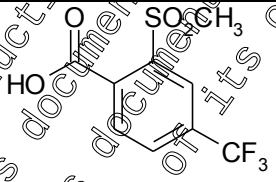
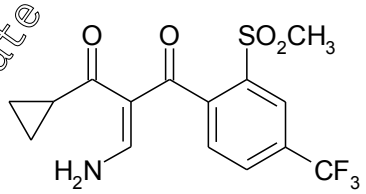
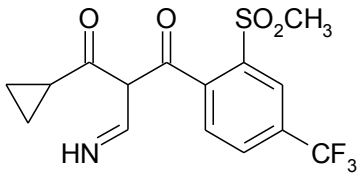
An Acute Reference Dose (ARfD) was not proposed in the initial EU review of isoxaflutole. The endpoint most appropriate to use for derivation of the ARfD is feto-/embryo-toxicity, as observed in the rat developmental toxicity study (full summary provided in this document at data point CA 5.6.2 although the study was reviewed during the first EU review). The ARfD can be derived from the fetal NOAEL in this study of 10 mg/kg bw/day, based on decreased fetal body weight and decreased ossification of specific structures, observed at the LOAEL of 100 mg/kg bw/day.

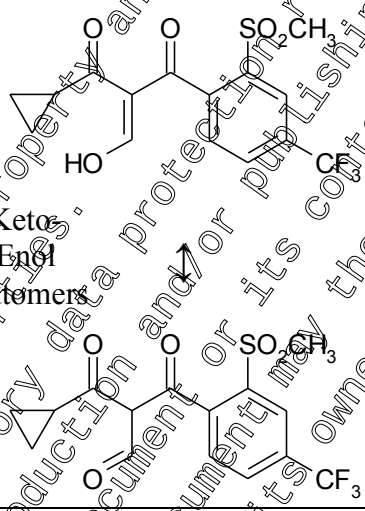
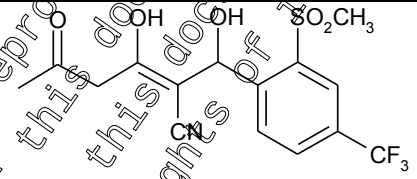
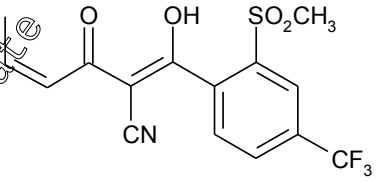
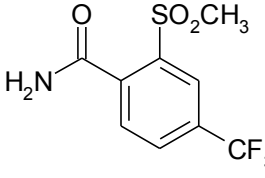
Furthermore, it is the position of Bayer CropScience, as supported by data included at data point CA 5.8.2 ([redacted], 2006), that increased maternal plasma tyrosine concentration is responsible for decreased ossification observed in the rat after administration of an HPPDase inhibitor such as isoxaflutole. Due to differences in ability to catabolize excess tyrosine after HPPDase inhibition, the rat is much more sensitive to the downstream effects of excess plasma tyrosine concentration than are humans, and thus effects related to tyrosine are non-relevant for human risk assessment of agrochemicals such as isoxaflutole. Therefore, the use of the NOAEL of 10 mg/kg bw/day from the rat developmental toxicity study will be conservative regarding human effects.

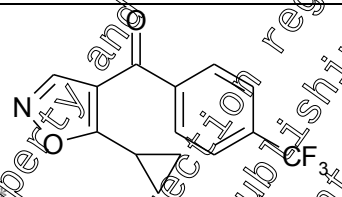
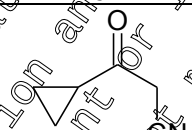
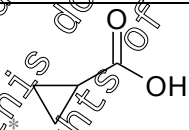
Safety factors of 100 are appropriate for calculation of the ARfD, yielding a final ARfD of 0.1 mg/kg bw/day.

The Table below lists isoxaflutole and metabolites, including structures, codes and synonyms.

Code Number (Synonyms)	Description	Compound found in:	Structure
IUPAC: 5-cyclopropyl-1,2-oxazol-4-yl α,α,α-trifluoro-2-mesyl-p-tolyl ketone **CAS:** (5-cyclopropyl-4-isoxazolyl)[2-(methylsulfonyl)-4-(trifluoromethyl)phenyl] methanone **CAS No.:** 141112-29-0	ISOXA-FLUTOLE $C_{15}H_{12}F_3NO_4S$ 359.3 g mol^{-1} [a] AE B197278 [b] RPA 201772, RPA 591428 [c] 94 BCS-AH21981	Active substance	

Code Number (Synonyms)	Description	Compound found in:	Structure
IUPAC: 3-cyclopropyl-2-[2-mesyl-4-(trifluoromethyl)benzoyl]-3-oxopropanenitrile CAS: α-(cyclopropyl-carbonyl)-2-(methylsulfonyl)-β-oxo-4-trifluoromethyl)-benzenepropanenitrile CAS No.: 143701-75-1	$C_{15}H_{12}F_3NO_4S$ 359.3 g mol^{-1} [a] AE 0540092 [b] RPA 202248 [c] 14733 BCS-AB59005 Isoxaflutole-diketonitrile, DKN	Soil Water/sediment Wheat Soybean Poppies Rat Goat Hen Rotational crop (minor) Radish leaf Sorghum grain	
IUPAC: 2-mesyl-4-trifluoromethylbenzoic acid **CAS:** 2-methane-sulfonyl-4-trifluoromethyl benzoic acid **CAS No.:** 142994-06-7	$C_9H_7F_3O_4S$ 268.2 g mol^{-1} [a] AE B197555 [b] RPA 203328 [c] 10069 BCS-AB49990 Pyrasulfotole benzoic acid IFT acid	Soil Wheat Soybeans Poppy seeds Rat Hen Rotational crop Radish Lettuce Sorghum	
IUPAC: 2-aminomethylene-l-cyclopropyl-3-(2-mesyl-4-trifluoromethylphenyl)-propan-1,3-dione **CAS**: n.a. **CAS No.:** n.a.	$C_{15}H_{14}F_3NO_4S$ 361.3 g mol^{-1} [a] AE 0692291 [b] RPA 205834 [c] 10361 BCS-BY16134	Soil Water/sediment Rat Goat Hen	 Amidine-Enamine tautomers ↕ 

Code Number (Synonyms)	Description	Compound found in:	Structure
IUPAC: 1-cyclopropyl-2-hydroxymethylene-3-(2-mesyl-4-trifluoromethylphenyl)propane-1,3-dione **CAS**: n.a. **CAS No.:** n.a.	$C_{15}H_{13}F_3O_5S$ 362.3 g mol^{-1} [a] AE 0893029 [b] RPA 207048 [c] 10054	Water/sediment Rat Goat Hen	
IUPAC: (2Z)-3-hydroxy-2-{hydroxy[2-(methylsulfonyl)-4-(trifluoromethyl)phenyl]methyl}-5-oxohex-2-enenitrile **CAS**: n.a. **CAS No**.: n.a.	$C_{15}H_{14}F_3NO_5S$ 377 g mol^{-1} [a] NA [b] NA [c] NA Met 14	Photolysis, buffer	
IUPAC: (2Z,4Z)-2-{hydroxy[2-(methylsulfonyl)-4-(trifluoromethyl)phenyl]methylene}-3-oxohex-4-enenitrile **CAS**: n.a. **CAS No**.: n.a.	$C_{14}H_{12}F_3NO_4S$ 359 g mol^{-1} [a] NA [b] NA [c] NA Met 20	Photolysis, buffer	
IUPAC: 2-(methylsulfonyl)-4-(trifluoromethyl) benzamide **CAS**: n.a. **CAS No.:** n.a.	$C_9H_8F_3NO_3S$ 267.2 g mol^{-1} [a] NA [b] NA [c] NA IFT-Amide	Soybean forage Soybean (minor)	 *

Code Number (Synonyms)	Description	Compound found in:	Structure
IUPAC: 5-cyclopropyl-1,2-oxazol-4-yl-α,α,α-trifluoro-p-tolyl ketone (IUPAC) **CAS:** (5-cyclopropyl-4-isoxazolyl)[4-(trifluoromethyl)phenyl] methanone **CAS No.:** 171187-01-2	$C_{14}H_{10}F_3NO_2$ 281.2 g mol^{-1} [a] AE 0893028 [b] RPA 205568 [c] 10053	Rat (tentatively identified)	
IUPAC: 3-cyclopropyl-3-oxopropionitrile **CAS**: 3-cyclopropyl-3-oxopropanenitril **CAS No.:** 118431-88-2	C_6H_7NO 109.1 g mol^{-1} [a] AE 1011789 [b] RPA 202304 [c] 10782	Corn (minor) (identified by co-chromatography)	 *
IUPAC: cyclopropanecarboxylic acid **CAS**: cyclopropanecarboxylic acid **CAS No.:** 1759-53-1	$C_4H_6O_2$ 86.09 g mol^{-1} [a] AE 1011990 [b] RPA 033853 [b] RPA 719259 [c] 10783	Artificial metabolite (RPA202304 in hot NaOH solution)	 *

*****Metabolite is not included in the risk assessment of any section. Their listing is to show the present knowledge about compounds deriving from degradation of isoxaflutole or tentiative appearance.

CA 5.1 Studies on absorption, distribution, metabolism and excretion in mammals

CA 5.1.1 Absorption, distribution, metabolism and excretion by oral route

According to the data requirements published in the Commission Regulation (EU) No 283/2013 of 1-March-2013 a "comparative in-vitro metabolism study" should be performed "on animal species to be used in pivotal studies and on human materials (microsomes or intact cell systems) in order to determine the relevance of the toxicological animal data …"
Therefore the following study was performed:

Report:	[redacted] 5; [redacted] ;2013;M-471593-01
Title:	[Phenyl-UL-14C]isoxaflutole: Metabolic Stability and Profiling in Liver Microsomes from Rats and Humans for Inter-Species Comparison
Report No:	M-471593-02-1
Document No:	M-471593-02-1
Guidelines:	**Regulation (EC) No 1107/2009 (Europe) amended by the Commission Regulation Regulation (EC) No 1107/2009 amended by the Commission Regulation (EU) No. 283/2013 (Europe); US EPA OCSPP 870.SUPP; Deviations: not specified**
GLP/GEP:	**yes**

The comparative metabolism of [Phenyl-UL-^{14}C]Isoxaflutole was investigated in animal *in-vitro* systems by incubating the test item with liver microsomes from male rats (RLM) and humans (HLM) in the presence of NADPH cofactor.

Materials and Method

The test item concentration was 15 µM and the protein concentration 1 mg/mL. The 15 µM test item concentration was chosen in order to have enough sample material for possible identification of metabolites by chromatographic or spectroscopic methods. The sampling times were 0 and 1 hour after test start. The test duration of 1 hour for the test item was considered as reasonable because positive results were obtained from the enzymatic reaction of Testosterone to Hydroxy-Testosterone already after 10 minutes. Samples were analysed following protein precipitation by reversed phase HPLC with radiochemical detection (HPLC-RAD).

The experiments were conducted with pooled liver microsomes from male Wistar rats (RLM, batch 1010126, pool of 200 individuals) and humans (HLM, batch 1110189, pool of 50 donors from both genders). Following incubation, samples were centrifuged, diluted with the HPLC mobile phase A and afterwards directly analysed in triplicate by HPLC-RAD system (150 µL injection volume). The chromatograms were recorded electronically and quantitatively evaluated using the MassLynx® Chromatography software (V4.0, Waters). The ^{14}C-trace of a chromatogram, which should be integrated, was divided into regions of interest (ROI's), corresponding to the separated radioactive peaks. The area counts from all regions of interest were used for the percentage calculation of the individual compounds

LLOQ and linearity of response were evaluated for the radiochemical detection.
The linearity of the methodology was determined as a tool to assess the correct performance of the radioactivity flow-through detector (6 levels for radioactivity detection). Peak area values at each level were plotted versus the respective nominal injected dpm, and linear regression was carried out. The correlation coefficient obtained was 0.999746.

Results

The mean recovery of radioactivity after microsome incubations and sample preparation (*i.e*. protein precipitation with AcN and centrifugation) at T=0 was found to be 101.7% and 99.3% in RLM and HLM, respectively, while after 1 hour incubation the recoveries were 101.7% in RLM and 104.6% in HLM.

Metabolite Profile of ^{14}C-Isoxaflutole

^{14}C-Isoxaflutole was found to be slightly unstable in the incubation buffer at 37 °C. One ^{14}C-containing degradation product (Iso-2) was detected accounting for 18.4% of the relative percentage

(calculated from peak area values) after 1 hour incubation period. It showed nearly the same HPLC retention time as a respective peak either in the testing solution, or in the incubations with RLM and HLM at 0 and 1 hour incubation periods.
Iso-2 accounted for 98.8% and 100% of the relative percentage in RLM and HLM, respectively after 1 hour incubation.
In addition, three further ^{14}C-labeled radioactivity regions were detected in very low amounts of the relative percentage in the 1 h incubates with RLM and HLM. These radioactivity regions were considered as non-relevant.

^{14}C-Isoxaflutole was completely metabolized when incubated with liver microsomes from rats and humans. The results of the tests demonstrated that the *in-vitro* metabolism of ^{14}C-Isoxaflutole when incubated with liver microsomes is almost similar between rats and humans.

Conclusion
The in-vitro metabolism of 14C-Isoxaflutole when incubated with liver microsomes was found to be very similar between rats and humans.

In rat microsomal incubations, 14C-Isoxaflutole was mainly transformed towards the metabolite Iso-2. Beside that a very minor radioactivity region with less than 2% (Iso-3) was detected.
In human microsomal incubations the only metabolite of 14C-Isoxaflutole formed was metabolite Iso-2.

No specific 14C-Isoxaflutole metabolites were formed by human liver microsomes when compared to rat liver microsomal incubations.

CA 5.1.2 Absorption, distribution, metabolism and excretion by other routes

No new scientific findings that influence the regulatory interpretation of the official evaluation of the active substance since the first Annex I inclusion.

CA 5.2 Acute toxicity

The acute toxicity of isoxaflutole was assessed in the first EU review of isoxaflutole, however these data are summarized here for reference in gray type. Further information is available in the Baseline Dossier provided by Bayer CropScience and in the Monograph. A full summary of the new phototoxicity study is appended below in section 5.2.7.

Type of study (Reference)	Species	Results	OECD classification (proposed)
Acute oral toxicity ([redacted], 1993a, M-158376-01-1)	Rat	No mortalities at up to 5000 mg/kg bw	Category 5 / unclassified
Acute dermal toxicity ([redacted], 1993b, M-158374-01-1)	Rabbit	No mortalities at 2000 mg/kg bw	Category 5 / unclassified
Acute inhalation toxicity ([redacted], 1994, M-158415-02-1)	Rat	No mortalities at 5.23 mg/L air	Category 5 / unclassified

Type of study (Reference)	Species	Results	OECD classification (proposed)
Dermal irritation ([redacted], 1993c M-158372-01-1)	Rabbit	Not irritating	Category 5 / unclassified
Eye irritation ([redacted], 1993d M-158370-01-1)	Rabbit	Not irritating	Category 5 / unclassified
Skin sensitization (modified Buehler) ([redacted], 1992 M-158233-01-1)	Guinea pig	Not sensitizing	Category 5 / unclassified
Skin sensitization (Magnusson-Kligman) ([redacted], 1996 M-209748-01-1)	Guinea pig	Not sensitizing	Category 5 / Unclassified
Phototoxicity in vitro ([redacted], 2013 M-471195-01-1)	BALB/c 3T3 cells	Negative	Not relevant

It is concluded that based on these data isoxaflutole needs not be classified for acute oral, dermal, and inhalation toxicity. Isoxaflutole is classified as non-irritating to skin and eyes and is not a skin sensitizer.

CA 5.2.1 Oral

No new scientific findings that influence the regulatory interpretation of the official evaluation of the active substance since the first Annex I inclusion.

CA 5.2.2 Dermal

No new scientific findings that influence the regulatory interpretation of the official evaluation of the active substance since the first Annex I inclusion.

CA 5.2.3 Inhalation

No new scientific findings that influence the regulatory interpretation of the official evaluation of the active substance since the first Annex I inclusion.

CA 5.2.4 Skin irritation

No new scientific findings that influence the regulatory interpretation of the official evaluation of the active substance since the first Annex I inclusion.

CA 5.2.5 Eye irritation

No new scientific findings that influence the regulatory interpretation of the official evaluation of the active substance since the first Annex I inclusion.

CA 5.2.6 Skin sensitization

No new scientific findings that influence the regulatory interpretation of the official evaluation of the active substance since the first Annex I inclusion.

CA 5.2.7 Phototoxicity

According to the new data requirements (Commission Regulation (EU) No. 283/2013 of 1 March 2013; Official Journal of the European Union, L 93/1, 3.4.2013), the conduct of a phototoxicity study is required under certain conditions.

The circumstances in which a phototoxicity study, according to the new data requirements, is required are "where the active substance absorbs electromagnetic radiation in the range 290-700 nm and is liable to reach the eys or light-exposed areas of the skin, either by direct contact or through systemic distribution. If the Ultraviolet / visible molar extinction / absorption coefficient of the active substance is less than 10 L x mol^{-1} x cm^{-1}, no toxicity testing is required.

As the Ultraviolet / visible molar extinction / absorptioin coefficient of the active substance exceeds the trigger of 10 L x mol^{-1} x cm^{-1}, a cytotoxicity study has been performed in vitro using BALB/c 3T3 cells.

Report:	[redacted]7;[redacted];2013;M-471195-01
Title:	Isoxaflutole: Cytotoxicity assay in vitro with BALB/c3T3 cells: Neutral red (NR) test during simultaneous irradiation with artificial sunlight
Report No:	1579700
Document No:	M-471195-01-1
Guidelines:	**Commission regulation (EC) No. 440/2008 B 41, dated May 30, 2008 Committee for Proprietary Medicinal Products (CPMP) Note for Guidance on Photosafety testing, EMEA, CPMP/SWP/398/01, adopted 27 June 2002, into operation in Dec 2002. OECD Guideline for Testing of Chemicals: Guideline: 432; In vitro 3T3 NRU phototoxicity test (Revised and approved by the National Co-ordinators in May 2002, approved by Council April 2004); Deviations: not specified**
GLP/GEP:	**Yes**

I. Materials and methods

A. Materials

1. Test material:

Name: Isoxaflutole TC
Synonyms: AE B197278
Description: White solild
Lot/Batch no: AE B197278-01-01 (origin batch no. 6464/5/8/9
Purity: 98.5% (w/w)
Stability of test compound: guaranteed for study duration; expiry date: 2015-07-26

2. Vehicle and or positive control: Solvent control: Earle´s Balanced Salt Solution (EBSS) containing 1% (v/v) dimethylsulfoxide (DMSO).
Positive control: chlorpromazine (Sigma) dissolved in EBSS

3. Test system: BALB/c 3T3 cell clone 31

Culture medium: Dulbecco´s Minimal Essential Medium (DMEM) supplemented with 10% (v/v) NCS.

Cell cultures: Thawed stock cultures were propagated at 37 ± 1.5 °C in 75 cm^2 plastic flasks. Seeding was done with about 1 x 10^6 cells per flask in 15 mL DMEM, supplemented with 10 % NCS.
Cells were sub-cultured twice weekly. The cell cultures were

incubated at 37 ± 1.5 °C in a 7.5 ± 0.5% carbon dioxide atmosphere.

B. Study design and methods

1. Treatment

Dose:

Test item	+/– UV	Final concentrations in µg/mL*
IFT*	+/–	0.49, 0.98, 1.95, 3.91, 7.81, 15.6, 31.3, 62.5
Positive control**	–	6.25, 12.5, 25, 37.5, 50, 75, 100, 200
	+	0.125, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 4.0
Solvent control	+/–	EBSS containing 1 % (v/v) DMSO

*isoxaflutole, ** chlorpromazine

The test item isoxaflutole was dissolved in DMSO. The final concentration of the solvent in EBSS was 1 % (v/v).

Seeding of cultures: 2×10^4 cells per well were seeded in 100 µL culture medium in two 96 well plates

Replicates: 2 (one for irradiation exposure, one for treatment in the dark)

Treatment & irradiation: 24 h after seeding the cultures were washed with EBSS. 100 µL solved test item added per well for pre-incubation of the plates for 1 hour in the dark. Afterwards one plate was irradiated at 1.65 mW/cm² (4.95 J/cm²) for 50 min ± 2 min at 26 °C, the other plate was stored for 50 min ± 2 min at 20-31 °C in the dark. Test item was removed and both plates were washed with EBSS. Fresh culture medium was added and the plates were incubated about 21.5 hours at 37 ± 1.5 °C and 7.5 ± 0.5 % CO_2.

Cytotoxicity determination: For measurement of Neutral Red uptake the medium was removed and 0.1 mL serum-free medium containing 50 µg Neutral Red / mL were added to each well. The plates were incubated for another 3 hours at 37°, before the medium was removed completely and the cells were washed with EBSS. For extraction of the dye 0.15 mL of a solution of 49% (v/v) deionised water, 50% (v/v) ethanol and 1% (v/v) acetic acid were added to each well. After approximately 10 minutes at room temperature and a brief agitation, the plates were transferred to a microplate reader (Versamax®, Molecular Devices) equipped with a 540 nm filter to determine the absorbance of the extracted dye. This absorbance showed a linear relationship with the number of surviving cells.

Number of measurements: Isoxaflutole and positive control: each concentration was measured 6 times
Solvent control: 12 times

2. Evaluation

The mean absorption (OD_{540}) value per concentration was calculated. The ED_{50}* values were determined by curve fitting by software. The Photo-irritancy factor (PIF), as well as the Mean Phototoxic effect (MPE) was calculated according to OECD guideline 432.

*ED_{50} = effective dose where only 50% of the cells survived

Evaluation criteria: If PIF < 2 or MPE < 0.1 no phototoxic potential is predicted

If PIF > 2 and < 5 or MPE > 0.1 and < 0.15 a probable phototoxic potential is predicted
If PIF > 5 or MPE > 0.15 a phototoxic potential is predicted.

II. Results and discussion

In the range finding experiment (RFE) no cytotoxic effects were observed after exposure of the cells to the test item isoxaflutole, neither in the presence nor in the absence of irradiation to artificial sunlight. Therefore, ED_{50}-values and PIF could not be calculated. The resulting MPE-value was -0.042.

In the main experiment (ME) no cytotoxic effects were observed after exposure of the cells to the test item isoxaflutole, neither in the presence nor in the absence of irradiation to artificial sunlight. Therefore, ED_{50}-values and PIF could not be calculated. The resulting MPE-value was -0.066.

The mean of solvent control values of the irradiated versus the non-irradiated group met the acceptance criteria. The positive control chlorpromazine induced phototoxicity in the expected range in the presence of irradiation.

The results are summarized in Table 5.2.7-1 and Table 5.2.7-2 below.

Table 5.2.7-1: OD_{540} values Neutral Red assay of the main experiment

Con-centration [µg/mL]	OD_{540} with artificial sunlight			Con-centration [µg/mL]	OD_{540} without artificial sunlight		
	Mean	SD	% of solvent control		Mean	SD	% of solvent control
Treatment with isoxaflutole							
Solvent control	0.5339*	0.0531	100.00	Solvent control	0.5403*	0.0384	100.00
0.49	0.5803	0.0187	108.70	0.49	0.5512	0.0308	102.02
0.98	0.5733	0.0196	107.39	0.98	0.5408	0.0197	100.10
1.95	0.5835	0.0297	109.30	1.95	0.5390	0.0123	99.77
3.91	0.5664	0.0298	106.09	3.91	0.5391	0.0307	99.78
7.81	0.5564	0.0248	104.22	7.81	0.5371	0.0234	99.41
15.6	0.5544	0.0414	103.85	15.6	0.5346	0.0426	98.95
31.3	0.5576	0.0410	104.45	31.3	0.5172	0.0234	95.72
32.5	0.5378	0.0477	100.73	62.5	0.5270	0.0693	97.54
Treatment with positive control chlorpromazine							
Solvent control	0.4810*	0.0365	100.00	Solvent control	0.5288*	0.0297	100.00
0.125	0.5149	0.0522	107.05	6.25	0.4489	0.0287	84.89
0.250	0.4249	0.0450	88.34	12.50	0.1264	0.0147	23.90
0.500	0.3042	0.0303	62.25	25.00	0.0477	0.0017	9.02
0.750	0.1478	0.0454	30.73	37.50	0.0483	0.0010	9.13
1.000	0.0674	0.0137	14.01	50.00	0.0474	0.0009	8.95
1.500	0.0627	0.0078	13.03	75.00	0.0478	0.0006	9.03
2.000	0.0626	0.0017	13.01	100.00	0.0483	0.0012	9.14
4.000	0.0684	0.0031	14.21	200.00	0.0508	0.0013	9.61

* mean OD_{540} out of 12 wells

Table 5.2.7-2: Summary of results of the Neutral Red assay

	Substance	ED_{50} (+UV) [µg/mL]	ED_{50} (−UV) [µg/mL]	PIF	MPE	% viability of solvent control of irradiated vs. non-irradiated plate
Range finding experiment	Isoxaflutole	--	--	--	-0.042	97.9
	Positive control	1.19	17.16	14.41	0.442	93.7
Main experiment	Isoxaflutole	--	--	--	-0.066	98.8
	Positive control	0.57	9.47	16.72	0.415	91.0

ED_{50} = effective dose where only 50% of the cells survived
PIF = Photo-Irritation Factor
MPE = Mean Phototoxic effect

III. Conclusions

Isoxaflutole is not phototoxic ton BALB/c 3T3 cells in this in vitro assay.

CA 5.3 Short-term toxicity

The sub-acute and subchronic toxicity of isoxaflutole was assessed in the first EU review of isoxaflutole, however these data are summarized here for reference. Further information is available in the Baseline Dossier provided by Bayer CropScience and in the Monograph.

Study duration (Reference)	Species	NOAEL mg/kg bw/day	LOAEL mg/kg bw/day	Critical effects
28-day dietary ([redacted], 1994; M-213066-01-1)	Mouse	< 29.4	29.4	- Increased levels of ALAT, ASAT, AP - Increased liver weight
6 week capsule or 3-week dietary ([redacted], 1994 M-213061-01-1)	Dog	< 1042	1042	- Increased AP
90-day dietary ([redacted], 1994 M-158457-01-1)	Rat	3	10	- Ocular lesions - Periacinar hypertrophy in liver
6-week dietary with 3-week reverse ([redacted], 1994 M-158400-01-1)	Rat	< 25	25	- Corneal opacities in males - Decreased AP, ASAT
90-day dietary ([redacted], 1994 M-158395-01-1)	Mouse	7.6	170.0	- Increased liver weight - Periacinar hypertrophy in liver - Increases in liver enzyme levels
21-day dermal ([redacted], 1994 M-158409-01-1)	Rat	100	1000	- Increased liver weight

CA 5.3.1 Oral 28-day study

No new scientific findings that influence the regulatory interpretation of the official evaluation of the active substance since the first Annex I inclusion.

CA 5.3.2 Oral 90-day study

No new scientific findings that influence the regulatory interpretation of the official evaluation of the active substance since the first Annex I inclusion.

CA 5.3.3 Other routes

No new scientific findings that influence the regulatory interpretation of the official evaluation of the active substance since the first Annex I inclusion.

CA 5.4 Genotoxicity testing

The genotoxicity of isoxaflutole was assessed in the first EU review of isoxaflutole, however these data are summarized here for reference. Further information is available in the Baseline Dossier provided by Bayer CropScience and in the Monograph.

Endpoint (Reference)	Test species / cell type	Result	Comments
In vitro			
Gene mutation ([redacted], 1993, M-158334-01-1)	TK +/- mouse lymphoma cells	Negative	Precipitation at 150, 300, and 600 µg/ml
Chromosome aberrations ([redacted], 1992, M-211242-02-1)	Human lymphocytes	Negative	Precipitation at 150, 300, 500, and 600 µg/ml
Chromosome aberrations ([redacted], 1993, M-158330-01-1)	Human lymphocytes	Negative	Precipitation at 600 µg/ml
Gene mutation ([redacted], 1992, M-158222-01-1)	Chinese hamster V79 cells	Negative	Precipitation at 50 and 100 µg/ml
Gene mutation ([redacted], 1993, M-162063-01-1)	*Salmonella typhimurium*	Negative	Precipitation at 500 and 1000 µg/ml
In vivo			
UDS test ([redacted], 1993, M-211245-01-1)	Rat liver, animals treated in vivo	Negative	No findings with Isoxaflutole
Micronucleus test ([redacted], 1993, M-158358-01-1)	Mouse bone marrow	Negative	No cytotoxicity

The overall weight of evidence from the *in vitro* and *in vivo* studies is that isoxaflutole is not genotoxic.

CA 5.4.1 In vitro studies

No new scientific findings that influence the regulatory interpretation of the official evaluation of the active substance since the first Annex I inclusion.

CA 5.4.2 In vivo studies in somatic cells

No new scientific findings that influence the regulatory interpretation of the official evaluation of the active substance since the first Annex I inclusion.

CA 5.4.3 In vivo studies in germ cells

No new scientific findings that influence the regulatory interpretation of the official evaluation of the active substance since the first Annex I inclusion.

CA 5.5 Long-term toxicity and carcinogenicity

The chronic toxicity and oncogenicity of isoxaflutole was assessed in the first EU review of isoxaflutole, however these data are summarized here for reference. Further information is available in the Baseline Dossier provided by Bayer CropScience and in the Monograph.

Endpoint (Reference)	Species	NOAEL mg/kg bw/day	LOAEL mg/kg bw/day	Critical effects
104 week dietary chronic toxicity oncogenicity ([redacted], 1995 M-213068-01-1)	Rat	3	12	Effect on weight and pathology of liver and thyroid
78 week dietary chronic toxicity ([redacted], 1995 M-213077-01-1)	Mouse	3.2	64.4	Histopathological effects in liver
52 week dietary chronic toxicity ([redacted], 1994 M-213081-01-1)	Dog	8.56	44.81	Increased liver weight

CA 5.6 Reproductive toxicity

The reprotoxicity of isoxaflutole was assessed in the first EU review of isoxaflutole, however these data are summarized here for reference. Further information is available in the Baseline Dossier provided by Bayer CropScience and in the Monograph and subsequent addenda.

Endpoint (Reference)	Species	NOAEL mg/kg bw/day	LOAEL mg/kg bw/day	Critical effects
2-generation reproduction ([redacted], 1995 M-213083-01-1)	Rat	2	20	- Increased liver weight - Histopathological effects in the liver - Decreased pup viability
Developmental toxicity ([redacted], 1995 M-158493-01-1)	Rat	Fetal: 10 Maternal: 100	Fetal: 100 Maternal: 500	Fetal: - delayed ossification - subcutaneous hemorrhages Maternal: - decreased body weight
Developmental toxicity ([redacted], 1995 M-158585-01-1)	Rabbit	Fetal: 5 Maternal: 20	Fetal: 20 Maternal: 100	Fetal: Delayed ossification Maternal: decreased body weight

Although it is not a new study, the rat developmental toxicity study is summarized in point CA 5.6.2 below to support derivation of the Acute Reference Dose.

CA 5.6.1 Generational studies

No new studies have been performed. See studies as listed under point CA 5.6.

CA 5.6.2 Developmental toxicity studies

Report:	[redacted]l; [redacted]1995;M-158493-01
Title:	RPA201772 (active ingredient): Teratology study in the rat - Final report
Report No:	500037
Document No(s):	Report includes Trial No's 94/RHA536/1203 - RHA/536 M-158493-01-1
Guideline:	**JMAF: (1985); OECD: (1981); USEPA (=EPA): 83-3;Deviation not specified**
GLP/GEP:	**yes**

Executive summary:
Female CD rats were paired with stock males and allocated to treatment groups following mating. RPA 201772 was administered on gestation days 9 through 15 inclusive by oral gavage at doses of 0, 10, 100 and 500 mg/kg bw/day. Body weight and food consumption were measured throughout gestation, and at necropsy on gestation day 20 the reproductive tracts were dissected and examined. Fetuses were weighed, examined externally, and then either dissected immediately for visceral examination followed by fixation and skeletal staining, or fixed for serial sectioning.

At 500 mg/kg bw/day, clinical signs noted were limited to increased salivation within the first 90 minutes following dosing; this sign was only observed in some animals on some occasions. Maternal cumulative body weight gain was decreased from gestation day 8. Body weight gain was similar to

that in controls from gestation day 12, however total body weight gain remained decreased throughout the remainder of gestation. Food consumption was decreased on gestation days 6 to 8. There was no effect on either pre- or post-implantation losses or on the number of resorptions observed. The number of viable young and the sex ratio was not affected.

Fetal body weight was decreased, with an increased incidence of the external observation "small fetus". The incidence of subcutaneous edema was noted only at serial sectioning to be increased at this dose level; subcutaneous edema was not observed at external examination of the fetuses. There were no visceral findings. Ossification of sternebrae, caudal vertebrae, and metacarpal and/or metatarsal bones was decreased, and there were increases in 14th ribs, 27 pre-sacral vertebrae, and incomplete ossification of the first thoracic vertebral centrum.

At 100 mg/kg bw/day, there were no effects on maternal body weight or food consumption, and no effect on any reproductive parameters.

Fetal body weight was decreased at 100 mg/kg bw/day. There were some decreases in the ossification of sternebrae and metacarpals or metatarsals.

There were no maternal or fetal findings at 10 mg/kg bw/day.

The maternal NOAEL for this study is 100 mg/kg bw/day based on the decreased body weight gain observed at 500 mg/kg bw/day.

The developmental NOAEL for the rat teratology study is 10 mg/kg bw/day, based on decreased fetal body weight and decreased ossification observed at 100 mg/kg bw/day.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material: BPA 201772
Description: fine white powder
Lot/Batch: 40ADM13
Purity: not stated in the report
CAS: 141112-29-0
Stability of test compound: 8 days at ambient temperature in methylcellulose

2. Control materials: **Negative:** 0.5% aqueous methylcellulose
Solvent: 0.5% aqueous methylcellulose
Positive: no positive control tested

3. Test animals:
Species: rat
Strain: CD
Age: approximately 10-11 weeks
Weight at dosing: 211-272g
Source: [redacted], England
Number of animals per dose: 25
Acclimation period: 5 days
Diet: LAD1 (E) SQC ([redacted], England)
Water: tap water

Housing: individually housed in TR18 stainless steel or RB3 polypropylene cages, with lids and floors of stainless steel grid

Environmental conditions –

Temperature: 21°C (range 19-25°C)
Humidity: 55% (range 40-70%)
Air changes: approximately 15 changes per hour
Photoperiod: 12-hour light, 12-hour dark cycle

4. Test compound concentrations: 0, 10, 100, 500 mg/kg bw/day

B. TEST PERFORMANCE

1. Animal assignment and treatment
Animals were not assigned to treatment groups until after mating. The females used for this study were paired on a one-to-one basis with stock males of the same strain. Each morning following pairing, the trays beneath the cages were checked for ejected copulation plugs, and a vaginal smear was prepared from each animal and examined for the presence of spermatozoa. Only females showing a sperm-positive vaginal smear or at least three copulation plugs were allocated to treatment groups. The day on which evidence of mating was found was designated day 0 of gestation.

Females which showed clear evidence of mating were allocated to groups in sequence. At the time of assignment, each animal was assigned a number and identified by tail tattoo.

Control and treated animals were administered either 0.5% aqueous methylcellulose or RPA 201772 in 0.5% aqueous methylcellulose by oral gavage, on gestation days 6 through 15 inclusive. The volume dosage administered was 10 mg/kg, with the volume administered to each animal based on that animal's body weight that day.

2. Test substance formulations and analysis
The test agent suspensions were prepared fresh daily in 0.5% methylcellulose in purified water (prepared by reverse osmosis of tap water). An aliquot of the test agent was dry ground in an agate mortar and pestle then an appropriate quantity of vehicle was gradually added to form a paste, which was then diluted with the remaining vehicle. Suspensions were mixed with a laboratory mixer for two minutes at low speed following this final dilution.

3. Statistics
The significance of apparent inter-group differences was tested using appropriate statistical tests, and differences with an associated probability of $p < 0.05$ were considered to be statistically significant.

C. METHODS

1. Clinical examination of females
All animals were checked daily for clinical signs of toxicity, and any signs were recorded with respect to details of type, severity, time of onset, and duration.

2. Body weight
Body weight was measured on gestation days 0, 3, daily from gestation days 6 to 16 inclusive, 18 and 20.

3. Food intake
Food consumption was measured for gestation days 0-2, 3-5, 6-8, 9-11, 12-15, 16-17 and 18-19.

4. Necropsy
On gestation day 20, animals were killed by inhaled carbon dioxide and examined macroscopically for indications of disease or an adverse reaction to treatment.

5. Investigation at Caesarean section
After the initial gross necropsy of each dam, the reproductive tract was then dissected out and examined for number of corpora lutea per ovary, number of implantation sites, number of early or late resorption sites, and number and distribution of fetuses in each uterine horn.

Each fetus was then weighed, sexed, and examined for external abnormalities. Each placenta was also weighed and any placental abnormalities were recorded. Fetuses were individually tagged with identity marks. Approximately half of the fetuses in each litter were dissected immediately and the neck and thoracic and abdominal cavities were examined. Fetal abnormalities were recorded and the fetuses were eviscerated then fixed in industrial methylated spirit prior to staining with Alizarin red for skeletal examination. The remaining fetuses in each litter were fixed in Bouin's fixative following external examination. Once fixed, the fetuses were then examined by Wilson free-hand serial sectioning.

II. RESULTS AND DISCUSSION

A. OBSERVATIONS AND MORTALITY
Clinical findings noted during the study were limited to sporadic incidents of increased salivation after dosing among 10 animals at 500 mg/kg bw/day. When increased salivation was noted, it ceased within 1.5 hours after dose administration.

There were no mortalities in any treatment group.

B. BODY WEIGHT
Cumulative body weight gain was statistically significantly decreased at 500 mg/kg bw/day from gestation day 8 onwards. From gestation day 12, the rate of body weight gain at 500 mg/kg bw/day was similar to that observed in controls, however the total body weight gain never reached that of control animals. There was no effect on body weight gain at either 10 or 100 mg/kg bw/day.

Table 5.6.2/1: Maternal body weight, in grams, in the rat developmental toxicity study with RPA 201772

	RPA 201772, dose in mg/kg bw/day			
Day	0	10	100	500
0	237	238	241	240
6	280	284	283	282
9	300	304	301	296***

	RPA 201772, dose in mg/kg bw/day			
Day	0	10	100	500
12	325	329	326	316***
15	350	354	352	340***
16	366	369	369	356**
20	440	442	446	430*

Statistically significant at : * p < 0.05; ** p < 0.01; *** p < 0.001

C. FOOD INTAKE

At 500 mg/kg bw/day, on gestation days 6 through 8, food consumption was decreased compared to control animals. At 100 and 500 mg/kg bw/day, after gestation day 16, food consumption was slightly increased compared to controls.

Table 5.6.2-2: Maternal food consumption, in grams per rat per day, in the rat developmental toxicity study with RPA 201772

	RPA 201772, dose in mg/kg bw/day			
Day	0	10	100	500
0-2	26	28*	27	27
3-5	29	29	28	30
6-8	30	31	30	27**
9-11	31	32	31	29
12-15	33	33	33	33
16-17	35	36	38**	39***
18-19	35	35	36	37

Statistically significant at : * p < 0.05; ** p < 0.01; *** p < 0.001

D. NECROPSY

There were no treatment-related observations at necropsy.

E. GENERAL REPRODUCTION DATA

There was no effect on the number of corpora lutea or on the incidence of either pre- or post-implantation losses or on resorptions.

F. EFFECTS ON INTRAUTERINE DEVELOPMENT

1. Gestation rate

Gestation rate was unaffected by administration of RPA 201772 on gestation days 6 through 15.

2. Post-implantation loss, number and sex of foetuses

There was no effect of administration of RPA 201772 on post-implantation loss, number of viable young, or sex ratio in any treatment group.

3. Fetal weight

At 100 and 500 mg/kg bw/day, there was a dose-related statistically significant decrease in fetal body weight.

4. Fetal external and visceral deviations

At 500 mg/kg bw/day, the incidence of the external observation “small fetus” was slightly increased compared to controls. Also at 500 mg/kg bw/day, an increased incidence of

subcutaneous edema was noted only at free-hand serial sectioning, but not at external observation immediately after necropsy. There were no other treatment-related findings.

5. Fœtal skeletal and cartilaginous deviations

Single-stained (Alizarin red) fetuses showed some treatment-related findings at 100 and 500 mg/kg bw/day. At 100 mg/kg bw/day, there were decreases in sternebral and metacarpal or metatarsal ossification. At 500 mg/kg bw/day, there were decreases in the ossification of sternebrae, caudal vertebrae, and metacarpal or metatarsal bones, and increased incidences of 14th ribs, incomplete ossification of the first thoracic vertebral centrum, 27 pre-sacral vertebrae and asymmetric pelvis. At 10 mg/kg bw/day, there was no effect on skeletal development.

Table IIA 5.6.10-3: Fetal body weight and external and skeletal observations in the rat developmental toxicity study with RPA 201772

		RPA 201772, dose in mg/kg bw/day				Control data
		0	10	100	500	
	Fetuses, N	403	381	410	393	16256
Observation	Litters, N	25	25	25	25	656
Fetal body wt, g	Male	3.77	3.79	3.52**	3.30**	3.75
	Female	3.62	3.55	3.35**	3.13**	3.55
	Combined	3.70	3.67	3.43**	3.21**	3.65
Small fetus (< 2.80g)	Fetuses	0.0	1.3	2.2	6.4	1.45
	Litters	0	4	7	11	0.0-4.9
Shiny fetus	Fetuses	0.0	0.5	1.0	0.3	0.33
	Litters	0	2	4	1	0.0-2.7
Subcutaneous edema	Fetuses	0.0	1.4	3.4	21.9	1.54
	Litters	0	2	5	17	0.0-6.1
Incompl. Ossif. 1 sternebra	Fetuses	45.2	30.1	12.4	12.0	32.17
	Litters	20	16	12	4	10.7-52.9
Incompl. Ossif. 3 sternebrae	Fetuses	8.7	13.8	23.3	54.7	15.38
	Litters	12	12	17	23	2.0-31.2
Incompl. Ossif. 4 sternebrae	Fetuses	1.9	2.5	6.2	12.9	3.04
	Litters	3	1	8	13	0.0-9.2
Ribs 13 / 13	Fetuses	84.3	88.7	79.2	44.6	87.27
	Litters	25	24	25	23	77.6-95.5
Ribs 13 / 14	Fetuses	7.7	8.2	16.1	22.8	7.65
	Litters	11	11	11	22	2.9-13.0
Ribs 14 / 14	Fetuses	11.1	9.2	6.6	32.7	5.04
	Litters	10	7	10	23	0.0-10.8
14th rib or ribs enlarged	Fetuses	1.8	1.3	0.5	6.4	0.32
	Litters	2	1	1	8	0.0-2.6
1st thoracic vert centrum unossified	Fetuses	1.0	0.5	1.4	9.4	1.00
	Litters	2	1	3	9	0.0-5.3
27 pre-sacral vertebrae	Fetuses	1.0	0.0	1.4	7.9	0.51
	Litters	1	0	3	11	0.0-7.0
Fewer than 5 caudal vert. ossified	Fetuses	1.0	1.1	5.7	12.9	1.80
	Litters	2	3	8	15	0.0-5.9
Metacarpals/ metatarsals 3/4	Fetuses	69.3	79.1	89.5	92.6	72.16
	Litters	22	24	25	25	55.2-88.5
Metacarpals/ metatarsals 4/4	Fetuses	33.7	20.4	8.1	4.0	26.63
	Litters	17	13	7	5	9.7-44.2
One or more phalangeal bones ossified	Fetuses	4.3	4.1	0.0	0.0	3.29
	Litters	3	2	0	0	0.0-17.1

Statistically significant at : * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

III. CONCLUSION

Maternal effects observed in this study were limited to decreased body weight gain and food consumption at 500 mg/kg bw/day, and some increased salivation immediately after dosing.

Fetal findings, including decreased body weight, decreased ossification of specific bones, and / or an increased incidence of 14th ribs and 27 presacral vertebrae, were observed from 100 mg/kg bw/day, with the incidence generally related to increasing maternal dose of RPA 201772.

The maternal NOAEL for this study is 100 mg/kg bw/day, based on the decreased body weight gain observed at 500 mg/kg bw/day.

The developmental NOAEL for the rat teratology study is 10 mg/kg bw/day, based on decreased fetal body weight and decreased ossification observed at 100 mg/kg bw/day.

CA 5.7 Neurotoxicity studies

The neurotoxicity of isoxaflutole, as examined in an acute and 90-day neurotoxicity study, was assessed in the first EU review of isoxaflutole, however these data are summarized here in grayed text for reference. Further information is available in the Baseline Dossier provided by Bayer CropScience and in the Monograph. A full summary of the developmental neurotoxicity study is presented under point 5.7.1, below.

Endpoint (Reference)	NOAEL mg/kg bw/day	LOAEL mg/kg bw/day	Adverse effects at LOAEL / target organs	
Acute neurotoxicity ([redacted], 1995 M-213088-01-1)	2000	>2000	None	
90-day neurotoxicity ([redacted], 1995 M-166843-01-2)	Systemic: 250 Neurotox.: 750	Systemic: 750 Neurotox.: >750	Decreased body weight, food consumption; no neurotoxicity observed	
Developmental neurotoxicity ([redacted], 1999, M-254881-01-1)	25	250	Maternal	Decreased body weight, body weight gain, food consumption
	25	250	Offspring systemic	Decreased survival PND 0-1, decreased body weight
	250	---	Offspring neurotoxicity	No neurotoxicity observed

In the rat developmental neurotoxicity study, pregnant dams were administered RPA 201772 by oral gavage at doses of 0, 5, 25, and 250 mg/kg bw/day from gestation day 6 through lactation day 10. Assessments of pup development and learning and memory were conducted on a regular basis, and brain measurements and neuropathology examinations were conducted at necropsy.

At 250 mg/kg bw/day, maternal body weight and body weight gain were reduced, as was food consumption. Pup survival was reduced from postnatal day 0 to 1, with a slight decrease in pup survival from PND 0 to 4 as well. Pup body weight and body weight gain were decreased. There was no effect on the acquisition of either balanopreputial separation or vaginal patency, acoustic startle, locomotor activity, swimming ability, or learning and memory. There were no biologically meaningful effects on brain weight, and brain width and length measurements were not affected. There were no histopathological findings in the brain of pups at this dose.

At 25 and 5 mg/kg bw/day, there were no biologically significant effects on any of the assessed parameters in either dams or pups.

The maternal NOAEL for this study is 25 mg/kg bw/day, based on decreased maternal body weight, body weight gain, and food consumption at 250 mg/kg bw/day. The offspring systemic NOAEL is 25 mg/kg bw/day, due to decreased pup survival, body weight, and body weight gain at 250 mg/kg bw/day. In the absence of any neurotoxic findings, the NOAEL for developmental neurotoxicity in the rat is 250 mg/kg bw/day, the highest dose tested.

CA 5.7.1 Neurotoxicity studies in rodents

Report:	[redacted]p;[redacted];2000;M-254881-01
Title:	An oral developmental neurotoxicity study of Isoxaflutole (IFT) in rats
Report No:	M-254881-01-1
Document No:	M-254881-01-1
Guidelines:	**US EPA; OPPTS 870.6300**
GLP/GEP:	**yes**

Executive Summary:

Female CD rats were mated on a one-to-one basis with males of the same strain, and assigned to treatment groups following confirmation of mating. Animals were dosed by oral gavage from gestation day 6 through lactation day 10, at doses of 0, 5, 25, and 250 mg/kg bw/day. Maternal and pup body weights, and maternal food consumption, were measured regularly during gestation and / or lactation, as appropriate. Ten dams per group were observed outside the home cage on two occasions during gestation and during lactation. Litters were culled on postnatal day 4 to equalize litters to 8 pups, with 4 males and 4 females where possible. The acquisition of balanopreputial separation and vaginal opening were assessed for each pup and body weight was measured on the day of acquisition. Pups were assessed on postnatal day 20 or 60 for acoustic startle response, for locomotor activity on postnatal days 13, 17, 21, or 61, and for swimming ability and learning and memory beginning on postnatal day 22 or 62. Selected pups were sacrificed on postnatal days 11, 28 or 29, and 72, and necropsied. Neuropathological examinations were carried out on the brains and / or peripheral nervous systems, as appropriate.

At 250 mg/kg bw/day, maternal body weight and body weight gain was decreased compared to controls during gestation. Food consumption was also decreased during gestation and early lactation at this dose. There were no treatment-related effects on maternal macroscopic observations, implantation sites, number of pups born, mean live litter size, or percentage of males per litter. Pup survival from postnatal day 0 to 1 was significantly reduced, and there was a statistically non-significant decrease in pup survival from postnatal day 0 to 4. Pup body weight gain was decreased relative to controls from PND 1 to 4, and from PND 4 to 7, and pup body weight was decreased throughout the pre-weaning phase when compared to controls. A decrease in body weight and body

weight gain was observed on occasions throughout the remainder of the study. There was no biologically significant effect on either balanopreputial separation or vaginal patency, acoustic startle, locomotor activity, swimming ability, or learning and memory. No macroscopic findings were noted at necropsy of the pups. Absolute brain weight at PND 11 was decreased, however with no effect on relative brain weight and no change in brain length or width measurements this observation is considered not to be biologically significant. There were no histopathological findings at 250 mg/kg bw/day.

At 25 and 5 mg/kg bw/day, there were no effects on maternal body weight, body weight gain, or food consumption, or on maternal reproductive parameters. Pup survival was not affected, and there were no adverse findings in any of the neurobehavioral assessments. At 5 mg/kg bw/day, pup brain width was reduced, however in the absence of a dose-response relationship or any effect on brain weight, it is not considered to be adverse. There were no effects at either 25 or 5 mg/kg bw/day on the histopathology of the pup brains.

Based on reduced body weight, body weight gain, and food consumption observed at 250 mg/kg bw/day, the maternal NOEL for this study is established at 25 mg/kg bw/day.

In pups, body weight gain and survival immediately following birth were decreased at 250 mg/kg bw/day. The offspring systemic NOAEL is established at 25 mg/kg bw/day based on these findings. The NOAEL for developmental neurotoxicity is established at 250 mg/kg bw/day in the absence of any biologically significant effect on neurological function.

I. MATERIALS AND METHODS

A. MATERIALS:

1. Test Material: Isoxaflutole
- **Description:** fine, off-white powder
- **Lot/Batch:** IFT98-196
- **Purity:** 99.15%
- **CAS:** 141112-29-0
- **Stability of test compound:** stable for 8 days at room temperature

2. Vehicle and /or positive control: 1% aqueous methylcellulose

3. Test animals:
- **Species**: rat
- **Strain:** CD
- **Age:** approximately 84 days at start of the study
- **Weight at dosing:** 226-302g
- **Source:** [redacted] USA
- **Acclimation period:** 14 days
- **Diet:** Certified Rodent LabDiet 5002, PMI Nutrition International Inc.
- **Water:** tap water
- **Housing:** plastic maternity cages with ground corn cob bedding through PND 28 or 29, then individually housed in wire-mesh cages
- **Environmental conditions –**
  - **Temperature:** 71.8-72.9F
  - **Humidity:** 40.8-66.9%
  - **Air changes:** approximately 10 changes per hour
  - **Photoperiod:** 12 hours light / 12 hours dark

B. STUDY DESIGN:

1. In life dates: 13 July 1999 to 1 November 1999

2. Animal assignment and treatment

Animals were mated with stock males by cohousing in the home cages of males of the same strain and from the same source. Each mating pair was examined daily, and positive evidence of mating was confirmed by the presence of a copulatory plug or the presence of sperm in a vaginal smear. The day on which evidence of mating was identified was termed day 0 of gestation, and the animals were separated. The bred females were assigned to groups containing 25 rats each using a computer program which randomized the animals based on body weight stratification in a block design. Body weight values on gestation day 0 ranged from 226 g to 302 g.

One control group and three treatment groups were established, at 0, 5, 25, and 250 mg/kg bw/day respectively.

Dosing preparations were administered orally using 16-gauge stainless steel gavage cannulas from gestation day 6 through lactation day 10. If parturition was ongoing for a given animal during dose administration, that animal was not dosed on that day. A dosage volume of 5 ml/kg bw/day was used for the treated groups. The control group animals received the vehicle, 1% aqueous methylcellulose, at 5 ml/kg bw/day. In all cases, individual doses were based on the most recently recorded body weight to provide the correct mg/kg bw/day dose. All animals were dosed at approximately the same time each day. The offspring of the P0 generation (the F1 litters) were potentially exposed to the test article in utero and through nursing during lactation.

3. Test compound preparation and analysis

For the treated groups, an appropriate amount of the test compound was weighed for each group into a storage container. A stir bar was added, and a sufficient amount of vehicle was added to bring the volume of the formulation to the calibration mark. The preparations were homogenized using an electronic homogenizer for approximately 10 minutes, until a uniform suspension was obtained. The formulations were stirred continuously throughout use. Dosing formulations were prepared approximately weekly and were stored at room temperature. The preparations were visually inspected for homogeneity prior to the start of dosing on each day.

Prior to the initiation of dosing, representative batches of dosing suspensions were prepared at each dosage level. Duplicate 10ml samples were taken from the middle level of the control suspension, and from the top, middle, and bottom of each treated group formulation. One set of samples was analyzed to confirm homogeneity of the test article formulations. The remaining samples at each dosage level were combined and stored under normal laboratory conditions for 8 days, then analyzed to determine stability of the test article in the vehicle.

During the in-life phase of the study, one 10ml sample was collected from the middle level of each weekly dosing formulation including the control and analyzed for test compound concentrations.

4. Statistics

All analyses were conducted for a minimum significance level of 5%, comparing each treated group to the vehicle control group. All tests for significance at the 5% probability level were two-tailed for the group comparisons. The litter was used as the experimental unit.

For maternal gestation and lactation body weights and weight gains, maternal food consumption, mean litter weights, length of gestation, implantation sites, unaccounted sites, numbers of pups born, live litter sizes, organ weights, startle response, Biel maze, pup body weights, day of

balanopreputial separation, and day of vaginal patency, two-tailed analysis of variance with Dunnett's test were used. For locomotor activity, the Multitest procedure was used. The Kruskal-Wallis test and the Mann-Whitney U-test were used for pup sex at birth (% males per litter) and postnatal survival, and the Kolmogorov-Smirnov test was used for histopathologic findings.

C. METHODS

1. MORTALITY AND CLINICAL SIGNS

The animals were observed twice daily for moribundity and mortality. Clinical observations were recorded daily from gestation day 0 through lactation day 21. Animals were also observed daily for signs of toxicity approximately one hour following dosing throughout the treatment period. Females that delivered were observed three times daily during the period of expected parturition and at parturition for dystocia, prolonged or delayed labor, or other difficulties.

Ten randomly selected dams per group were observed outside the home cage on gestation days 6 and 12, and on lactation days 4, and 7.

All pups were examined daily for moribundity and mortality from the day of parturition through euthanasia. Individual clinical observations regarding general appearance, behavior, and overt toxicity were recorded on postnatal days 1, 4, 7, 11, 14, 17, and 21, and at weekly intervals thereafter until euthanasia.

Ten pups per sex per dose group were observed outside of the home cage on postnatal days 4, 11, 21, 35, 45, and 60.

2. BODY WEIGHTS

Maternal body weights were measured on gestation days 0, 3, 6, 9, 12, 15, and 20, and on lactation days 1, 4, 7, 10, 16, and 21.

Pup body weights were measured on postnatal days 1, 4, 7, 11, 14, 17, and 21, and at weekly intervals thereafter until euthanasia, and whenever pups were removed from their cages for behavioral testing.

3. FOOD CONSUMPTION

Maternal food consumption was measured on gestation days 0, 3, 6, 9, 12, 15, and 20, and on lactation days 1, 4, 7, 10, 16, and 21.

The determination of food consumption for the post-weaning pups is not specifically described in the study report.

4. PARTURITION

All females from each dose group were allowed to litter naturally and rear their young to post-natal day 21. During the period of expected parturition, the dams were observed three times daily for initiation and completion of parturition and for signs of dystocia. The day on which delivery was complete was designated lactation day 0. When parturition was judged complete, pups were sexed and examined for gross malformations, and the numbers of live and stillborn pups were recorded.

5. MACROSCOPIC OBSERVATIONS

Females that did not deliver with 25 days following mating were euthanized, and the thoracic and abdominal cavities were opened and examined. Uteri with no evidence of implantation were opened and placed in a 10% ammonium sulfide solution for the detection of implantation sites.

For females which delivered litters, on lactation day 21, each P0 female was euthanized and examined by gross necropsy. The number of former implantation sites was recorded.

6. LITTER PARAMETERS

Each litter was examined twice daily for survival, and all deaths were recorded. A daily record of litter size was maintained. Pups which were found dead or were sacrificed in extremis were examined externally and sexed, and the sex was confirmed by internal examination, and stomachs were examined for the presence of milk. A detailed gross necropsy was performed on any pup dying after postnatal day 4.

Eight pups per litter were randomly selected on postnatal day 4 to continue on study; wherever possible, the male:female ratios were to be held at 4:4, 5:3, or 3:5. The remaining pups were weighed, euthanized, and discarded.

Pups were individually sexed on postnatal days 0, 4 (prior to culling), 11, and 21.

7. POSTWEANING DEVELOPMENTAL LANDMARKS, SENSORY FUNCTION, AND NEUROBEHAVIORAL TESTING

7.1 BALANOPREPUTIAL SEPARATION

Each male pup was observed for balanopreputial separation beginning on postnatal day 35, and the day on which balanopreputial separation was first observed was recorded for each pup. Examination of the pups continued daily until balanopreputial separation was present. The body weight of each male was recorded on the day of acquisition of balanopreputial separation.

7.2 VAGINAL PATENCY

Each female pup was observed for vaginal patency beginning on postnatal day 25, and the day on which the vaginal lumen was first observed to open was recorded for each pup. Examination of the females was continued daily until vaginal patency was present. The body weight of each female was recorded on the day of acquisition of vaginal patency.

7.3 ACOUSTIC STARTLE RESPONSE

The acoustic startle response was assessed for ten rats per sex per dose group on postnatal days 20 and 60 using the SR-Lab Startle Response System. Acoustic startle response testing was performed in a room equipped with a white noise generation system set to operate at 70 decibels. Each test session consisted of a five-minute acclimation period with a 65-decibel broadband background white noise. The startle stimulus for each trial was a 115-db mixed-frequency noise burst stimulus of approximately 20 milliseconds in duration. Responses were recorded during the first 100 milliseconds following onset of the startle stimulus for each trial. Each test session consisted of 50 trials, with an eight-second intertrial interval. Startle response data were analyzed in five blocks of 10 trials each. Startle response measurements obtained were maximum response amplitude (V_{max}), average response amplitude (V_{ave}), and latency to Vmax (T_{max}).

7.4 LOCOMOTOR ACTIVITY

Motor activity observations were made on ten rats per sex per dose group on postnatal days 13, 17, 21, and 61, with the same animals monitored at each interval. Motor activity was measured automatically using the SDI Photobeam Activity System. The testing of treatment groups was conducted according to replicate sequence, and each animal was tested separately. Data were collected in 5-minute intervals over a test session duration of 60 minutes.

Data for ambulatory and total motor activity were tabulated. Total motor activity was defined as a combination of fine motor skills and ambulatory motor activity.

7.5 BIEL MAZE SWIMMING TRIALS

Swimming ability and learning and memory were assessed for 10 rats per sex per dose group using a water-filled eight-unit T maze and standard methodology. Animals were placed in the maze and were required to traverse the maze and escape by locating a platform hidden beneath the surface of the water. The amount of time required to traverse the maze and the number of errors were recorded, with an error defined as any instance in which an animal deviated from the correct channel with all four feet. The first testing interval was initiated for each animal on postnatal day 22. A second test interval using different animals was initiated on postnatal day 62. Each testing interval consisted of three phases conducted over seven consecutive days.

8. MACROSCOPIC EXAMINATIONS OF OFFSPRING

On postnatal day 11, one male or one female pup was removed from each litter, to a maximum of 10 pups per sex per dose group, and macroscopically examined for neuropathology following euthanasia and in situ perfusion. The brains were removed, including the olfactory bulbs, and weighed, and the length and width of the brain was recorded.
On postnatal day 28 or 29, all F1 offspring not selected for behavioral evaluations were euthanized and subjected to gross pathology evaluations.

Offspring scheduled for euthanasia on postnatal day 72, but not allocated for neuropathology or brain weight measurements, were euthanized and subjected to a gross pathology examination.

On postnatal day 72, one male or one female from each litter was randomly selected from those pups which had been used for assessment of locomotor activity, auditory startle, or learning and memory tests. The animals were euthanized and perfused in situ, and the central and peripheral nervous system and tissues were dissected and preserved. The brains including olfactory bulbs were removed and weighed, and the length and width recorded.

9. MICROSCOPIC EXAMINATIONS OF OFFSPRING

Of the pups perfused in situ on postnatal day 11 and 72, the brain tissues from ten randomly selected pups per sex per dose group and time point were prepared for microscopic evaluation, but only the control and high dose groups were evaluated. Sections from all major brain regions were prepared.

Table 5.7.1-1: Tissues prepared and examined by histopathology from pups after maternal dosing with isoxaflutole.

	Postnatal day 11	Postnatal day 72
Brain	X	X
Olfactory bulbs	X	X
Cerebral cortex	X	X
Hippocampus	X	X
Basal ganglion	X	X
Thalamus	X	X
Hypothalamus	X	X
Midbrain	X	X
Brainstem	X	X
Cerebellum	X	X
Spinal cord		X
At cervical swellings C3-C7		X
At lumber swellings T13-L4		X
Gasserian ganglion / trigeminal nerves		X
Lumbar dorsal root ganglion and fibers at T13-L4		X
Lumbar ventral root fibers at T13-L4		X
Cervical dorsal root ganglion and fibers at C3-C7		X
Cervical ventral root fibers at C3-C7		X
Sciatic nerves		X
Sural nerves		X
Tibial nerves		X
Peroneal nerves		X
Optic nerves		X
Eyes		X
Skeletal muscle (calf)		X

As evaluation of brains by light microscopy did not reveal any structural abnormalities, nor were there any clear functional differences between the control or treated groups, morphometric analyses were not conducted on brains from either PND 11 or PND 72 pups.

II. RESULTS AND DISCUSSION

A. MORTALITY AND CLINICAL SIGNS

There were no mortalities among the dams in any treatment groups. No treatment-related clinical signs were noted during the study.

B. BODY WEIGHTS

During gestation, mean body weights and body weight gains were statistically significantly decreased at 250 mg/kg bw/day compared to controls. During lactation, body weight gain at 250 mg/kg bw/day was statistically significantly increased compared to controls during the post-treatment period. Also at 250 mg/kg bw/day, body weight on lactation days 1, 4, 7, and 10 was statistically significantly reduced compared to controls.

Table 5.7.1-2: Maternal body weights and body weight gains during gestation and lactation in the developmental neurotoxicity study with isoxaflutole.

		RPA 201772, dose in mg/kg bw/day			
Phase	Day	0	5	25	250
Gestation	0	261	258	256	258
	3	279	278	271	276
	6	294	292	286	291
	9	305	304	297	292*
	12	323	321	314	303**
	15	341	334	332	320**
	20	411	408	403	392
	6-20	117	116	116	101**
	0-20	150	149	146	133*
Lactation	1	309	305	297	279**
	4	326	321	315	300**
	7	332	324	318	307**
	10	351	344	337	325**
	15	340	347	340	327
	21	345	341	336	333
	1-10	43	40	40	46
	10-21	-6	-3	-1	8**

Statistically significant at : * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

C. FOOD CONSUMPTION
At 250 mg/kg bw/day, food consumption was statistically significantly reduced compared to controls throughout gestation and the first four days of lactation.

D. PARTURITION
There was no effect of treatment on gestation length or on the incidence of dystocia in any treatment group. The incidence of dams which failed to deliver and were found to be non-gravid was not affected by administration of isoxaflutole.

E. MATERNAL MACROSCOPIC OBSERVATIONS
Two females in the control group, one at 25 mg/kg bw/day, and one at 250 mg/kg bw/day, did not litter and were sacrificed at 25 days after mating. These animals were found to be non-gravid, however this is considered not to be related to treatment.

At necropsy of the F0 females on lactation day 21, there were no macroscopic findings of note. The mean numbers of implantation sites, numbers of pups born, and numbers of unaccounted sites recorded at the scheduled necropsy did not differ between control and treated groups.

F. LITTER DATA, POSTNATAL SURVIVAL, AND GENERAL PUP GROWTH
The mean number of pups born, mean live litter size, and percentage of males per litter at birth were not affected by treatment at any dose.

At 250 mg/kg bw/day, pup survival in the interval of postnatal day (PND) 0 to postnatal day 1 was statistically significantly reduced compared to controls. For the interval of postnatal day 0 to postnatal day 4, there was a biologically significant (although statistically not significant) reduction in pup survival at 250 mg/kg bw/day.

Table 5.7.1-3: Pup survival, as % during each interval, in the developmental neurotoxicity study with RPA 201772

	RPA 201772, dose in mg/kg bw/day			
Day	0	5	25	250
0	99.0	98.5	98.2	98.9
0-1	98.3	97.8	100.0	93.2
1-4	98.9	99.	99.5	98.2
4-7	100.0	100.0	100.0	99.5
7-14	99.4	98.4	100.0	99.4
14-21	100.0	99.4	100.0	100.0

At 250 mg/kg bw/day, pup body weight gain was statistically significantly reduced compared to controls for the intervals of PND 1-4 and PND 4-7. Pup body weight at 250 mg/kg bw/day was statistically significantly reduced throughout the pre-weaning phase. Following weaning, body weight gains and body weights were slightly and occasionally decreased in a statistically significant manner compared to controls at 250 mg/kg bw/day; a greater effect was generally seen in male pups than in females.

Table 5.7.1-4: Pup body weight, in grams, in the developmental neurotoxicity study with RPA 201772

	RPA 20177, dose in mg/kg bw/day							
	Males				Females			
Day	0	5	25	250	0	5	25	250
1	7.0	7.1	7.0	6.4*	6.6	6.6	6.5	6.0*
4	9.8	9.9	9.8	8.7*	9.2	9.3	9.2	8.2*
7	15.4	15.4	15.4	13.5**	14.5	14.6	14.5	12.8**
11	23.8	24.3	23.4	21.0**	22.8	23.4	22.3	20.5*
21	53.2	54.4	51.1	49.0*	51.5	52.3	48.7	48.2
28	88.6	90.8	86.3	82.4**	82.0	83.6	79.9	77.6**
42	209.1	218.3	213.2	198.8	165.5	167.5	166.6	161.3
56	332.7	343.1	337.7	315.2*	217.5	218.6	222.0	215.0
72	426.2	439.6	435.3	408.9	258.8	259.9	265.2	257.2

Statistically significant at : * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

LITTER PARAMETERS – POSTWEANING DEVELOPMENTAL LANDMARKS, SENSORY FUNCTION, AND NEUROBEHAVIORAL TESTING

1. BALANOPREPUTIAL SEPARATION

All male pups were observed to have balanopreputial separation on or before PND 51. Although there was a slight increase in the day of acquisition at 250 mg/kg bw/day (PND 44.0) compared to control (PND 42.6), both values are below the laboratory's historical control data of PND 44.5 days. There was no effect on mean body weight at the time of acquisition.

Table 5.7.2-4: Day of attainment, and body weight on day of attainment, of balanopreputial separation in the developmental neurotoxicity study with RPA 201772

	RPA 201772, dose in mg/kg bw/day				
	0	5	25	250	HCD
Day	42.6	41.9	42.3	44.1*	44.5
Body wt, g	211.8	216.1	213.5	214.4	225.9

Statistically significant at : * p < 0.05; ** p < 0.01; *** p < 0.001

2. VAGINAL PATENCY

There was no effect of maternal administration of isoxaflutole on day of acquisition of vaginal patency, or on body weight at time of acquisition.

Table 5.7.2-5: Day of attainment, and body weight on day of attainment, of vaginal opening in the developmental neurotoxicity study with RPA 201772

	RPA 201772, dose in mg/kg bw/day				
	0	5	25	250	HCD
Day	33.2	33.6	33.2	33.2	33.2
Body wt, g	117.0	119.9	114.7	110.7	111.8

Statistically significant at : * p < 0.05; ** p < 0.01; *** p < 0.001

3. ACOUSTIC STARTLE RESPONSE

There were no treatment-related trends in any group on responses to the auditory startle test in terms of maximum response amplitude (V_{max}), latency to maximum response amplitude (T_{max}), or average response amplitude (V_{ave}). No changes in habituation were observed when the treated groups were compared to the control group. At 5 mg/kg bw/day on PND 60, there was a reduction in the T_{max} value but in the absence of a dose-response relationship this observation is considered not related to treatment.

Table 5.7.2-6: Acoustic startle response in pups on postnatal day 20 or 60 in the developmental neurotoxicity study with RPA 201772

		RPA 201772, dose in mg/kg bw/day			
		Males			
PND	Measure	0	5	25	250
20	V_{max}, mv	161.7	193.7	168.5	161.7
	T_{max}, ms	26.5	24.3	25.2	26.1
	V_{ave}, mv	35.4	44.4	37.7	37.3
60	V_{max}, mv	195.5	169.5	207.4	154.2
	T_{max}, ms	30.3	34.4	30.7	32.4
	V_{ave}, mv	42.7	36.7	42.3	32.8
		Females			
20	V_{max}, mv	198.3	173.7	204.1	171.1
	T_{max}, ms	23.2	23.2	22.7	23.3
	V_{ave}, mv	44.4	38.9	44.1	38.0
60	V_{max}, mv	106.8	171.7	86.2	112.4
	T_{max}, ms	33.6	28.6*	34.5	32.7
	V_{ave}, mv	21.5	35.1	18.6	22.4

4. LOCOMOTOR ACTIVITY

No treatment-related trends were apparent at any dose level when either total or ambulatory motor activity were evaluated, and no changes in habituation were apparent. On PND 17, increases in the total and ambulatory locomotor activity were noted at 250 mg/kg bw/day in both males and females. These observations were due to one pup per sex, and were not noted on PND 13, 21, and / or 61, and thus are considered not to be related to treatment.

Table 5.7.5-7: Pup locomotor activity during pre-weaning and post-weaning phases in the developmental neurotoxicity study with RPA 201772

		RPA 201772, dose in mg/kg bw/day			
		Males			
PND	Measure	0	5	25	250
13	Total	336	443	418	387
	Ambulatory	49	79	105	64
17	Total	425	324	489	860
	Ambulatory	146	98	162	395
21	Total	682	568	467	549
	Ambulatory	239	181	128	184
61	Total	1534	2070	1931	1909
	Ambulatory	411	604	587	607
Females					
13	Total	247	368	461	449
	Ambulatory	46	77	136	84
17	Total	190	294	509	876
	Ambulatory	38	88	184	376
21	Total	408	618	627	586
	Ambulatory	131	169	196	204
61	Total	1403	1781	1966	1695
	Ambulatory	509	646	755	657

5. BIEL MAZE SWIMMING TRIALS

Evaluation of data from the Biel maze trials showed no effects of treatment on swimming ability or motivation, and no effects on learning and memory ability on either PND 22 or 62. There was a slight, statistically significant increase in swimming ability on day 1 of the test procedure at 250 mg/kg bw/day in males only. In the absence of an effect in females, and in the absence of a relationship to dose, this observation was considered to be not related to treatment.

Table 5.7.2-8: Biel maze data for pups in the developmental neurotoxicity study with RPA 201772

			RPA 201772, dose in mg/kg bw/day				
			Males				
PND	Type	Trials	Measure	0	5	25	250
22	Biehl	1-10	Seconds	93.50	91.02	90.58	92.58
			Errors	16	16	16	18
	Probe	11-12	Seconds	62.63	60.44	53.34	45.82
			Errors	17	16	14	12
62	Biehl	1-10	Seconds	63.85	63.87	66.39	67.85
			Errors	11	12	11	13
	Probe	11-12	Seconds	38.77	55.76	60.23	49.04
			Errors	8	11	13	12
Females							
22	Biehl	1-10	Seconds	77.78	78.95	94.57	91.47
			Errors	13	15	17	18
	Probe	11-12	Seconds	74.11	49.60	78.16	56.10
			Errors	19	14	21	15
62	Biehl	1-10	Seconds	59.95	58.27	61.79	77.55
			Errors	10	10	10	15
	Probe	11-12	Seconds	45.05	55.15	47.74	32.83
			Errors	10	10	8	7

G. MACROSCOPIC EXAMINATIONS OF OFFSPRING
On PND 28 / 29, and on PND 72, there were no macroscopic findings which were considered to be related to treatment in any dose group or time point. In the examinations of pups selected for brain weight measurements on PND 11 or PND 72, there were no gross observations noted.

H. BRAIN WEIGHTS AND BRAIN MEASUREMENTS
At PND 11, terminal body weights and absolute brain weights were reduced in both males and females in a statistically significant manner compared to controls. As relative brain weights, and brain length and width measurements were no different between control and 250 mg/kg bw/day, however, the decreases in absolute brain weights at 250 mg/kg bw/day were considered not to be treatment-related. At 5 mg/kg bw/day, brain width was statistically significantly reduced compared to controls. However, in the absence of a dose-response relationship or any effect on brain weight, this observation is considered not to be treatment-related.

On PND 72, there was no treatment-related effect on either absolute or relative brain weight or on brain width or length. At 250 mg/kg bw/day in males, relative brain weight was statistically significantly increased, however this is related to decreased body weight in that group and is nota direct effect of treatment.

Table 5.7.2-9: Pup brain weight, length, and width in the developmental neurotoxicity study with RPA 201772

		RPA 201772, dose in mg/kg bw/day							
		Males				Females			
PND	Measure	0	5	25	250	0	5	25	250
11	Brain wt, g	1.18	1.16	1.10	1.05*	1,08	1.09	1.08	0.95*
	Brain wt, % body wt	4.70	4.79	4.64	4.89	4.83	4.67	4.91	5.23
	Length, mm	15.4	15.0	14.8	15.2	14.8	14.5	14.7	14.8
	Width, mm	12.7	12.2*	12.6	12.2	12.2	12.1	12.3	11.9
72	Brain wt, g	1.95	1.99	1.94	1.85	1.82	1.81	1.78	1.75
	Brain wt, % body wt	0.435	0.454	0.438	0.479*	0.723	0.715	0.729	0.687
	Length, mm	20.3	20.7	20.3	20.4	19.9	20.0	19.8	19.8
	Width, mm	14.7	15.1	15.1	14.6	14.6	14.5	14.2	14.3

Statistically significant at: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

I. QUALITATIVE HISTOPATHOLOGY AND BRAIN MORPHOMETRY

There were no treatment-related effects on any region of the brain or central or peripheral nervous systems in either PND 11 or PND 72 animals in any treatment group.

In the absence of histopathological effects in either group of offspring, brain morphometry measurements were not conducted.

III. CONCLUSION

In this study, maternal toxicity was exhibited at a dose level of 250 mg/kg bw/day by reductions in mean body weight gain and food consumption. No maternal toxicity was observed at 5 or 25 mg/kg bw/day. Maternal reproductive toxicity was not observed at any dose level. Thus, the maternal systemic NOAEL for this study is established at 25 mg/kg bw/day.

Developmental and / or neonatal toxicity was exhibited at a dose level of 250 mg/kg bw/day by a reduction in postnatal survival for the PND 0-1 interval, and reductions in mean offspring body weight gains during the pre-weaning and post-weaning periods; these effects on body weight gains occurred in the presence of maternal toxicity. The offspring systemic NOAEL is established at 25 mg/kg bw/day based on these findings.

The NOAEL for developmental neurotoxicity was established at 250 mg/kg bw/day in the absence of effects on any of the neurotoxicological parameters examined.

CA 5.7.2 Delayed polyneuropathy studies

Not relevant for this class of chemical substances.

CA 5.8 Other toxicological studies

CA 5.8.1 Toxicity studies of metabolites

The toxicity of the benzoic acid metabolite (RPA 203328) of isoxaflutole was assessed in a limited manner in the first EU review of isoxaflutole, however these data are summarized here for reference. Further information is available in the Baseline Dossier provided by Bayer CropScience and in the Monograph. A full summary of each of the studies conducted since that time is presented below.

Endpoint (Reference)	Species	Results	Comments
Acute oral toxicity ([redacted], 1995 M-170815-01-1)	Rat	LD50 > 5000 mg/kg bw	Category 5 / unclassified
14-day gavage study ([redacted], 1994 M-212732-01-1)	Rat	NOAEL = 30 mg/kg bw/day	LOAEL = 300 mg/kg bw/day
28-day dietary study ([redacted], 1995 M-170705-01-1)	Rat	NOAEL = 15000 ppm (1113.3 / 1268.7 mg/kg bw/day, M / F)	No toxicity observed
90-day dietary study ([redacted], 1998 M-240662-01-1)	Rat	NOAEL = 12000 ppm (769 / 952 mg/kg bw/day, M / F)	No toxicity observed
Bacterial mutagenicity ([redacted], 1994 M-170668-01-1)	*Salmonella typhimurium*	Negative	Cytotoxicity observed from 2500 µg/plate
Chromosomal aberrations ([redacted], 1998 M-157884-01-1)	Chinese hamster ovary cells	Negative	Slight reductions in mitotic index, no indications of cytotoxicity
Gene mutation ([redacted], 1998 M-189726-01-2)	Chinese hamster ovary cells	Negative	Cytotoxicity observed only in range-finding assay
Micronucleus in vivo ([redacted], 1998 M-211247-01-1)	Mouse	Negative	No micronuclei observed at up to 2000 mg/kg bw
Developmental toxicity ([redacted] 1999 M-189848-01-1)	Rat	Fetal NOAEL = 750 mg/kg bw/day, highest dose tested Maternal NOAEL = 75 mg/kg bw/day	No fetal effects observed; dams showed decreased body weight gain

Report:	[redacted] ;1994;M-212732-01
Title:	RPA 203328 - Exploratory 14-day toxicity study in the rat by gavage
Report No:	C027126
Document No:	M-212732-01-1
Guidelines:	**Deviation: not specified**
GLP/GEP:	**no**

Executive summary:
Male and female Sprague Dawley rats were administered RPA 203328 by oral gavage at doses of 0, 30, 100, 300, and 1000 mg/kg bw/day for a period of 14 days. Clinical signs, body weight, food consumption, hematology, and clinical chemistry were monitored during the study, organs were weighed at necropsy, and gross pathological examination was carried out.

The only treatment-related clinical sign was increased salivation from 300 mg/kg bw/day. Body weight and body weight gain were slightly decreased in males from 300 mg/kg bw/day, but with no effect in females. Increased red blood cell count, hemoglobin, and hematocrit were noted in males at the top doses, but not in females. Cholesterol concentration was decreased in females at 1000 mg/kg bw/day, but not in males.

There was no effect on organ weights in any dose group. Slight to moderate pale abnormal color of the liver was the only gross finding noted, and was seen in females of all treatment groups and in some males at 300 mg/kg bw/day.

The NOAEL for this study was established at 30 mg/kg bw/day.

I. MATERIALS AND METHODS

A. MATERIALS:

1. Test Material: RPA 203328
Description: white powder
Lot/Batch: BM 1262
Purity: 100%
CAS:
Stability of test compound: not stated in report

2. Vehicle and/or positive control: 0.5% methylcellulose in distilled water

3. Test animals:
Species: rat
Strain: Sprague-Dawley
Age: 42-49 days of age
Weight at dosing: 258-299g (males); 193-229g (females)
Source: [redacted], France
Acclimation period: 14 days
Diet: Certified Rodent Pellet diet A04C (U.A.R., Villemoisson-sur-Orge, Epinay-sur-Orge, France)
Water: tap water
Housing: individually in suspended stainless steel wire mesh cages

Environmental conditions –

Temperature:	22 ± 2°C
Humidity:	55 ± 15%
Air changes:	average 15 air changes per hour
Photoperiod:	12 hours light / 12 hours dark

B. STUDY DESIGN:

1. In life dates: 2 March – 16 March 1994

2. Animal assignment and treatment

On the day before treatment, animals were assigned permanent identification numbers within groups using a randomization procedure that ensured a similar body weight distribution among groups for each sex.

Groups of 5 male and 5 female rats were administered either vehicle (0.5% methylcellulose in water) or RPA 203328 by oral gavage for 14 days, at 0, 10, 100, 300, and 1000 mg/kg bw/day. The volume dosage for all animals was 5 ml/kg bw/day, based on the most recently recorded body weight.

3. Statistics

For body weight and food intake data, Dunnett's test was used to determine statistical significance of any observed changes. For clinical pathology and organ weights, variables were intercompared for the treated and control groups by use of Bartlett's test followed by either ANOVA and Dunnett's test or by a modified t-test.

A. METHODS:

1. Observations

Animals were inspected at least twice daily on weekdays and once daily on weekends and holidays for clinical signs, and any observations were recorded in terms of nature and severity, date and time of onset, and duration and progress of the observation. A detailed examination including palpation was conducted once per week. Cages and cage trays were inspected daily for signs of ill health.

2. Body weight

Body weights were measured on the first day of treatment and on a weekly basis through the study.

3. Food consumption

The amount of food provided and the amount remaining at the end of the week were recorded for each animal.

4. Clinical pathology

Ophthalmological examinations were conducted using an indirect ophthalmoscope after the instillation of an atropic agent. Examinations were conducted on both eyes of all animals prior to the start of treatment, and in the control and 1000 mg/kg bw/day groups during the second week of the study.

On study day 15, blood samples were taken from all surviving animals in all groups by puncture of the retro-orbital venous plexus. Animals were fasted overnight prior to bleeding, and were anesthetized by inhalation of ether. Blood was collected on EDTA (0.5ml) for hematology and

on lithium heparin (2.5ml) for plasma chemistry parameters.

5. Sacrifice and pathology

On study day 15, all surviving animals from all groups were sacrificed by exsanguination under deep anesthesia (pentobarbital, intraperitoneal injection of 50 or 60 mg/kg bw). Animals were fasted overnight prior to sacrifice. Necropsy included the examination of all major organs, tissues, and body cavities. Kidney, liver, spleen, ovary, and testis were weighed fresh, with paired organs weighed together. Kidney, liver, spleen, thyroid gland with parathyroid, ovary, and testis were fixed in 10% neutral buffered formalin for potential histological examination.

II. RESULTS AND DISCUSSION

A. OBSERVATIONS:

Mortality: No animals died during the study.

Clinical signs and ophthalmology: The only treatment-related clinical sign was increased salivation observed after dosing in animals at 300 and 1000 mg/kg bw/day. At 300 mg/kg bw/day, this was noted on occasion from day 9 in three females and on day 10 in all males. At 1000 mg/kg bw/day, increased salivation was noted from day 6 in all animals.

B. BODY WEIGHT AND BODY WEIGHT GAIN:

From 300 mg/kg bw/day in males, there was a slight but statistically non-significant decrease in body weight gain. There was no effect on body weight gain at 30 or 100 mg/kg bw/day in males, and no effect at any dose in females.

Table 5.8.1-1: Body weight and body weight gain in rats in a 14-day study with RPA 203328

	RPA 203328, dose in mg/kg bw/day				
	Males				
Week	**0**	**30**	**100**	**300**	**1000**
1	47.2	43.4	47.8	40.8	40.2
2	92.4	83.4	95.4	83.0	77.4
	Females				
1	13.8	15.6	19.4	17.6	19.2
2	29.6	38.6	31.2	31.6	30.4

C. FOOD CONSUMPTION AND COMPOUND INTAKE:

There was no effect on food consumption at any dose level in either males or females.

D. CLINICAL PATHOLOGY:

RPA 203328 induced higher mean red blood cell count in males at 300 and 1000 mg/kg bw/day. Also at 1000 mg/kg bw/day, in males, hemoglobin and hematocrit were also increased.

In females at 1000 mg/kg bw/day, cholesterol concentration was decreased, although the decrease was not statistically significant. Slight increases in aspartate aminotransferase and alanine aminotransferase activities were noted in one female at 1000 mg/kg bw/day.

Table 5.8.1-2: Treatment-related hematology and clinical chemistry parameters in rats in a 14-day study with RPA 203328

	RPA 203328, dose in mg/kg bw/day				
	Males				
	0	**30**	**100**	**300**	**1000**
Red cell count, 1012/L	7.66	8.25	7.86	8.47*	8.56*
Hemoglobin, g/100ml	14.68	15.30	15.05	15.16	16.16*
Hematocrit, %	44.62	46.92	45.95	46.58	49.06*
Total cholesterol, mmol/L	0.694	0.692	0.772	0.780	0.794
ASAT, IU/L	55.80	48.80*	51.20	52.00	47.00*
ALAT, IU/L	23.80	23.60	21.20	21.80	21.00
	Females				
Red cell count, 1012/L	8.44	8.16	8.67	8.35	8.33
Hemoglobin, g/100ml	15.58	15.25	16.00	15.36	15.40
Hematocrit, %	48.03	46.43	48.68	46.56	46.64
Total cholesterol, mmol/L	0.956	0.892	0.806	0.826	0.664
ASAT, IU/L	50.80	50.80	47.60	50.20	54.60
ALAT, IU/L	18.40	19.40	15.40	17.80	22.40

Statistically significant at : * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

E. SACRIFICE AND PATHOLOGY

There were no treatment-related effects on organ weights in either males or females at any dose.

At gross examination, slight to moderate pale abnormal color of the liver was noted in some females of all groups administered RPA 203328, and in males at 300 mg/kg bw/day only.

Table 5.8.1-3: Incidence of pale abnormal color of the liver in rats in a 14-day study with RPA 203328

	RPA 203328, dose in mg/kg bw/day				
	0	**30**	**100**	**300**	**1000**
Male	0	0	0	2	0
Female	0	1	3	2	3

III. CONCLUSION:

Based on increased salivation, slightly decreased body weight gain, and decreased red blood cell count, the NOAEL for this study could be established at 30 mg/kg bw/day.

Report:	[redacted]u; [redacted];1998;M-240662-01
Title:	RPA 203328: 90-Day Toxicity Study in the Rat by Dietary Administration
Report No:	B003642
Document No(s):	Report includes Trial Nos.: SA 98129 M-240662-01-1
Guidelines:	**OECD 408 (1981); EEC 92/69, Annex V, Method B26 (1992); EPA / FIFRA 82-1 (1984); MAFF in Japan 59 NohSan 4200 (1985);** **Deviation: not specified**
GLP/GEP:	**yes**

Executive summary:
Male and female Sprague Dawley rats were fed diets containing RPA 203328 at concentrations of 0, 1200, 4800, and 12000 ppm for 90 days. These concentrations provided doses of 0, 73.21, 306.1, and 768.9 mg/kg bw/day in males and 0, 93.10, 371.4, and 952.4 mg/kg bw/day in females. Clinical signs, body weights, and food consumption were monitored. Blood and urine were collected near or at the end of the study for hematological, clinicochemical, and urinalysis determinations. Ophthalmological examinations were conducted near the end of the study in the control and 12000 ppm animals. At the end of the study, selected organs were weighed and histopathological examinations were conducted.

There were no mortalities, clinical signs, or changes in body weight or body weight gain in either males or females. No effects were observed on hematological or clinicochemical parameters. From 4800 ppm, urine pH was increased in females, but in the absence of any other findings the toxicological significance of this finding is unclear. There were no effects of administration of RPA 203328 over 28 days on organ weights. At gross necropsy, dark or yellowish liver, marked lobular liver, and / or dark kidneys were noted in some animals. In the absence of histological changes, these were not considered to be related to treatment.

The NOAEL of this 90-day dietary study with RPA 203328 was 12000 ppm (768.9 mg/kg bw/day in males, 952.4 mg/kg bw/day in females).

III. MATERIALS AND METHODS

A. MATERIALS:

1. Test Material: RPA 203328
Description: white powder with small aggregates
Lot/Batch: NMI874
Purity: 99%
CAS:
Stability of test compound: 1 week at ambient temperature in diet

2. Vehicle and /or positive control: none

3. Test animals:
Species: rat
Strain: Sprague-Dawley
Age: approximately 7 weeks
Weight at dosing: 219-249g (males); 159-189g (females)
Source: [redacted], France

Acclimation period:	7 days
Diet:	Certified Rodent Pellet diet A04C (U.A.R., Villemoisson-sur-Orge, France)
Water:	tap water
Housing:	individually in suspended stainless steel wire mesh cages
Environmental conditions –	
Temperature:	20-24°C
Humidity:	40-70%
Air changes:	average 10 to 15 air changes per hour
Photoperiod:	12 hours light, 12 hours dark

B. STUDY DESIGN:

1. In life dates: 25 March – 25 June 1998

2. Animal assignment and treatment
On the day before treatment, animals were assigned permanent identification numbers within groups using a randomization procedure that ensured a similar body weight distribution among groups for each sex. Groups of 10 male and 10 female rats were fed diets containing RPA 203328 at constant concentrations of 0, 1200, 4800, and 12000 ppm for 90 days.

3. Diet preparation and analysis
The test substance was incorporated into the diet by dry mixing to provide the required dietary concentrations. The test substance was ground into a fine powder before being incorporated into the diet by dry mixing. The four preparations of the test substance formulations were prepared approximately every three weeks, and diets were stored below -15C when not in use.

Diet samples were taken from the highest and lowest concentrations and frozen for four weeks, then thawed, held at room temperature for a week, and finally analyzed to verify the stability of the test substance in the conditions of utilization.

The homogeneity of RPA 203328 was verified at the first preparation for the lowest and highest concentrations to demonstrate adequate formulation procedures. The dietary levels of the test substance were verified for each concentration at the first and last preparations. In addition, samples of diet preparation at each concentration were frozen for possible future analysis.

3. Statistics
For body weight and body weight gain, food consumption, clinical pathology parameters, and absolute and relative organ weights, Bartlett's test was followed by either ANOVA and Dunnett's test, or by a combination of the Kruskal-Wallis one-way analysis of variance and the Mann-Whitney test.

C. METHODS:

2. Observations
Animals were inspected at least twice daily on weekdays and once daily on weekends and holidays for clinical signs, and any observations were recorded in terms of nature and severity, date and time of onset, and duration and progress of the observation. A detailed examination including palpation was conducted once per week. Cages and cage trays were inspected daily for signs of ill health.

2. Body weight

Body weights were measured on the first day of treatment and on a weekly basis through the study.

3. Food consumption

The amount of food provided and the amount remaining at the end of the week were recorded for each animal.

4. Clinical pathology

Ophthalmological examinations were conducted using an indirect ophthalmoscope after the instillation of an atropic agent. Examinations were conducted on both eyes of all animals prior to the start of treatment, and in the control and 12000 ppm groups during week 12 of the study.

On study day 86 or 87, blood samples were taken from all surviving animals in all groups by puncture of the retro-orbital venous plexus. An approximately equal number of animals randomly selected in all groups were sampled on each day. Animals were fasted overnight prior to bleeding, and were anesthetized by inhalation of isoflurane. Blood was collected on EDTA (0.5ml) for hematology, on lithium heparin (2.5ml) for plasma chemistry parameters, and on sodium citrate (0.9 ml) for coagulation parameters. In addition, a blood sample was collected on the first 5 animals per group per sex, into tubes containing heparin just before necropsy from the abdominal aorta. Plasma samples were then kept frozen at -80C for possible future analysis.

On study days 91, 92, or 93, in the morning, prior to sacrifice, overnight urine samples were collected from all surviving animals in all groups. An approximately equal number of animals randomly selected in all groups were sampled on each day. Feed and water were not accessible during urine collection. After centrifugation of the urine, the remaining supernatant was kept frozen at -80C for possible future analysis.

5. Neurotoxicity assessment

During the acclimatization phase and during week 12 of the study, the grasping, righting, corneal, pupillary, auditory startle, and head-shaking reflexes were tested according to established methods.

5. Sacrifice and pathology

On study day 91, 92, or 93, all surviving animals from all groups were sacrificed by exsanguination under deep anesthesia (pentobarbital, intraperitoneal injection of 50 or 60 mg/kg bw). Animals were fasted overnight prior to sacrifice. Necropsy included the examination of all major organs, tissues, and body cavities.

Table of organs weighed, fixed, and / or examined by histopathology

Organ / Tissue	Weighed	Fixed	Exam.	Organ / Tissue	Weighed	Fixed	Exam.
Adrenals	x	x	x	Pancreas		x	x
Aorta		x	x	Pituitary	x	x	x
Brain	x	x	x	Prostate	x	x	x
Caecum		x	x	Rectum		x	x
Colon		x	x	Salivary gland – submandibular		x	x
Duodenum		x	x	Sciatic nerves		x	x
Epididymides	x	x	x	Seminal vesicles	x	x	x
Eyes and optic nerves		x	x	Skeletal muscle: thigh		x	x
Femoral bone and articular surface		x	x	Skin		x	x
Harderian glands		x	x	Spinal cord		x	x
Heart	x	x	x	Spleen	x	x	x
Ileum		x	x	Sternum and marrow		x	x
Jejunum		x	x	Stomach – keratinized		x	x
Kidneys	x	x	x	Testes	x	x	x
Liver	x	x	x	Thymus	x	x	x
Lungs with mainstem bronchi		x	x	Thyroid with parathyroids	x1	x	x
Lymph nodes – submaxillary		x	x	Tongue		x	x
Lymph nodes – mesenteric		x	x	Trachea		x	x
Mammary gland		x	x	Urinary bladder		x	x
Oesophagus		x	x	Uterus with cervix	x	x	x
Ovaries	x	x	x	Vagina		x	x

Tissues noted in the table above were examined in all animals of the control and 12000 ppm groups. Additionally, sections of the liver, kidneys, and lung from all animals of all intermediate dose groups were examined.

IV. RESULTS AND DISCUSSION

D. MORTALITIES
There were no mortalities in any group during the study.

E. CLINICAL SIGNS
There were no treatment-related clinical signs.

F. BODY WEIGHT AND BODY WEIGHT GAIN:
There was no effect on body weight or body weight gain at any dose of RPA 203328.

G. FOOD CONSUMPTION AND COMPOUND INTAKE:
There was no effect on food consumption in any group.

Table 5.8.1-4: Consumption of RPA 203328 added to the diet in rats in a 90-day study with RPA 203328

	RPA 203328, dietary concentration in ppm		
	1200	**4800**	**12000**
Males	73.21	306.1	768.9
Females	93.10	371.4	952.4

H. OPHTHALMOLOGICAL INVESTIGATIONS
There were no treatment-related observations at ophthalmological investigation.

I. CLINICAL PATHOLOGY:
There were no effects of dietary administration of RPA 203328 on either hematological or clinicochemical parameters.

From 4800 ppm, only in females, urine pH was statistically significantly decreased. However, in the absence of any histopathological or other correlates, these changes are considered not to be toxicologically significant.

J. SACRIFICE AND PATHOLOGY
There were no treatment-related effects on organ weights. Dark or yellowish liver color, a marked lobular liver pattern, and / or dark kidneys were observed in a small number of treated rats. In view of the low incidence, the lack of an obvious dose relationship, and the lack of corroborative histological changes, they were considered not to be related to dietary administration of RPA 203328.

There were no treatment-related histopathological findings in either males or females.

Table 5.8.1-5: Treatment-related macroscopic findings in rats in a 90-day study with RPA 203328

	RPA 203328, dietary concentration in ppm							
	Males				**Females**			
Observation	**0**	**1200**	**4800**	**12000**	**0**	**1200**	**4800**	**12000**
Liver								
Dark	0	2	2	2	0	0	0	0
Yellowish	0	0	0	0	0	0	1	1
Marked lobular pattern	0	2	1	2	0	0	0	1
Kidney								
Dark	0	0	0	0	0	0	1	1

IV. CONCLUSION:

The dietary administration of RPA 203328 at up to 12000 ppm, over a period of 90 days, did not induce any gross, microscopic, or organ weight changes, nor were there any biologically significant effects on ophthalmology, clinicochemical parameters, or behavior. The NOAEL in this study was therefore considered to be 12000 ppm (768.9 mg/kg bw/day in males, 952.4 mg/kg bw/day in females).

Report:	[redacted]$;[redacted];1998;M-157884-01
Title:	Mutagenicity test on RPA203328 - Measuring chromosomal aberrations in Chinese hamster ovary (CHO) cells
Report No:	R000093
Document No:	M-157884-01-1
Guidelines:	**USEPA (=EPA): FIFRA 84-2;** **Deviation: not specified**
GLP/GEP:	**yes**

Executive summary:
In this *in vitro* assessment of the clastogenic potential of RPA 203328, Chinese hamster ovary cells were exposed to RPA 203328 at concentrations up to 2700 ug/ml, diluted in DMSO, for up to 17.8 hours.

Slight reductions in the mitotic index were observed in some of the assays, but there were no visual indications of cytotoxicity.

None of the cultures treated with RPA 203328 either in the presence or in the absence of S9 mix showed biologically relevant increased numbers of aberrations.

The positive controls mitomycin C and cyclophosphamide induced clastogenic effects and demonstrated the sensitivity of the test system and the activity of the used S9 mix.

RPA 203328 was considered not to be clastogenic for mammalian cells *in vitro*.

I. MATERIALS AND METHODS

A. MATERIALS:

1. Test Material: RPA 203328
Description: white powder
Lot/Batch: NMI874
Purity: 99.0%
CAS:
Stability of test compound: not stated in report

2. Control materials: **Negative:** dimethylsulfoxide
Solvent: dimethylsulfoxide
Positive: cyclophosphamide, mitomycin C

3. Test organisms:
Cell line: Chinese Hamster ovary cells, CHO-WBL
Source: cell line maintained at performing laboratory, originally obtained from [redacted] San Francisco

4. Test compound concentrations: initial chromosomal aberrations assay: 0, 18.3, 26.2, 37.4, 53.4, 76.3, 109, 156, 223, 319, 456, 652, 931, 1330, 1900, and 2710 ug/ml; confirmatory chromosomal aberrations study without metabolic activation: 0, 317, 453, 647, 924, 1320, 1890,

and 2700 ug/ml; confirmatory chromosomal aberrations study with metabolic activation: 0, 647, 924, 1320, 1890, and 2700 ug/ml.

B. STUDY DESIGN AND METHODS:

1. Treatment protocol:

Aberration assay without metabolic activation: Cultures were initiated by seeding approximately 1.2 x 106 cells per 75 cm2 flask into 10 ml of complete McCoy's 5a medium. One day after culture initiation, the cells were incubated at approximately 37C with the test article at predetermined concentrations for approximately 3 hours for the initial assay, or 17.8 hours in the confirmatory assay. The cultures were then washed with buffered saline. In the initial assay, the cells were then refed with complete McCoys' 5a medium and incubated for the rest of the culture period up to the time of harvest with 0.1 ug/ml Colcemid present during the last 2.0 hours of incubation. In the confirmatory assay, cells were refed with complete McCoy's 5a medium with 0.1 ug/ml Colcemid and harvested 2.0 hours later.

Aberration assay with metabolic activation: Cultures were initiated by seeding approximately 1.2 x 106 cells per 75 cm2 flask into 10 ml of complete McCoy's 5a medium. One day after culture initiation, the cells were incubated at approximately 37C with the test article at predetermined concentrations for approximately 3 hours in the presence of the test article and the S9 reaction mixture in McCoy's 5a medium without FBS. After the three hour exposure period, the cells were washed twice with buffered saline and then refed with complete McCoy's 5a medium. The cells were incubated for the rest of the culture period up to the time of harvest with 0.1 ug/ml colcemid present during the last two hours of incubation.

2. Harvest procedures:

Prior to the harvest of the cultures, visual observations of toxicity were made. These observations included an assessment of the percent confluence of the cell monolayer within the culture flasks. The cultures were also evaluated for the presence of mitotic or dead cells floating in the medium. The cultures were then trypsinized to collect mitotic and interphase cells and were treated with 0.075M KCl hypotonic solution. The cultures were then fixed with absolute methanol: glacial acetic acid before slide preparation.

Slides were prepared by dropping the harvested cultures on clean slides which were then stained with 5% Giemsa solution.

3. Analysis of aberrations

Cells were selected for good morphology and only cells with the number of centromeres equal to the modal number 21 ± 2 were analyzed. One hundred cells, if possible, from each replicate culture at four concentrations of the test article, and from the negative, solvent, and one dose of the positive control cultures were analyzed for the different types of chromosomal aberrations. At least 25 cells were analyzed from those cultures which had greater than 25% of cells with one or more aberrations. Mitotic index was evaluated from the negative control, vehicle control, and a range of concentrations by analyzing the number of mitotic cells in 1000 cells and expressing the ratio as a percentage of mitotic cells. Percent polyploidy and endoreduplication were also analyzed by evaluating 100 metaphases, if available.

4. Assessment criteria:

An assay was considered acceptable for evaluation of test results only if:

- The negative and vehicle control cultures must contain fewer than approximately 5% cells with aberrations;
- The positive control must be significantly ($p < 0.01$) higher than the vehicle controls; and

- If the aberration results are negative and there is no significant reduction (approximately ≥ 50%) in confluence or mitotic index, the assay must include the highest applicable dose (target dose of 10 mM or 5 mg/ml, whichever is lower) or a dose exceeding the solubility limit of the test substance in the culture medium.

A test article was considered positive for inducing chromosomal aberrations if a significant increase ($p < 0.01$) in the number of cells with chromosomal aberrations is observed at one or more concentrations. A test article was considered negative for inducing chromosomal aberrations if no significant increase in the number of cells with chromosomal aberrations was observed at any of the concentrations tested.

i. Statistics:

Statistical analysis employed a Cochran-Armitage test for linear trend and Fisher's Exact Test to compare the percentage of cells with aberrations in treated cells to the results obtained for the vehicle controls.

II. RESULTS AND DISCUSSION

A. Chromosomal aberrations assay without metabolic activation

In the initial assay, no visual signs of toxicity were observed in any of the test cultures. Reductions of 2% and 22% were observed in the mitotic indices of the cultures treated with 931 and 1900 ug/ml, respectively as compared with the solvent control cultures. Chromosomal aberrations were analyzed from the cultures treated with 931, 1330, 1900, and 2710 ug/ml. No significant increases in cells with chromosomal aberrations, polyploidy, or endoreduplication were observed at the concentrations analyzed.

In the confirmatory assay, no visual signs of toxicity were observed in any of the test cultures. A reduction of 21% was observed in the mitotic index of the cultures treated with 2700 ug/ml as compared with the solvent control cultures. Chromosomal aberrations were analyzed from the cultures treated with 924, 1320, 1890, and 2700 ug/ml. No significant increases in cells with chromosomal aberrations, polyploidy, or endoreduplication were observed at the concentrations analyzed.

The sensitivity of the cell cultures for induction of chromosomal aberrations is shown by the increasing frequency of aberrations in the cells exposed to mitomycin C, the positive control agent. The test article is considered negative for inducing chromosomal aberrations, polyploidy, and endoreduplication under nonactivation conditions.

B. Chromosomal aberrations assay with metabolic activation:

In the initial assay, no visual signs of toxicity were observed in any of the test cultures. No reductions were observed in the mitotic indices of the culture analyzed as compared with the solvent control cultures. Chromosomal aberrations were analyzed from the cultures treated with 931, 1330, 1900, and 2710 ug/ml. No significant increases in cells with chromosomal aberrations, polyploidy, or endoreduplication were observed at the concentrations analyzed, except for a weak increase in endoreduplication at 1330 ug/ml. This is a statistical anomaly due to the solvent control cultures with 0.5% endoreduplication; the historical control data range for solvent controls is 0-9%, and thus the value observed at 1330 ug/ml of 6.0% is well within the historical control data. In addition, the two higher concentrations analyzed did not show any increase in endoreduplication, and this increase was not observed at any of the concentrations analyzed in the confirmatory assay. Thus the increase observed in this assay is not deemed relevant.

In the confirmatory assay, no visual signs of toxicity were observed in any of the test cultures. A reduction of 8% was observed in the mitotic index of the cultures treated with 924 ug/ml as compared with the solvent control cultures. Chromosomal aberrations were analyzed from the cultures treated with 924, 1320, 1890, and 2700 ug/ml. No significant increases in cells with chromosomal aberrations, polyploidy, or endoreduplication were observed at the concentrations analyzed.

The successful activation by the metabolic system is illustrated by the increased incidence of cells with chromosomal aberrations in the cultures induced with cyclophosphamide, the positive control agent. The test article is considered negative for inducing chromosomal aberrations, polyploidy, and endoreduplication under activation conditions.

III CONCLUSION

RPA 203328 was considered negative for inducing chromosome aberrations in CHO cells, either with or without metabolic activation.

Report:	[redacted]n; [redacted];1998;M-189726-01
Title:	Mutagenicity test on RPA203328 in the CHO/HGPRT forward mutation assay with duplicate cultures and a confirmatory assay
Report No:	M-189726-01-2
Document No:	M-189726-01-2
Guidelines:	**USEPA (=EPA): FIFRA, 84-2;** **Deviations: not specified**
GLP/GEP:	**yes**

Executive summary:
Chinese hamster ovary cells were incubated for four hours, in either the presence or the absence of S9 metabolic activating mix, with RPA 203328, then washed and cultured for an appropriate expression time and further for colony development, then cultures were evaluated for colony development after selection with 6-thioguanidine.

Some cytotoxicity was observed in the range-finding assay, but not in either of the mutation assays. RPA 203328 was tested at a top concentration of 2700 ug/ml, equivalent to the testing limit of 10 mM for this assay.

There was no consistent, dose-related increase in the incidence of mutant colonies in any of the assays. RPA 203328 was thus considered negative for inducing forward mutations at the HGPRT locus in CHO cells.

I. MATERIALS AND METHODS

A. MATERIALS:

1. Test Material: RPA 203328
Description: white powder
Lot/Batch: MBI874
Purity: 99.0%
CAS:
Stability of test compound: not stated in report

2. Control materials: **Negative:** DMSO
Solvent: DMSO
Positive: 5-Bromo-2'-deoxyuridine (BrdU) and 20-methylcholanthrene (MCA)

3. Test organisms:
Cell line: hypodiploid CHO cell line, clone CHO-K1-BH4
Source: established at performing laboratory, originally obtained from [redacted], Tennessee

4. Test compound concentrations: Preliminary cytotoxicity study: 5.30 to 2700ug/ml; main study without metabolic activation: 84.5, 169, 338, 675, 1350, and 2700 ug/ml; main study with metabolic activation, 338, 675, 1350, 1600, 1800 or 1900, 2000, 2300, 2500, and 2700 ug/ml.

B. STUDY DESIGN AND METHODS:

1. Determination of cytotoxicity:
For the preliminary cytotoxicity study, THMG medium cleansed cells were plated at 2 x 106 to 3 x 106 cells per 75cm2 tissue culture flask on the day before initiation of treatment. At the time of dosing, cell cultures were treated with the test or control article for about four hours at 37C in a humidified atmosphere with about 5% CO_2. At the end of the treatment period, the cells were trypsinized and replated in triplicate dishes at approximately 200 cells per dish in 60mm dishes. The cells were then incubated for seven days for colony development. Colonies were fixed in methanol, stained with Giemsa, and counted manually, excluding those with approximately 50 cells or fewer. Cytotoxicity is expressed as a percentage of mean colony counts in each concentration as compared to the vehicle controls.

2. Treatment protocol without metabolic activation:
THMG medium cleansed cells were plated at 2 x 106 to 3 x 106 cells per 75cm2 tissue culture flask on the day before initiation of treatment. At the time of dosing, cell cultures were treated with the test or control article for about four hours at 37C in a humidified atmosphere with about 5% CO_2. After treatment, the cells were washed with phosphate-buffered saline, trypsinized, and suspended in medium. Cell suspensions from each concentration were counted by hemacytometer or Coulter Counter and replated at about 1.5 x 106 cells into each of two 150mm dishes and approximately 200 cells into each of three 60mm dishes. The 60mm dishes were incubated for seven days for colony development and determination of the cytotoxicity associated with each concentration. The large dishes were incubated for seven days to permit growth and expression of induced mutants. The large dishes were subcultured every two to three days to maintain logarithmic growth conditions. At each subculture, the cells from the two 150mm dishes of each concentration were trypsinized, combined, counted, and reseeded at approximately 1.5 x 106 cells into each of two 150mm dishes.

Each assay was initiated with vehicle controls in duplicate, a single positive control, and six different test article conditions using two cultures per test article concentration.

At the end of the 7-day phenotypic expression period, each culture was reseeded at approximately 2 x 105 cells per 100mm dish (12 dishes total) in mutant selection medium. Also, three 60mm dishes were seeded at approximately 200 cells per dish in normal culture medium to determine the cloning efficiency of each culture. Cells were incubated for 7 to 10 days at 37C in a humidified incubator with about 5% CO_2.

After incubation of the cells for 7-10 days to allow for colony development, the colonies were fixed with methanol, stained with Giemsa, and counted to determine the number of TG-resistant colonies in the mutant colony dishes. The colonies were counted manually, excluding those with approximately 50 cells or fewer. The mutant frequency is expressed as the number of mutants per 106 clonable cells. The number of clonable cells was determined from the number of cells plated, with adjustments for the absolute cloning efficiency at the time of selection.

A confirmatory assay was performed without metabolic activation after completion and analysis of the results of the first mutation assay.

3. Treatment protocol with metabolic activation:
Two assays were performed with metabolic activation, each with its own set of negative and positive controls. THMG medium cleansed cells were plated at 2 x 106 to 3 x 106 cells per

75cm2 tissue culture flask on the day before initiation of treatment. At the time of dosing, cell cultures were treated with the test or control article for about four hours at 37C in a humidified atmosphere with about 5% CO_2, and S9 homogenate was added to a final concentration in the cultures of 10 ul/ml; necessary cofactors were also added. After treatment, the cells were washed with phosphate-buffered saline, trypsinized, and suspended in medium. Cell suspensions from each concentration were counted by hemacytometer or Coulter Counter and replated at about 1.5 x 106 cells into each of two 150mm dishes and approximately 200 cells into each of three 60mm dishes. The 60mm dishes were incubated for seven days for colony development and determination of the cytotoxicity associated with each concentration. The large dishes were incubated for seven days to permit growth and expression of induced mutants. The large dishes were subcultured every two to three days to maintain logarithmic growth conditions. At each subculture, the cells from the two 150mm dishes of each concentration were trypsinized, combined, counted, and reseeded at approximately 1.5 x 106 cells into each of two 150mm dishes.

Each assay was initiated with vehicle controls in duplicate, a single positive control, and six different test article conditions using two cultures per test article concentration.

At the end of the 7-day phenotypic expression period, each culture was reseeded at approximately 2 x 105 cells per 100mm dish (12 dishes total) in mutant selection medium. Also, three 60mm dishes were seeded at approximately 200 cells per dish in normal culture medium to determine the cloning efficiency of each culture. Cells were incubated for 7 to 10 days at 37C in a humidified incubator with about 5% CO_2.

After incubation of the cells for 7-10 days to allow for colony development, the colonies were fixed with methanol, stained with Giemsa, and counted to determine the number of TG-resistant colonies in the mutant colony dishes. The colonies were counted manually, excluding those with approximately 50 cells or fewer. The mutant frequency is expressed as the number of mutants per 106 clonable cells. The number of clonable cells was determined from the number of cells plated, with adjustments for the absolute cloning efficiency at the time of selection.

4. Parameters assessed:
In order to determine cytotoxicity, plating efficiency was assessed in both the preliminary cytotoxicity study, and in each of the main mutation assays. The frequency of colonies resistant to 6-thioguanine was assessed as a measure of mutagenicity of the test compound.

5. Acceptance criteria:
An assay was considered acceptable for evaluation of test results only if:

- The average absolute cloning efficiency of negative controls was between 50% and 115%;
- The background mutant frequency was between 0 and 15 x 10-6;
- The positive control mutant frequency is significantly elevated over the concurrent negative control ($p \leq 0.01$);
- The greatest concentration of the test agent either reduces the clonal survival in an appreciable manner, or is the lowest insoluble concentration of the test article in the culture medium, or is either 5 mg/ml, 5 ul/ml, or 0.01M; and
- A minimum of four concentrations of the test article are available for determination of mutant frequencies.

6. Assessment criteria:
For an assay to be considered positive,

- The mutant frequency must meet or exceed 15 x 106 and should be statistically significantly different from those of negative controls; and
- There should be a concentration- or dose-related increase in mutant frequency which should be observed in both the initial and confirmatory assay.

7. Statistical analysis:
Statistical significance of the results was determined using the Fischer Exact Test to determine if the mutant frequencies in each treated culture were significantly elevated compared to the mutant frequencies of the concurrent negative controls.

II. RESULTS AND DISCUSSION

1. Cytotoxicity:
In the dose-rangefinding assay without metabolic activation, the test article was noncytotoxic at all concentrations of up to 2700 ug/ml. In the presence of metabolic activation, RPA 203328 was noncytotoxic from 5.30 to 1350 ug/ml, and was lethal at 2700 ug/ml. A top concentration of 2700 ug/ml was chosen for both assays as this concentration was equivalent to 10 mM, the testing limit for this assay.

Table 5.8.1-6: Cytotoxicity data for culture of RPA 203328 in the presence of S9 with CHO cells in a mammalian mutagenesis assay

Applied concentration, ug/ml	Average count	Relative survival (%)
0	157	100
5.30	172	109.6
10.6	176	112.1
21.2	156	99.4
42.3	155	98.7
84.5	142	90.4
169	165	105.1
338	143	91.1
675	165	105.1
1350	165	105.1
2700	0	0.0

2. Mutation assay without metabolic activation:
Two trials of the mutation assay without metabolic activation were performed, using concentrations of 84.5, 169, 338, 675, 1350, and 2700 ug/ml. These concentrations did not induce any cytotoxicity. None of the assayed cultures in either trial induced mutant frequencies that were significantly elevated over the vehicle control cultures.

Table 5.8.1-7: Cell survival and mutant frequency after culture with RPA 203328 in the absence of S9 with CHO cells in a mammalian mutagenesis assay.

Treatment	Dose, ug/ml	Test	Survival, % vehicle control	Total mutant colonies	Mutant frequency x 10-6 units
Vehicle	---	1	95.1	24	8.0
		2	113.8	10	4.2
		1	104.9	15	5.3
		2	86.2	13	5.9
BrdU	50	1	69.0	277	108.4*
		2	96.8	210	103.8*
		1	81.4	461	150.9*
		2	98.8	229	124.2*
RPA 203328	84.5	1	101.7	5	1.8
		1	100.0	8	3.8
		2	118.7	7	3.5
		2	129.5	1	0.5
	169	1	106.1	15	5.8
		1	104.7	18	7.2
		2	114.7	6	2.7
		2	129.8	3	1.3
	338	1	107.8	15	5.9
		1	101.3	7	2.6
		2	109.6	13	6.0
		2	135.2	0	0.0
	675	1	97.5	9	3.4
		1	94.5	9	3.4
		2	111.8	3	2.4
		2	102.9	6	2.4
	1350	1	107.8	00	3.6
		1	82.5	26	9.7
		2	100.5	2	1.2
		2	110.6	3	1.4
	2700	1	103.8	12	6.0
		1	92.6	16	6.6
		2	96.0	14	7.1
		2	109.9	6	2.8

3. Mutation assay with metabolic activation:

Two mutation assays were initiated with the test article with metabolic activation. The first trial was conducted using concentrations of 338, 675, 1350, 1600, 1900, 2300, and 2700 ug/ml. Unlike the dose-rangefinding assay, no cytotoxicity was observed and all seven concentrations were analyzed for mutant induction. Sporadic increases were observed, however none of the increases reached the threshold frequency of 15 x 106, which is required for a positive response. A confirmatory trial was performed.

In the second trial, concentrations of 169, 338, 675, 1350, 1600, 1800, 2000, 2300, 2500, and 2700 ug/ml were initiated. The treatments of 169 and 338 ug/ml were not analyzed for mutation frequency. No cytotoxicity was observed at up to the testing limit of 2700 ug/ml. Two significant

increases in single cultures at 2300 and 2500 ug/ml were observed, but replicates at the same concentrations were not positive and no dose-related response were observed. Additionally, mutant frequencies at all doses were within historical control values.

Table 5.8.1-8: Cell survival and mutant frequency after culture with RPA 203328 in the presence of S9 with CHO cells in a mammalian mutagenesis assay.

Treatment	Dose, ug/ml	Test	Survival, % vehicle control	Total mutant colonies	Mutant frequency x 10-6 units
Vehicle	---	1	115.1	8	4.5
		2	81.5	20	6.9
		1	84.9	4	2.3
		2	112.6	17	7.0
MCA	5	1	95.5	218	132.0*
		2	95.2	195	119.1*
		1	83.0	243	80.9*
		2	86.0	198	85.8*
RPA 203328	338	1	126.8	8	4.8
		1	138.3	4	2.2
	675	1	131.7	15	9.4a
		1	121.3	13	6.4a
		2	105.2	20	8.7
		2	112.7	24	9.3
	1350	1	133.7	4	2.0
		1	114.6	17	8.6a
		2	117.7	31	11.6
		2	159.0	12	5.2
	1600	1	115.3	15	7.3a
		1	64.6	18	8.9a
		2	167.3	30	10.9
		2	142.6	17	7.7
	1800	2	145.7	29	9.8
		2	156.9	9	3.1
	1900	1	105.5	5	2.6
		1	101.3	1	0.6
	2000	2	119.8	21	6.9
		2	115.7	29	10.5
	2300	1	89.8	18	10.6b
		1	123.7	8	4.6
		2	139.6	40	15.8*
		2	176.3	17	6.8
	2500	2	143.4	30	13.3b
		2	112.3	23	10.2
	2700	1	114.6	5	2.8
		1	96.6	5	3.0
		2	129.2	18	7.2
		2	158.2	30	11.1

* statistically significant at $p < 0.01$ and mutant frequency > 15 x 10-6 units
a significant increase compared to one vehicle control but not both; biologically not significant
b statisitically significant at $p < 0.05$ but mutant frequency < 15 x 10-6

III. CONCLUSION

The test article RPA 203328 was evaluated as negative for inducing forward mutations at the HGPRT locus in CHO cells with and without metabolic activation.

Report:	[redacted] §; [redacted] ;1998;M-211247-01
Title:	Mutagenicity test on RPA 203328 in the in vivo mouse micronucleus assay
Report No:	C026351
Document No(s):	Report includes Trial Nos.: 19201 M-211247-01-1
Guidelines:	**USEPA (=EPA): 84-2;** **Deviation: Not specified**
GLP/GEP:	**yes**

Executive summary:
RPA 203328 was administered by oral gavage to male mice at doses of 0, 500, 1000, or 2000 mg/kg bw on one occasion, and mice were sacrificed at either 24 hours (all concentrations) or 48 hours (control 2000 mg/kg bw) for collection of bone marrow and quantification of micronucleus induction.

The polychromatic:normochromatic erythrocyte ratio (PCE:NCE) was determined, and the micronucleus freuqency was determined by analyzing at least 2000 polychromatic erythrocytes per animal.

There were no clinical signs or indications of toxicity in any dose group, and no effect on the PCE:NCE ratio with either RPA 203328 or the positive control agent cyclophosphamide. RPA 203328 did not increase the incidence of micronucleated polychromatic erythrocytes. An expected increase in micronuclei formation was observed with cyclophosphamide.

Under the conditions of the study, RPA 203328 was negative for the induction of micronucleated polychromatic erythrocytes.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material: RPA 203328
Description: white powder with small aggregates
Lot/Batch: NMI874
Purity: 99.0%
CAS: 142994-06-7
Stability of test compound: not stated in report

2. Control materials: **Negative:** 0.5% methylcellulose
Solvent: 0.5% methylcellulose
Positive: cyclophosphamide

3. Test animals:
Species: mouse
Strain: CD-1
Age: approximately 8 weeks (preliminary and main study)
Weight at dosing: approximately 21.8-34.4g (preliminary study); approximately 30.6-37.1g (main study)
Source: [redacted], NC

Number of animals per dose:	Preliminary test: 3 male s and 3 females main study: 6 males per group per timepoint
Acclimation period:	at least 5 days
Diet:	Certified Rodent Diet #5002 pellets (PMI Feeds, Inc.)
Water:	tap water
Housing:	polycarbonate cages on hardwood chip bedding
Environmental conditions –	
Temperature:	64-79°F
Humidity:	30-70%
Air changes:	at least 10 changes per hour
Photoperiod:	12 hours light / 12 hours dark

4. Test compound doses: **Range-finding test:** 200, 500, 800, 1500, 2000 mg/kg bw
Micronucleus assay: 200, 1000, 2000 mg/kg bw

B. TEST PERFORMANCE

1. Treatment and sampling times
For the preliminary cytotoxicity study, three males and three females per dose level were administered RPA 203328 once by oral gavage in 0.5% methylcellulose at a dose volume of 10 ml/kg. Following dosing, all animals were examined daily throughout the duration of the study for toxic signs or mortalities. All animals appeared normal immediately after dosing and remained healthy until the end of the observation period. At the termination of the study after two days, all surviving animals were sacrificed.

In the main study, 6 male mice per group were administered RPA 203328 at doses of 500, 1000, or 2000 mg/kg bw, or 0.5% methylcellulose, or cyclophosphamide at 80 mg/kg bw. An additional 6 male mice were administered RPA 203328 at 2000 mg/kg bw or 0.5% methylcellulose, and these two groups were used for an additional later sacrifice point.

2. Tissues and cells examined
At either 24 hours (vehicle and positive control, and RPA 203328 at 500, 1000, and 2000 mg/kg bw) or 48 hours (vehicle control and RPA 203328 at 2000 mg/kg bw), main-study animals were euthanized by CO2 inhalation and incision of the diaphragm. The hind limb bones were removed from the first 5 animals for marrow extraction. The marrow was flushed from the bone and transferred to centrifuge tubes containing 3-5ml bovine serum.

3. Details of slide preparation
Following centrifugation to pellet the tissue, the supernatant was removed by aspiration and portions of the pellet were spread on slides and air-dried. The slides were fixed in methanol, stained in May-Grunwald solution followed by Giemsa, and coverslipped.

4. Evaluation
The slides from the first five surviving animals in each treatment and control group were scored for micronuclei and the polychromatic to normochromatic erythrocyte (PCE:NCE) ratio. The micronucleus frequency, expressed as percent micronucleated cells, was determined by analyzing the number of micronucleated polychromatic erythrocytes from at least 2000 polychromatic erythrocytes per animal. The PCE:NCE ratio was determined by scoring the number of PCEs and NCEs observed in the optic fields while scoring at least the first 200 erythrocytes on the slide.

5. Statistical methods

Assay data analysis was performed using an analysis of variance on untransformed proportions of cells with micronuclei per animal and on untransformed PCE:NCE ratios when the variances were homogeneous. Ranked proportions were used for heterogeneous variances. If the analysis of variance was statistically significant ($p < 0.05$), a Dunnett's t-test was used to determine which dose groups, if any, were statistically significantly different from the vehicle control. Additionally, parametric or nonparametric tests for trend may have been employed to identify any dose-related response.

II. RESULTS AND DISCUSSION

A. RANGE-FINDING TEST

There were no signs of clinical toxicity in any of the range-finding animals at doses of RPA 203328 up to the limit dose of 2000 mg/kg bw, and there was no indication of toxicity to the bone marrow, as there was no change in the PCE:NCE ratio.

B. MICRONUCLEUS ASSAY:

1. Toxicity

No toxic effects were noted in animals administered RPA 203328 at up to 2000 mg/kg bw in the main study.

2. PCE ratio

There was no effect on the PCE ratio in any group.

3. Micronucleated polychromatic erythrocytes

There were no increases in the incidence of micronucleated polychromatic erythrocytes after oral gavage administration of RPA 203328 at doses of up to 2000 mg/kg bw. Cyclophosphamide at 80 mg/kg bw/day induced an expected increase in the incidence of micronucleated polychromatic erythrocytes.

Table 5.8.1-9: Induction of micronucleated polychromatic erythrocytes after administration of RPA 203328 to mice.

Treatment	Dose	Harvest time, hr	% micronucleated PCEs	Ratio PCE:NCE
Vehicle	0.5% MC	24	0.02	0.52
		48	0.04	0.42
RPA 203328	500 mg/kg bw	24	0.03	0.35
	1000 mg/kg bw	24	0.05	0.47
	2000 mg/kg bw	24	0.02	0.47
		48	0.03	0.43
Cyclophosphamide	80 mg/kg bw	24	3.74*	0.39

Statistically significant at : * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

III. CONCLUSION

Under the conditions of the study, there was no indication of induced chromosomal or other damage leading to micronucleus formation in polychromatic erythrocytes of treated mice after oral administration of RPA 203328.

Report:	[redacted]o;[redacted];1999;M-189848-01
Title:	Developmental toxicology study in the rat by gavage RPA203328
Report No:	R014875
Document No:	M-189848-01-1
Guidelines:	**EU (=EEC): 92/69/EEC, V, B31, (1992); JMAF: 59 Nohsan No.4200, (1985); OECD: 414, (Draft August 1996); USEPA (=EPA): OPPTS 870.3700, (1998); Deviation: not specified**
GLP/GEP:	**yes**

Executive summary:
Female rats were mated one-to-one with males of the same strain and supplier, then on gestation days 6 through 20 were administered RPA 203328 by oral gavage at doses of 0, 75, 250, and 750 mg/kg bw/day. Body weight and food consumption were monitored throughout the study. On day 21, dams were sacrificed, the uterus was weighed, and the fetuses were weighed, then examined externally and fixed for either free-hand serial sectioning or skeletal staining and examination.

Maternal corrected body weight gain was decreased at 250 and 750 mg/kg bw/day, and decreased body weight gain was seen in some intervals in these groups. Food consumption was decreased from 250 mg/kg bw/day.

There was no effect of administration of RPA 203328 on gestation rate, implantation rate, the number of viable young, sex ratio, or fetal body weight. On examination of the fetuses, there was no effect of treatment on external, visceral, or skeletal observations.

Based on the decreased body weight gain and food consumption from 250 mg/kg bw/day, the maternal NOAEL was 75 mg/kg bw/day. The fetal NOAEL was 750 mg/kg bw/day, the highest dose tested.

I. MATERIALS AND METHODS

A. MATERIALS:

1. Test Material: RPA 203328
Description: white powder with small aggregates
Lot/Batch: NMI 874
Purity: 99.0%
CAS:
Stability of test compound: at least 21 days at ambient temperatures

2. Control materials:
Negative: 0.5% aqueous methylcellulose
Solvent: 0.5% aqueous methylcellulose
Positive: no positive control tested

3. Test animals:
Species: rat
Strain: CD
Age: not stated
Weight at dosing: 237-306g at mating
Source: [redacted]France
Acclimation period: 14 days

Diet:	A04C Certified rodent pellet diet (U.A.R., Villemoisson-sur-Orge, France)
Water:	tap water
Housing:	individually housed in suspended stainless steel wire-mesh cages
Environmental conditions –	
Temperature:	20-24°C
Humidity:	40-70%
Air changes:	approximately 10 changes per hour
Photoperiod:	12-hour light, 12-hour dark cycle

4. Test compound concentrations: 0, 75, 250, 750 mg/kg bw/day

B. TEST PERFORMANCE

1. Animal assignment and treatment
Animals were not assigned to treatment groups until after mating. The females used for this study were paired on a one-to-one basis with stock males of the same strain. Each morning following pairing, the trays beneath the cages were checked for ejected copulation plugs, and a vaginal smear was prepared from each animal and examined for the presence of spermatozoa. Only females showing a sperm-positive vaginal smear or sperm plug in situ were allocated to treatment groups. The day on which evidence of mating was found was designated day 0 of gestation.

Females which showed clear evidence of mating were allocated to groups using a body weight dependent procedure.

Control and treated animals were administered either 0.5% aqueous methylcellulose or RPA 203328 in 0.5% aqueous methylcellulose by oral gavage, on gestation days 6 through 20 inclusive. The volume dosage administered was 10 ml/kg, with the volume administered to each animal based on that animal's most recent body weight.

2. Test substance formulations and analysis
The appropriate amount of test substance was periodically suspended in an aqueous solution of 0.5% methylcellulose and stored at approximately 5C.

Homogeneity of the suspensions was checked during the first formulation for the lowest and highest concentrations. Stability of the test substance in suspension in the vehicle was determined in a previous study. All concentrations were checked for each new formulation.

3. Statistics
For maternal body weight gain, corrected body weight gain, food consumption, and mean litter weight, statistical evaluation was conducted by Bartlett's test for homogeneity of variances, followed by analysis of variance and Dunnett's test if needed, or by Kruskal-Wallis non-parametric one-way analysis of variance and the Mann-Whitney test if indicated.

Litter data were statistically treated using the Kruskal-Wallis test followed by the Mann-Whitney test when the Kruskal-Wallis test was significant.

C. METHODS

6. Clinical examination of females
All animals were checked daily for clinical signs of toxicity, and any signs were recorded with respect to details of type, severity, time of onset, and duration.

7. Body weight
Body weight was measured on gestation days 0, 3, 6, 8, 10, 12, 14, 16, 18, and 21.

8. Food intake
Food consumption was measured for gestation days 1-3, 3-6, 6-8, 8-10, 10-12, 12-14, 14-16, 16-18, and 18-21.

9. Necropsy
On gestation day 21, animals were killed by inhaled carbon dioxide and examined macroscopically for indications of disease or an adverse reaction to treatment.

10. Investigation at Caesarean section
After the initial gross necropsy of each dam, the reproductive tract was then dissected out and examined for number of corpora lutea per ovary, number of implantation sites, number of early or late resorption sites, and number and distribution of fetuses in each uterine horn.

All viable fetuses were sacrificed by subcutaneous injection of sodium pentobarbital. Each fetus was then weighed, sexed, and examined for external abnormalities. Approximately half of the viable fetuses from each litter were immersed in Bouin fluid for subsequent internal examination following free-hand sectioning. The remaining half was eviscerated and then placed in absolute ethanol before staining for skeletal examination.

II. RESULTS AND DISCUSSION

A. OBSERVATIONS AND MORTALITY
At 250 and 750 mg/kg bw/day respectively 6 and 18 females had at least one occurrence of transient salivation upon treatment. In some animals at 750 mg/kg bw/day, this was associated with red nasal discharge within a few minutes following dosing. These observations disappeared approximately one hour after treatment and were probably linked with the acidic nature of the test substance (pH approximately 2.5).

There were no mortalities in any treatment group.

B. BODY WEIGHT
Maternal body weight during the pre-dosing period was similar between all the experimental groups. At 250 and 750 mg/kg bw/day, the overall corrected body weight changes were statistically significantly reduced, indicating maternal toxicity. Additionally, significantly reduced body weight changes occurred in the 750 mg/kg bw/day group during the interval GD 8-10 and in the 250 mg/kg bw/day group during the interval GD 10-14. Body weight and body weight gain were comparable to the control at 75 mg/kg bw/day.

Table 5.8.1-10: Maternal body weight and body weight change in a rat teratology study with RPA 203328

		RPA 203328, dose in mg/kg bw/day			
	Day	**0**	**75**	**250**	**750**
Maternal body weight, g	0	270.5	270.1	267.2	266.6
	6	306.8	305.7	300.4	301.0
	8	314.9	311.4	306.1	303.6
	10	325.3	321.5	313.4	309.2
	14	346.8	342.0	329.4	325.2
	18	390.7	385.1	372.0	367.3
	21	445.8	439.9	423.7	415.7
Corrected body weight change, g		68.2	63.9	46.8**	43.1**

C. FOOD INTAKE

Mean food consumption was significantly reduced throughout the treatment period at 750 mg/kg bw/day, and from GD 8 at 250 mg/kg bw/day. Food consumption at 75 mg/kg bw/day was comparable to control.

Table 5.8.1-11: Maternal food consumption in a rat teratology study with RPA 203328

	RPA 203328, dose in mg/kg bw/day			
Gestation day	**0**	**75**	**250**	**750**
1-3	27.7	27.0	26.8	26.9
3-6	28.7	28.3	27.5	27.6
6-8	29.5	28.3	27.6	25.9**
8-10	29.5	29.0	26.4*	24.7**
10-12	30.2	28.6	25.7**	25.4**
12-14	28.9	27.6	25.2**	24.2**
14-16	29.0	27.7	25.7*	24.7**
16-18	29.4	28.4	26.3*	26.2*
18-21	28.9	28.1	25.2**	25.4**

Statistically significant at : * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

D. NECROPSY

There were no treatment-related observations at necropsy.

E. GENERAL REPRODUCTION DATA

There was no effect on the number of corpora lutea or on the incidence of either pre- or post-implantation losses or on resorptions.

F. EFFECTS ON INTRAUTERINE DEVELOPMENT

1. Gestation rate

Gestation rate was unaffected by administration of RPA 203328 on gestation days 6 through 20.

2. Post-implantation loss, number and sex of foetuses
There was no effect of administration of RPA 203328 on post-implantation loss, number of viable young, or sex ratio in any treatment group.

3. Fœtal weight
There was no effect of treatment on fetal body weight at any dose level.

4. Fœtal external and visceral deviations
There were no effects related to treatment at either external or visceral examination.

5. Fœtal skeletal and cartilaginous deviations
At skeletal examination of the fetuses, there was no treatment-related effect observed.

IV. CONCLUSION

Administration of RPA 203328 by gavage to pregnant rats from gestation days 6 through 20 resulted in signs of maternal toxicity at 250 and 750 mg/kg bw/day, including decreased body weight changes, decreased corrected body weight changes, and decreased food consumption during the treatment period. None of the litter parameters recorded during cesarean section were considered affected by treatment. External, internal, and skeletal examinations of the fetuses did not reveal any findings which were considered to be related to treatment.

On the basis of these findings, the maternal NOAEL for RPA 203328 was 75 mg/kg bw/day, while the developmental NOAEL was 750 mg/kg bw/day, the highest dose tested.

CA 5.8.2 Supplementary studies on the active substance

A number of studies were conducted to investigate various aspects of the toxicity of the active substance, and were assessed in the first EU review of isoxaflutole. However these data are summarized here for reference in greyed text. Further information is available in the Baseline Dossier provided by Bayer CropScience and in the Monograph. Additional studies have been conducted since the first EU review of isoxaflutole and are summarized in the following sections.

Study type (Reference)	Species	Critical effects
2-week dietary and liver enzyme induction ([redacted], 1994 158466-01-1)	Rat	Induction of specific cytochrome P450 isozymes
2-week dietary and liver enzyme induction ([redacted], 1994 M-158462-01-1)	Mouse	Induction of specific cytochrome P450 isozymes
2-week dietary and thyroid investigative study ([redacted], 1995 M-166853-01-1)	Rat	- induction of hepatic UDPGT - decrease of circulating thyroid hormones - increased degradation of thyroid hormones

Study type (Reference)	Species	Critical effects
2- and 13-week investigative study on cell proliferation in liver ([redacted], 2001 M-240441-01-1)	Rat	- increased cell proliferation in the liver - increased liver weight - rapid and complete reversibility of effects NOAEL: 50 mg/kg bw/day LOAEL: 200 mg/kg bw/day
Investigative study on plasma tyrosine concentration after tyrosine or NTBC ([redacted], 2005 M-258468-01-1)	Rat	Increased plasma tyrosine observed when NTBC administered at ≥ 10 µg/kg bw/day in conjunction with 2% dietary tyrosine
Investigative study on tyrosine effects on selected organs ([redacted], 2006 M-275329-01-1)	Rat	Increased plasma tyrosine observed in animals receiving NTBC + dietary tyrosine, and NTBC alone; increased liver weight, treatment-related findings in pancreas, thyroid, and eye
Investigative study on tyrosine effects on selected organs ([redacted], 2006 M-275336-01-1)	Rat	Animals receiving NTBC + dietary tyrosine showed: increased plasma tyrosine, increased liver weight, treatment-related effects in pancreas, thyroid, eye
Investigative study on maternal tyrosine effects on fetal skeletal development ([redacted], 2006 M-263626-01-1)	Rat	Dams receiving NTBC + dietary tyrosine had increased plasma tyrosine; fetuses in this grop showed decreased fetal body weight, decreased or delayed ossification, increase in extra ossification points on 14th thoracic vertebra
Investigative in vitro study on metabolism of tyrosine in various species ([redacted], 2006 M-264099-01-1)	Rat, mouse, rabbit, dog, human	Mouse shows constitutive metabolism of tyrosine to HPLA; this metabolic pathway is inducible in mouse and human, less so in rat and rabbit; no activity observed in dog; humans are similar to mice, different from rats, in response to inhibition of HPPDase enzyme
Immunotoxicity study ([redacted], 2010 M-390522-01-1)	Rat	- slightly decreased body weight after isoxaflutole administration - no effect on spleen or thymus weight, no effect on SRBC-specific IgM - immunotoxicity NOAEL: 279 mg/kg bw/day, highest dose tested

Supplementary studies conducted with isoxaflutole or a functional analog were aimed at:

- Exploring the effect of isoxaflutole on hepatic cell proliferation in the female rat,
- Determining the effect of an HPPDase inhibitor on tyrosine concentrations in the male and female rat,
- Determining the effect of increased plasma tyrosine concentrations on selected organs in the male and female rat,
- Determining the effect of maternal increased plasma tyrosine concentrations on fetal skeletal development in the rat,

- Examining the ability of hepatocytes from various species to metabolize tyrosine in the absence and presence of an HPPDase inhibitor, and
- Determining the effect of isoxaflutole on immune function in the male rat.

A cell proliferation study (KCA 5.8.2/17, [redacted], 2001) conducted with isoxaflutole administered for 2 or 13 weeks, with or without a 2-week reversibility period for control and high dose animals, was conducted to determine whether the increased liver weight seen after dietary administration of isoxaflutole was related to increased cell proliferation, and further whether this cell prolilferation was reversible when isoxaflutole administration ceased. The study, in which isoxaflutole was administered in the diet to female rats at concentrations ranging from 2 to 500 mg/kg bw/day, clearly showed that increased liver weight and increased cell proliferation were high dose phenomena and were clearly reversible within a short period of time after cessation of isoxaflutole administration. Thus, the increased liver weight observed in the rat chronic / oncogenicity study was due to increased cell proliferation as observed in this mechanistic study.

The first study conducted on the effects of HPPDase inhibitors (KCA 5.8.2/18, [redacted], 2005) was an exploratory study to determine the concentrations of NTBC and tyrosine which alone would have little to no effect on plasma tyrosine concentrations but which when combined would suffice to increase plasma tyrosine concentrations in the rat to the level at which putative tyrosine-linked effects could be examined. A dietary concentration of 2% tyrosine was used, either with or without gavage dosing of NTBC at doses of 0 to 40 ug/kg bw/day, and plasma tyrosine concentrations were measured. The study showed that a dose of 10 ug/g bw/day could be expected to have a minimal effect on plasma tyrosine concentrations when administered in conjunction with normal diet.

Two 28-day studies were then conducted (KCA 5.8.2/19, [redacted], 2006; KCA 5.8.2/20, [redacted], 2006) in which rats were administered control or 2% tyrosine diet, and either distilled water or NTBC by oral gavage at 10 ug/kg bw/day. Plasma tyrosine concentrations and organ weights were measured, and histopathology was conducted on selected organs. It had been hypothesized that the findings observed in common target organs such as the liver, thyroid, pancreas, and kidney after administration of an HPPDase inhibitor such as isoxaflutole were not due to the specific toxic effects of the test compound, but to the increased plasma tyrosine concentration resulting from inhibition of the HPPDase inhibitor, and that furthermore there existed a threshold concentration of tyrosine below which effects would not be observed. The findings observed in these studies were more frequent in the group receiving both NTBC and dietary tyrosine, and thus which had a greatly increased plasma tyrosine concentration, and included corneal opacities, increased liver weight, and histopathological findings especially in the pancreas, liver, and thyroid. This data supported the hypothesis that certain findings were related to increased plasma tyrosine concentration and that a threshold for such effects could be defined.

Similarly, a developmental toxicity study was conducted to examine the effects of increased plasma tyrosine concentration on fetal skeletal development (KCA 5.8.2/21, [redacted], 2006). In this study, pregnant female rats were administered normal or tyrosine supplemented diets, and either distilled water or NTBC by oral gavage. Tyrosine concentrations were measured and specific fetal skeletal parameters were examined. The results showed that neither NTBC alone, nor tyrosine alone, had a biologically significant effect on plasma tyrosine concentrations, corneal opacities, fetal body weight, or ossification of specific fetal skeletal structures, which co-administration of NTBC and dietary tyrosine markedly increased maternal plasma tyrosine concentrations and the incidence of corneal opacities in the dams, as well as decreasing fetal body weight and the ossification of specific fetal skeletal structures. This study supports the hypothesis that the fetal findings observed in the developmental toxicity study with isoxaflutole are due to increased maternal plasma tyrosine concentrations, will not occur below a defined threshold, and are rat- and rabbit-specific and thus are not relevant for human risk assessment.

Finally, in an in vitro study (KCA 5.8.2/22, [redacted], 2006), hepatocyte preparations from rat, mouse, rabbit, dog, and human were incubated with tyrosine in the absence or presence of NTBC, and after incubation concentration of tyrosine and hydroxyphenyl lactic acid (HPLA, a metabolite of tyrosine) were measured. Production of HPLA in the absence of NTBC was noted only in the mouse, while in the presence of NTBC mouse and human showed a rapid response with a greater extent than the slow response observed in rabbit or rat. These results demonstrate that the species of interest for risk assessment can be divided into two groups, based on their ability to produce an alternate metabolite of tyrosine when the HPPDase enzyme is inhibited. Furthermore, humans are shown in vitro to be more like mice, which in vivo show a lower maximum tyrosine concentration after administration of an HPPDase inhibitor and show few if any responses to HPPDase inhibitors in the key target organs (eye, liver, pancreas, thyroid, kidney, fetal skeletal development).

The United States Environmental Protection Agency required that an immunotoxicity study be conducted with isoxaflutole during the re-registration process there (KCA 5.8.2/23, [redacted], 2010). Male rats were administered diet containing isoxaflutole at up to 4000 ppm for 28 days, then inoculated with sheep red blood cells (SRBC) four days prior to necropsy. Positive control animals were administered cyclophosphamide by oral gavage for 28 days, inoculated with sheep red blood cells, and sacrificed four days after inoculation. The concentration of specific anti-SRBC IgM was measured in all animals. In the group receiving isoxaflutole, body weight and body weight gain were reduced at 4000 ppm. There was no effect on IgM production after SRBC inoculation at any dietary concentration of isoxaflutole. In animals administered cyclophosphamide by oral gavage, there was a slight reduction in body weight and body weight gain, spleen and thymus weight were reduced, and the IgM response to SRBC injection was markedly reduced. These results show that isoxaflutole is not immunotoxic under the conditions of this study.

Report:	[redacted]8;[redacted];2001;M-240441-01
Title:	Cell Proliferation Study in Sprague-Dawley Rats Administered Isoxaflutole (IFT) in the Diet for 2 or 13 Weeks
Report No:	B003410
Document No:	M-240441-01-1
Guidelines:	**Deviation: not specified**
GLP/GEP:	**yes**

Executive Summary

To examine the potential for isoxaflutole to cause hepatic cell proliferation in the rat, and to determine the reversibility of any effects observed, groups of 10 female rats were administered isoxaflutole at varying concentrations to provide constant doses of 0, 2, 20, 50, 200, and 500 mg/kg bw/day for either 2 or 13 weeks. Reversibility groups of 10 animals were fed diets which provided 0 or 500 mg/kg bw/day for either 2 or 13 weeks, then transferred to control diets for a further 2 weeks. For the week prior to sacrifice, animals were administered bromodeoxyuridine in the drinking water to assess cell proliferation.

Body weights and body weight gains were decreased at 200 mg/kg bw/day and above, and food consumption was also decreased. Both absolute and relative liver weight were increased from 200 mg/kg bw/day, and in the groups treated for either 2 or 13 weeks there was an increase in hepatic labeling index from 200 mg/kg bw/day.

In the recovery groups, liver weight and labeling index were similar to controls after either 2 or 13 weeks dietary administration of isoxaflutole followed by 2 weeks control diet, indicating that changes induced by isoxaflutole are rapidly and completely reversible.

Isoxaflutole demonstrated dose- and time-dependent effects on hepatocyte proliferation similar to those produced by other non-genotoxic hepatocarcinogens such as phenobarbital. Hepatocyte proliferative effects were observed only at the highest dose of isoxaflutole, were correlated to changes in liver weight, and were shown to be reversible upon cessation of isoxaflutole exposure.

These data strongly support the hypothesis that cell proliferation is the non-genotoxic mode of action for isoxaflutole tumorigenicity, and that this response is dose-dependent and reversible.

I . MATERIALS AND METHODS

A. MATERIALS:

1. Test Material: Isoxaflutole
- **Description:** cream / light yellow crystalline powder
- **Lot/Batch:** 05ADM95
- **Purity:** 99.43%
- **CAS:** 141112-29-0
- **Stability of test compound:**

2. Vehicle and/or positive control: none

3. Test animals:
- **Species:** female rat
- **Strain:** Sprague-Dawley
- **Age:** 10 weeks at start of dosing
- **Weight at dosing:** approximately 225g
- **Source:** [redacted], NC
- **Acclimation period:** two weeks
- **Diet:** Certified Rodent Diet #5002 (PMI Feeds, Inc., St Louis, Missouri, US)
- **Water:** tap water
- **Housing:** two per cage on hardwood bedding in polycarbonate cages
- **Environmental conditions –**
  - **Temperature:** 22 ± 4°C
  - **Humidity:** 50 ± 20%
  - **Air changes:** at least 10
  - **Photoperiod:** 12 hours light / 12 hours dark

B. STUDY DESIGN

1. In life dates: 2 November 1998 – 16 February 1999

2. Animal assignment and treatment

Rats were assigned to treatment groups using a stratified (by body weight) randomization. Groups of 10 female rats were administered IFT in the diet at varying concentrations which provided

constant doses of 0, 2, 20, 50, 200, or 500 mg/kg bw/day for either 2 or 13 weeks. In addition, groups of 10 female rats received IFT at at concentrations which provided doses of either 0 or 500 mg/kg bw/day in the diet for 2 weeks followed by a 14-day recovery period, or for 13 weeks followed by a 15-day recovery period.

Table 5.8.2-1: Design of a study to examine incidence and reversibility of hepatocyte proliferation in female rats fed diets containing isoxaflutole for 2 or 13 weeks

	Isoxaflutole, dose in mg/kg bw/day					
	0	**2**	**20**	**50**	**200**	**500**
2 wks treatment	10	10	10	10	10	10
2 wks treatment + 2 wks reverse	10					10
13 wks treatment	10	10	10	10	10	10
13 wks treatment + 2 wks reverse	10					10

All animals were administered bromodeoxyuridine in the drinking water at a concentration of 40 ug/100ml for the last 7 or 8 days prior to sacrifice.

3. Test substance formulations and analysis
Diets containing IFT were prepared on a weekly basis, with concentrations varying each week to provide constant doses of IFT.

4. Statistics
For all quantitative data, mean and standard deviation were calculated at each time point. Group means were tested for statistical significance using the one-way ANOVA followed by Dunnett's test. Because the Labeling Index data was not normally distributed, it was transformed by the arcsine of the square root before testing by a one-way ANOVA followed by the Dunnett's test. The Dunnett's test was weighted for the total number of nuclei evaluated. For clinical observations, data were evaluated by Fisher's Exact Test.

C. METHODS

1. Clinical examination.
Each animal was observed twice daily on weekdays and once daily on weekends and holidays for mortality or moribundity. Prior to dosing on the first day of the study and weekly thereafter, each animal was also removed from its cage and examined thoroughly.

2. Body weight
Body weights were recorded weekly during acclimation and treatment, and at necropsy.

3. Food intake
Food consumption in grams per rat per day was measured for one day each week. Individual animal food consumption was determined by dividing the total amount of feed consumed for each cage by the number of rats per cage.

4. Clinical Chemistry

At terminal sacrifice, blood was drawn from the caudal vena cava, and the serum was analyzed for serum alanine aminotransferase, lactate dehydrogenase, and sorbitol dehydrogenase.

5. Necropsy and Histopathology

At necropsy, the liver and right kidney were excised, and the liver was weighed. Sections of the left, right median, and anterior lobes of the liver, approximately 5 mm in thickness, as well as a 5-mm section of the duodenum, were fixed in 10% neutral buffered formalin. The kidney and the remaining liver from each animal were minced, flash frozen in liquid nitrogen, and stored at approximately -70°C for possible future analysis.

Sections of liver and duodenum were cut and put on slides; one section was stained for traditional microscopic examination, while the other was used for BrdU immunohistochemistry.

6. Hepatocyte proliferation

Sections of liver and duodenum were stained via immunohistochemistry for BrdU incorporation, with the duodenal sections serving as an internal verification for the delivery of BrdU to the animal and as a positive control for immunohistochemical staining in each animal.

A minimum of 2000 hepatocellular nuclei per animal were counted, with nuclei counted as either labeled or unlabeled. The number of unlabeled hepatocyte nuclei per field was counted in the first five microscopic fields. The mean number of unlabelled hepatocytic nuclei in these five microscopic fields was multiplied by the total number of examined fields to calculate the total number of unlabeled hepatocellular nuclei evaluated. The Labeling Index, as a percent, was calculated by dividing the total number of labeled nuclei by the total number of hepatocellular nuclei counted, and multiplied by 100.

II. RESULTS AND DISCUSSION

A. OBSERVATIONS

1. Mortality

There were no mortalities during the study.

2. Clinical signs

There were no treatment-related clinical signs during the study.

3. Body Weight

At 500 mg/kg bw/day, body weight gains were statistically significantly reduced after either 2 or 13 weeks of treatment, while at 200 mg/kg bw/day, body weight gains were statistically significantly reduced only after 13 weeks of treatment. Mean body weights were also decreased at these doses relative to controls. During the respective recovery periods, body weight gains were equal to or greater than those in control animals.

Table 5.8.2-2: Body weight and body weight gain in female rats fed diets containing isoxaflutole for 2 or 13 weeks

		Isoxaflutole, dose in mg/kg bw/day					
Phase	**Parameter**	**0**	**2**	**20**	**50**	**200**	**500**
2 wks IFT	Initial BW, g	234.8	232.8	232.5	232.2	230.6	235.0
	Terminal BW, g	253.1	258.7	252.9	248.6	247.2	242.6
	BW gain, g	18.3	25.9	20.4	16.4	16.6	7.6*
2 wks IFT + 2 wks rev.	Initial BW, g	236.2					228.4
	Terminal BW, g	267.2					249.8
	BW gain, g	31.0					21.4
13 wks IFT	Initial BW, g	234.3	234.1	233.5	230.4	229.5	227.1
	Terminal BW, g	304.4	305.9	314.7	302.9	271.9*	271.9*
	BW gain, g	70.1	71.8	81.2	72.5	42.4*	44.8*
13 wks IFT + 2 wks rev.	Initial BW, g	233.3					226.6
	Terminal BW, g	298.9					268.9*
	BW gain, g	65.6					42.3

Statistically significant at : * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

4. Food Consumption

Overall food consumption was statistically and biologically significantly decreased at 200 and 500 mg/kg bw/day. Although there was also a decrease at 50 mg/kg bw/day, in the absence of an effect on body weight, this decrease is considered to be not biologically relevant.

Table 5.8.2-3: Body weight and body weight gain in female rats fed diets containing isoxaflutole for 2 or 13 weeks

	Isoxaflutole, dose in mg/kg bw/day					
	0	**2**	**20**	**50**	**200**	**500**
2 wks treatment	17.1	16.6	15.7	16.0	15.1	14.3
2 wks treatment + 2 wks reverse	16.5					14.9
13 wks treatment	17.1	16.5	17.1	15.7*	14.7*	14.7*
13 wks treatment + 2 wks reverse	16.8					14.5*

Statistically significant at : * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

B. NECROPSY AND ORGAN WEIGHT

Terminal body weight was unaffected by treatment after either two weeks dietary administration of IFT, or after two weeks of IFT plus 2 weeks reversibility. At 200 mg/kg bw/day, terminal body weight was significantly decreased after 13 weeks, while at 500 mg/kg bw/day, terminal body weight was significantly decreased relative to control both after 13 weeks treatment, and after 13 weeks treatment plus two weeks reversibility.

Absolute and relative liver weights were increased from 200 mg/kg bw/day in the animals administered IFT in the diet for either 2 or 13 weeks. In animals fed IFT for two weeks followed by a two-week recovery period, liver weight was similar to control. In animals fed IFT for 13 weeks followed by a two-week recovery period, relative liver weight was still slightly increased relative to control, however absolute liver weight did not differ from control in this group.

Table 5.8.2-4: Terminal body weight and liver weight in female rats fed diets containing isoxaflutole for 2 or 13 weeks

		Isoxaflutole, dose in mg/kg bw/day					
Phase	**Parameter**	**0**	**2**	**20**	**50**	**200**	**500**
2 wks IFT	Terminal BW, g	253.1	258.7	252.9	248.6	247.2	242.6
	Absolute liver wt, g	10.5	10.3	10.3	10.6	12.2*	12.2*
	Relative liver wt, %	4.1	4.0	4.1	4.2	4.9*	5.0*
2 wks IFT + 2 wks rev.	Terminal BW, g	267.2					249.8
	Absolute liver wt, g	10.8					10.6
	Relative liver wt, %	4.0					4.2
13 wks IFT	Terminal BW, g	304.4	305.9	314.7	302.9	271.9*	276.9*
	Absolute liver wt, g	11.4	12.5	12.3	12.8	14.2*	17.6*
	Relative liver wt, %	3.7	4.1	3.9	4.2	5.2*	6.5*
13 wks IFT + 2 wks rev.	Terminal BW, g	298.9					268.9*
	Absolute liver wt, g	11.3					12.1
	Relative liver wt, %	3.8					4.5

Statistically significant at : * p < 0.05; ** p < 0.01; *** p < 0.001

There were no differences between treatment groups or time points in alanine aminotransferase or sorbitol dehydrogenase. Lactate dehydrogenase was not measured as some of the serum samples had a reddish color suggestion that hemolysis had occurred, and it was likely that this would have resulted in spurious readings.

C. HEPATOCYTE PROLIFERATION

The mean hepatic labeling index was statistically significantly increased from 200 mg/kg bw/day after either 2 or 13 weeks of dietary administration of IFT. After 2 weeks treatment and 2 weeks reversibility, there was no difference in labeling index between 500 mg/kg bw/day animals and controls. After 13 weeks treatment and 2 weeks reversibility, the hepatic labeling index was markedly decreased compared to controls, most likely a compensatory response consistent with the return toward normal weight of the liver of these animals.

Table 5.8.2-5: Mean labeling index, in %, in livers from female rats fed diets containing isoxaflutole for 2 or 13 weeks

	Isoxaflutole, dose in mg/kg bw/day					
Phase	**0**	**2**	**20**	**50**	**200**	**500**
2 wks treatment	1.71	1.80	1.61	2.74	4.98*	5.96*
2 wks treatment + 2 wks reverse	0.96					0.71
13 wks treatment	0.90	1.24	1.00	0.80	2.04*	2.10*
13 wks treatment + 2 wks reverse	1.68					0.18*

Statistically significant at : * p < 0.05; ** p < 0.01; *** p < 0.001

III. CONCLUSION

The results of this study show that isoxaflutole in the diet to provide doses of 200 and 500 mg/kg bw/day produced a rapid and sustained increase in hepatocyte proliferation in the absence of elevated serum enzymes that would have indicated gross hepatotoxicity. The increased cell proliferation was accompanied by statistically significant increases in absolute and relative liver weights.

The IFT-induced increases in hepatic labeling index and liver weight in the absence of hepatotoxicity strongly suggest that IFT induces liver tumors through a mitogenic mode similar to that of non-genotoxic hepatocarcinogens such as phenobarbital. As shown in other studies previously assessed in the initial EU review, IFT is a phenobarbital-like microsomal enzyme inducer and, similar to phenobarbital, IFT produces increased liver weight and hepatocellular hypertrophy. Like phenobarbital, the effects of IFT only occur above a threshold, are dose-dependent, and are reversible.

The results of this study which show a lack of cell proliferation effects at 30 mg/kg bw/day allows a linkage to be drawn to the results of the chronic rat study in which there was no increase in hepatic tumors at this dose. These findings indicate that a dose of isoxaflutole which does not cause cell proliferation does not result in hepatic tumors.

Report:	[redacted]ü;[redacted];2005;M-258468-01
Title:	Effect on blood tyrosine levels in the rat - Following administration of NTBC by oral gavage and diet supplemented with 2 percent w/w L-tyrosine
Report No:	SA05186
Document No:	M-258468-01-1
Guidelines:	**not specified;** **Deviations: not specified**
GLP/GEP:	**yes**

Executive Summary

It was hypothesized following the development of several herbicides sharing an HPPDase inhibiting mode of action that a number of effects (organ weight and histopathology in liver, kidney, pancreas, thyroid; histopathology in eye; fetal skeletal development) were not primary toxic effects of the compounds, but were secondary to the increased plasma tyrosine concentrations observed in the rat.

Previous experiments had shown that dietary administration of tyrosine at concentrations greater than approximately 2-3% reduced dietary palatability, and other means of administration of sufficient tyrosine to increase plasma tyrosine markedly were unfeasible. A dietary concentration of 2% was thus selected as a concentration which in itself would not be expected to alter plasma tyrosine concentrations, but which when combined with an HPPDase inhibitor would result in increased plasma tyrosine concentrations.

The archetype HPPDase inhibitor NTBC was chosen based on its potency and free availability. A study was designed to find a concentration of NTBC which when administered alone would have little or no effect on plasma tyrosine concentration, but which when combined with 2% dietary tyrosine would increase plasma tyrosine concentrations above the expected threshold for biological effects.

Gavage dosing of NTBC was therefore conducted at 0, 5, 10, 20, and 40 ug/kg bw/day for 19 days. For the first 14 days of the study, all animals were fed standard control diets. On study days 15 through 19, all animals in the study were fed diets containing 2% tyrosine. Plasma tyrosine concentrations were then measured on study day 15, before transfer to the tyrosine-supplemented diets, and study day 19, after feeding diets with excess tyrosine.

On study day 15, plasma tyrosine was increased from 10 ug/kg bw/day, with a greater increase observed at 40 ug/kg bw/day. On study day 19, a dose-related increase in plasma tyrosine concentrations was observed from 10 ug/kg bw/day.

Based on these data, 10 ug/kg bw/day NTBC was chosen for future mechanistic studies with NTBC and tyrosine.

I. MATERIALS AND METHODS

A. MATERIALS:

1. **Test Material:**	2-(2-nitro-4-trifluoromethyl-benzoyl)-1,3-cyclohexanedione (NTBC)
Description:	light beige powder
Lot/Batch:	MKH13222-3-2
Purity:	99.7%
CAS:	104206-65-7

Stability of test compound:

Test Material:	L-tyrosine
Description:	white powder
Lot/Batch:	111K0888
Purity:	> 99%
CAS:	60-18-4
Stability of test compound:	

2. Vehicle and/or positive control: demineralized water (vehicle for NTBC)

3. Test animals:

Species:	female rat
Strain:	Sprague-Dawley
Age:	8-9 weeks at start of treatment
Weight at dosing:	236-261g
Source:	[redacted], France
Acclimation period:	8 days
Diet:	A04C-10 P1 (Scientific Animal Food and Engineering, Augy, France)
Water:	tap water
Housing:	individual housing in suspended stainless steel wire-mesh cages
Environmental conditions –	
Temperature:	20-24°C
Humidity:	40-70%
Air changes:	approximately 15 air changes per hour
Photoperiod:	12 hours light, 12 hours dark

B. STUDY DESIGN

1. In life dates: 8-26 July, 2005

2. Animal assignment and treatment

An automatic randomization procedure was used to select animals for the study from the middle of the weight range of the available animals, ensuring a similar body weight distribution among groups for each sex. On the day before treatment began, animals were assigned permanent identification numbers within groups.

Five groups of 3 rats were established. The control animals were gavaged on study day 1 through 18 inclusive with demineralized water, while four treatment groups received NTBC by oral gavage at 5, 10, 20, or 40 ug/kg bw/day. On study days 15 through 19, all groups including controls were transferred to diets supplemented with 2% L-tyrosine.

Table 5.8.2-6: Design of a study intended to find the optimal concentration of NTBC for further mechanistic studies in rats

Group	N	Gavage, day 1-18	Diet, day 1-14	Diet, day 15-19
1	3	Vehicle	Standard	2% tyrosine diet
2	3	NTBC, 5 ug/kg bw/day		
3	3	NTBC, 10 ug/kg bw/day		
4	3	NTBC, 20 ug/kg bw/day		
5	3	NTBC, 40 ug/kg bw/day		

3. Test substance formulations and analysis

Dosing formulations of NTBC were prepared by suspending NTBC in demineralized water to produce the required dosing concentrations. When not in use, the solutions were stored at approximately 5°C. Three formulations were prepared at each dose level.

L-Tyrosine was ground to a fine powder before being incorporated into the diet by dry mixing to provide the required dietary concentration. When not in use, the formulated diet was stored at room temperature.

4. Statistics

For body weight gain, the Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant, means were compared using ANOVA. If the ANOVA was not significant, the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant, means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant, group means were compared using the non-parametric Kruskal-Wallis test. If the Kruskal-Wallis test was not significant, the group means were considered to be homogenous and no further analysis was performed. If the Kruskal-Wallis test was significant, means of the exposed groups were compared to the means of the control group using the two-sided Dunn test.

For body weight and average food consumption per day, the Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant, means were compared using ANOVA. If the ANOVA was not significant, the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant, means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant, data were transformed using the log transformation. If the Bartlett test on log transformed data was not significant, means were compared using ANOVA on log transformed data. If the ANOVA on log transformed data was not significant, the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA on log transformed data was significant, means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test on log transformed data. If the Bartlett test was significant even after log transformation, group means were compared using the non-parametric Kruskal-Wallis test. If the Kruskal-Wallis test was not significant, the group means were considered to be homogenous and no further analysis was performed. If the Kruskal-Wallis test was significant, means of the exposed groups were compared to the means of the control group using the two-sided Dunn test.

If one or more group variance(s) equal 0, means were compared using non-parametric procedures.

C. METHODS

1. Clinical examination
Animals were checked for moribundity and mortality twice daily on weekdays and once daily on weekends and holidays. The nature, onset, severity, reversibility, and duration of any clinical signs were recorded. Detailed physical examinations were conducted on all animals on study days 1, 8, 15, and 19.

2. Body weight
Body weight was measured on the first day of test substance administration and then on study days 4, 8, 12, 15, and 19.

3. Food intake
The weight of food supplied and of that remaining at the end of the food consumption period was recorded on days 1, 5, 8, 12, and 15, and mean daily food consumption was calculated.

4. Blood sampling
On study days 15 and 19, blood samples were taken from all surviving animals in all groups by removing the extreme tip of the tail of each rat using a scalpel. Two 20ul samples were collected on 7% perchloric acid for HPLC determination of plasma tyrosine concentrations.

5. Necropsy
On study day 19, all surviving animals from all groups were sacrificed by inhalation of carbon dioxide. All animals were necropsied, including the examination of the external surfaces and all major organs, tissues, and body cavities. Macroscopic abnormalities were recorded. The weight of the pancreas and thyroid gland were measured, and tissue samples were fixed in 10% neutral buffered formalin for possible histological examination.

II. RESULTS AND DISCUSSION

A. OBSERVATIONS

1. Mortality

There were no mortalities during the study.

2. Clinical signs

From 10 ug/kg bw/day NTBC, one or more animals in each group were noted with white areas on the eye (bilateral) on study day 10. There were no clinical findings at 5 ug/kg bw/day of NTBC.

3. Body Weight

Treatment-related effects on body weight or body weight gain were limited to a body weight loss of 4g over the study period at 40 ug/kg bw/day of NTBC, and statistically significantly decreased body weight on study day 19 in this group.

Although the final body weight and body weight gain at 10 ug/kg bw/day of NTBC are lower than control, the absence of a dose-related effect at 20 ug/kg bw/day suggests that this observation is not treatment-related.

Table 5.8.2-7: Body weight and body weight change after administration of NTBC and dietary tyrosine

	NTBC, dose in ug/kg bw/day				
	Control	**5**	**10**	**20**	**40**
Body weight, g, day 1	247	247	252	246	250
Body weight, g, day 19	268	270	260	265	246*
Body weight gain, g	21	23	8	21	-4*

Statistically significant at : * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

4. Food Consumption

At 40 ug/kg bw/day, food consumption was decreased by 22% compared to controls between study days 15 and 19, when 2% L-tyrosine was added to the diet. There was no effect on food consumption at up to 20 ug/kg bw/day NTBC.

B. PLASMA TYROSINE CONCENTRATION

On study day 15, prior to addition of 2% tyrosine to the diet, plasma concentrations at 5 ug/kg bw/day NTBC were similar to those measured in the control group, while plasma tyrosine was increased from 10 ug/kg bw/day NTBC.

On study day 19, after the animals were fed diets containing 2% tyrosine from study day 15 to 19 while also treated daily with NTBC by oral gavage on study days 15 through 18, plasma tyrosine concentrations were increased in all groups including controls. The increase was dose-related in the groups administered NTBC.

Table 5.8.2-8: Plasma tyrosine concentration, in nmol/ml, after administration of NTBC and dietary tyrosine

	NTBC, dose in ug/kg bw/day				
	Control	**5**	**10**	**20**	**40**
Study day 15	44.5	53.3	408.7	142.4	921.9
Study day 19	113.8	182.0	1680.6	2136.9	2307.3

C. GROSS PATHOLOGY

Minimal to slight mottled kidney (bilateral) was observed in one animal each at 5 and 20 ug/kg bw/day, and in two animals at 40 ug/kg bw/day NTBC.

III. CONCLUSION

These results show that oral gavage administration of NTBC, together with administration of diet containing 2% tyrosine, provoked tyrosinemia in a dose-related manner and was associated with an increase in the incidence of white area on the eyes.

Report:	[redacted]o;[redacted];2006;M-275329-01
Title:	Effects of diets enriched with Tyrosine on elected organs in rats
Report No:	SA 05207
Document No:	M-275329-01-1
Guidelines:	**not specified**
GLP/GEP:	**yes**

Executive Summary
To determine the effects of elevated plasma tyrosine concentration on selected organs in the male and female rat, animals were administered diets supplemented with 2% tyrosine, NTBC by oral gavage at 10 ug/kg bw/day, or both diet containing 2% tyrosine and NTBC by oral gavage, for a period of 28 days. Plasma tyrosine concentrations were measured, organ weights were determined, and selected organs were examined by histopathology.

White areas in the eye were noted in males of the NTBC + Tyrosine group. At ophthalmological investigation, these were shown to be snowflake corneal opacities characteristic of elevated plasma tyrosine; corneal opacities were also noted in some females in the NTBC + Tyrosine group. Histopathological examination revealed corneal keratitis in the affected areas. Liver weight was increased in this group, with no effects observed in other groups. At histopathology, treatment-related findings were observed in the pancreas and thyroid.

Plasma tyrosine concentrations were only measured after overnight fasting of the animals at the end of the study. While plasma tyrosine was not increased in the group receiving only dietary tyrosine, and was increased as expected in the group receiving both NTBC and dietary tyrosine, it was unexpectedly increased in the group receiving only NTBC at 10 ug/kg bw/day. As this did not agree with results seen elsewhere, the effects on organ weight and histopathology were seen as supportive for the overall hypothesis of effects related to tyrosine, but it was decided to repeat the study (see below).

I . MATERIALS AND METHODS

A. MATERIALS:

1. **Test Material:** 2-(2-nitro-4-trifluoromethyl-benzoyl)-1,3-cyclohexanedione (NTBC)
   Description: light beige powder
   Lot/Batch: MKH1322-3-2 or MKH1322-4-1
   Purity: 99.7% or 99.9%
   CAS: 104206-65-7
   Stability of test compound:

   Test Material: L-tyrosine
   Description: white powder
   Lot/Batch: 078H06822 or 123K0376
   Purity: > 99%
   CAS: 60-18-4
   Stability of test compound:

2. **Vehicle and/or positive control:** demineralized water (NTBC)

3. Test animals:

Species:	rat
Strain:	Wistar
Age:	6-7 weeks
Weight at dosing:	172-199g (males); 150-175g (females)
Source:	[redacted] France
Acclimation period:	5 days
Diet:	A04C-10P1 (Scientific Animal Food and Engineering, Augy, France)
Water:	tap water
Housing:	individual housing in suspended stainless steel wire mesh cages
Environmental conditions –	
Temperature:	20-24°C
Humidity:	40-70%
Air changes:	approximately 10-15 air changes per hour
Photoperiod:	12 hours light, 12 hours dark

B. STUDY DESIGN

1. In life dates: 22 August 2005 – 20 September 2005

2. Animal assignment and treatment

The day before the test substance administration, all animals were weighed. An automatic randomization procedure was used to select animals for the study from the middle of the weight range, to ensure a similar body weight distribution among groups.

Groups of 10 male and 10 female rats were fed diets with or without 2% tyrosine, and were gavaged at a dose volume of 10 ml/kg bw with either demineralized water or 10 ug/kg bw/day NTBC, for 28 days.

Table 5.8.2-9: Design of a study intended to examine the effects of NTBC, tyrosine, or co-administration of NTBC + Tyrosine in rats

Group	**Diet**	**Gavage**	**Males**	**Females**
Control	Normal	Water	10	10
TYR	2% tyrosine	Water	10	10
NTBC	Normal	10 ug/kg bw/day NTBC	10	10
NTBC + TYR	2% tyrosine	10 ug/kg bw/day NTBC	10	10

3. Test substance formulations

Dosing formulations were prepared by suspending NTBC in demineralized water to produce the required dosing concentrations. When not in use, the solutions were stored at approximately 5°C.

L-Tyrosine was ground to a fine powder before being incorporated into the diet by dry mixing to provide the required dietary concentration. When not in use the formulated diet was stored at room temperature.

4. Statistics

For terminal body weight, body weight gain, and absolute and relative organ weight, the Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant, means were compared using ANOVA. If the ANOVA was not significant, the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant, means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant, group means were compared using the non-parametric Kruskal-Wallis test. If the Kruskal-Wallis test was not significant, the group means were considered to be homogenous and no further analysis was performed. If the Kruskal-Wallis test was significant, means of the exposed groups were compared to the means of the control group using the two-sided Dunn test.

For body weight and average food consumption per day, the Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant, means were compared using ANOVA. If the ANOVA was not significant, the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant, means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant, data were transformed using the log transformation. If the Bartlett test on log transformed data was not significant, means were compared using ANOVA on log transformed data. If the ANOVA on log transformed data was not significant, the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA on log transformed data was significant, means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test on log transformed data. If the Bartlett test was significant even after log transformation, group means were compared using the non-parametric Kruskal-Wallis test. If the Kruskal-Wallis test was not significant, the group means were considered to be homogenous and no further analysis was performed. If the Kruskal-Wallis test was significant, means of the exposed groups were compared to the means of the control group using the two-sided Dunn test.

If one or more group variance(s) equal 0, means were compared using non-parametric procedures.

C. METHODS

1. Clinical examination

Animals were checked twice daily on weekdays and once daily on weekends and holidays for morbidity or moribundity. The nature, onset, severity, reversibility, and duration of clinical signs was recorded. Detailed physical examinations were conducted at least weekly during the treatment period.

2. Body weight

Each animal was weighed on the first day of test substance administration and at weekly intervals throughout the treatment period, and at necropsy.

3. Food intake

The weight of food supplied and that remaining at the end of the food consumption period were recorded weekly for all animals during the treatment period, and any food spillage was noted.

4. Ophthalmological examination

During the acclimation period, all animals were subjected to ophthalmological examination. After instillation of mydriaticum, each eye was examined by an indirect ophthalmoscope. During week 4, all animals were re-examined in the same manner.

5. Blood sampling

On study days 29 or 30, blood samples were taken from all surviving animals in all groups by puncture of the retro-orbital venous plexus prior to sacrifice. An approximately equal number of animals randomly distributed among all groups were sampled on each day. Animals were diet fasted overnight prior to bleeding, and anesthetized by inhalation of isoflurane. Two 20ul aliquots from each animal were collected into eppendorf tubes containing 7% perchloric acid. After centrifugation at approximately 5000 rpm for 5 minutes, aliquots of the supernatant were frozen at -20C until determination by HPLC of tyrosine concentration.

6. Necropsy

On study days 29 or 30, all animals from all groups were sacrificed by exsanguination under deep anesthesia induced by inhalation of isoflurane. An approximately equal number of animals randomly distributed among all groups were sacrificed on each day. Animals were diet fasted overnight prior to sacrifice.

All animals were necropsied. The necropsy included the examination of the external surfaces, all orifices, and all major organs, tissues, and body cavities.

Selected organs were weighed, preserved for histopathology, and / or examined by light microscopy.

Table 5.8.2-10: Table of organs weighed, fixed and / or examined by histopathology.

Organ / Tissue	Weighed	Fixed	Exam.	Organ / Tissue	Weighed	Fixed	Exam.
Adrenals	x	x		Oesophagus		x	
Aorta		x		Ovaries	x	x	
Articular surface (femoro-tibial joint)		x		Pancreas		x	x
Bone (sternum)		x		Pituitary	x	x	
Brain	x	x		Prostate	x	x	
Caecum		x		Rectum		x	
Colon		x		Salivary gland – submaxillary		x	
Duodenum		x		Sciatic nerves		x	
Epididymides	x	x		Seminal vesicles		x	
Eyes and optic nerves		x	x	Skeletal muscle, thigh		x	
Harderian glands		x		Skin		x	
Heart	x	x		Spinal cord		x	
Ileum		x		Spleen	x	x	
Jejunum		x		Stomach		x	
Kidneys	x	x		Testes	x	x	
Lachrymal glands		x		Thymus	x	x	
Larynx / pharynx		x		Thyroid with parathyroids	x1	x	x
Liver	x	x	x	Tongue		x	
Lungs		x		Trachea		x	
Lymph nodes – submaxillary		x		Urinary bladder		x	
Lymph nodes – mesenteric		x		Uterus with cervix	x	x	
Mammary gland		x		Vagina		x	
Nasal cavities		x					

II. RESULTS AND DISCUSSION

A. OBSERVATIONS

1. Mortality

One female in the NTBC group died on study day 3 of accidental trauma. There were no other mortalities.

2. Clinical signs

In the NTBC + TYR group, treatment-related white areas on the eye were noted in nine of the 10 males between study days 23 and 26 on one or more occasions. There were no treatment-related clinical signs in females in this group, or in either males or females in either the NTBC or the TYR groups.

3. Body Weight

There was no effect of treatment on body weight or body weight gain during the study.

4. Food Consumption

There was no effect of treatment on food consumption during the study.

B. OPHTHALMOLOGICAL EXAMINATION

In the NTBC + TYR group, at the end of the study nine of 10 male rats were noted with corneal edema, all ten males were noted with snowflake corneal opacity, and three of 10 females were noted to have snowflake corneal opacity. These are considered to be treatment-related as they are linked with increased plasma tyrosine concentration. There were no treatment-related ophthalmological findings in either the NTBC or the TYR groups.

C. BLOOD TYROSINE CONCENTRATIONS

When measured on study day 29 or 30 after overnight fasting, plasma tyrosine concentrations were similar to controls in the TYR group, and markedly increased relative to controls in both the NTBC and the NTBC + TYR groups.

Table 5.8.2-11: Plasma tyrosine concentration, in nmol/ml, after administration of NTBC, tyrosine, or co-administration of NTBC + Tyrosine in rats

	Control	TYR	NTBC	NTBC + TYR
Males	70.17	77.71	1302.05	1477.45
Females	66.73	63.06	1531.85	1474.14

These results were not those which had been expected in advance of the study. Rather, it had been expected that the plasma tyrosine concentrations in the group administered 10 ug/kg bw/day

NTBC would be similar to those observed in the groups receiving either control diet, or diet supplemented with 2% tyrosine.

D. ORGAN WEIGHTS

At necropsy, terminal body weight was slightly but statistically non-significantly decreased in both males and females in the NTBC + TYR group when compared to controls.

In the NTBC + TYR group, relative liver weight was significantly increased in both males and females relative to controls.

Table 5.8.2-12: Terminal body weight and organ weight after administration of NTBC, tyrosine, or co-administration of NTBC + Tyrosine in rats

	Males				Females			
	Control	**TYR**	**NTBC**	**NTBC + TYR**	**Control**	**TYR**	**NTBC**	**NTBC + TYR**
Terminal body weight	340.3	342.6	342.4	333.9	211.9	213.0	211.6	208.0
Brain wt, g	2.01	2.01	2.04	1.97	1.86	1.92	1.89	1.88
Liver weight, g	9.41	9.59	9.78	10.07	5.42	5.39	5.43	6.07
Liver wt, % body wt	2.760	2.800	2.853	3.021**	2.557	2.536	2.563	2.904**
Liver wt, % brain wt	469.2	478.0	478.0	512.5	292.3	281.7	287.9	322.8

Statistically significant at : * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

E. GROSS PATHOLOGY

At necropsy, in the NTBC + TYR group, three of 10 males were noted with eye opacity. There were no other treatment-related macroscopic findings.

F. HISTOPATHOLOGY

Findings which are considered to be treatment-related were observed in the pancreas, thyroid, and eye in the male and female rats in the NTBC + TYR group.

Findings in the pancreas were acinar atrophy / fibrosis and / or acinar degeneration / apoptosis of the exocrine pancreas, and interstitial inflammation. In the thyroid gland, treatment related findings were colloid alteration and potentially follicular cell hypertrophy based on increased severity in one animal in the NTBC + TYR group. In the eye, the treatment-related change was unilateral or bilateral keratitis.

Table 5.8.2-13: Histopathology of selected organs after administration of NTBC, tyrosine, or co-administration of NTBC + Tyrosine in rats

	Males				**Females**			
	Control	**TYR**	**NTBC**	**NTBC + TYR**	**Control**	**TYR**	**NTBC**	**NTBC + TYR**
N examined	10	10	10	10	10	10	9	10
Pancreas								
Acinar atrophy / fibrosis: focal / multifocal	1	2	0	3	0	0	0	5
Acinar degeneration / apoptosis: focal / multifocal	0	1	0	2	0	2	0	5
Interstitial inflammation: focal / multifocal	0	1	0	3	0	0	0	5
Interstitial inflammation: diffuse	0	0	0	0	0	0	0	1
Thyroid								
Follicular cell hypertrophy: diffuse	2	3	1	3	0	1	0	0
Colloid alteration	0	1	1	6	0	0	0	0
Eye								
Keratitis: diffuse: unilateral and bilateral	1	0	0	9	0	0	0	1

IV. CONCLUSION

Administration of 10 ug/kg bw/day NTBC plus 2% dietary tyrosine markedly increased plasma tyrosine concentrations and produced findings in the eye, pancreas, and thyroid. These findings were not observed after administration of either tyrosine or NTBC alone, despite an increase in plasma tyrosine after gavage administration of 10 ug/kg bw/day NTBC alone.

As the plasma tyrosine concentration, measured on study day 29 or 30 following overnight fasting, in the NTBC group was markedly higher than that expected, further investigations were carried out.

Report:	[redacted]8;[redacted];2006;M-275336-01
Title:	Effects of Tyrosinaemia on selected organs in rats
Report No:	SA 05330
Document No:	M-275336-01-1
Guidelines:	**not applicable**
GLP/GEP:	**yes**

Executive Summary
To determine the effects of elevated plasma tyrosine concentration on selected organs in the male and female rat, animals were administered diets supplemented with 2% tyrosine, NTBC by oral gavage at 10 ug/kg bw/day, or both diet containing 2% tyrosine and NTBC by oral gavage, for a period of 28 days. Plasma tyrosine concentrations were measured, organ weights were determined, and selected organs were examined by histopathology.

White areas in the eye were noted in animals of the NTBC + Tyrosine group. At ophthalmological investigation, these were shown to be snowflake corneal opacities characteristic of elevated plasma tyrosine. Histopathological examination revealed corneal keratitis in the affected areas. Body weight and body weight gain were slightly decreased in the NTBC + Tyrosine group. Liver weight was increased in this group, with no effects observed in other groups. At histopathology, treatment-related findings were observed in the pancreas and thyroid.

Plasma tyrosine concentrations, as measured in non-fasted animals, were markedly increased in the groups receiving both NTBC and tyrosine, with slight but biologically non-significant increases observed in the tyrosine group as well as the NTBC group.

These findings show that sustained systemic tyrosinemia provokes effects in the eye, pancreas, and thyroid, and support the hypothesis that a threshold plasma tyrosine concentration exists below which tyrosine-related findings will not be observed.

I. MATERIALS AND METHODS

A. MATERIALS:

1. **Test Material:** 2-(2-nitro-4-trifluoromethyl-benzoyl)-1,3-cyclohexanedione (NTBC)
   Description: light beige powder
   Lot/Batch: MKH13222-4-1
   Purity: 99.9%
   CAS: 104206-65-7
   Stability of test compound:

   Test Material: L-tyrosine
   Description: white powder
   Lot/Batch: 114K0375
   Purity: 98.9%
   CAS: 60-18-4
   Stability of test compound:

2. **Vehicle and/or positive control:** demineralized water (NTBC)

3. Test animals:

Species:	rat
Strain:	Wistar
Age:	6-7 weeks at start of treatment
Weight at dosing:	217-264g (males); 178-195g (females)
Source:	R[redacted] France
Acclimation period:	6 days
Diet:	A04C-10P1 (Scientific Animal Food and Engineering, Augy, France)
Water:	tap water
Housing:	individual housing in suspended stainless steel wire mesh cages
Environmental conditions –	
Temperature:	20-24°C
Humidity:	40-70%
Air changes:	approximately 10-15 changes per hour
Photoperiod:	12 hours light, 12 hours dark

B. STUDY DESIGN

1. In life dates: 31 January – 1 March 2006

2. Animal assignment and treatment

The day before the test substance administration, all animals were weighed. An automatic randomization procedure was used to select animals for the study from the middle of the weight range, to ensure a similar body weight distribution among groups.

Groups of 5 male and 10 female rats were fed diets with or without 2% tyrosine, and were gavaged at a dose volume of 10 ml/kg bw with either demineralized water or 10 ug/kg bw/day NTBC, for 28 days.

Table 5.8.2-14: Design of a study intended to examine the effects of NTBC, tyrosine, or co-administration of NTBC + Tyrosine in rats

Group	Diet	Gavage	Males	Females
Control	Normal	Water	5	5
TYR	2% tyrosine	Water	5	5
NTBC	Normal	10 ug/kg bw/day NTBC	5	5
NTBC + TYR	2% tyrosine	10 ug/kg bw/day NTBC	5	5

3. Test substance formulations

Dosing formulations were prepared by suspending NTBC in demineralized water to produce the required dosing concentrations. When not in use, the solutions were stored at approximately 5°C.

L-Tyrosine was ground to a fine powder before being incorporated into the diet by dry mixing to provide the required dietary concentration. When not in use the formulated diet was stored at room temperature.

4. Statistics

For terminal body weight, body weight gain, and absolute and relative organ weight, the Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant, means were compared using ANOVA. If the ANOVA was not significant, the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant, means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant, group means were compared using the non-parametric Kruskal-Wallis test. If the Kruskal-Wallis test was not significant, the group means were considered to be homogenous and no further analysis was performed. If the Kruskal-Wallis test was significant, means of the exposed groups were compared to the means of the control group using the two-sided Dunn test.

For body weight and average food consumption per day, the Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant, means were compared using ANOVA. If the ANOVA was not significant, the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA was significant, means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test. If the Bartlett test was significant, data were transformed using the log transformation. If the Bartlett test on log transformed data was not significant, means were compared using ANOVA on log transformed data. If the ANOVA on log transformed data was not significant, the group means were considered to be homogeneous and no further analysis was performed. If the ANOVA on log transformed data was significant, means of the exposed groups were compared to the mean of the control group using the two-sided Dunnett test on log transformed data. If the Bartlett test was significant even after log transformation, group means were compared using the non-parametric Kruskal-Wallis test. If the Kruskal-Wallis test was not significant, the group means were considered to be homogenous and no further analysis was performed. If the Kruskal-Wallis test was significant, means of the exposed groups were compared to the means of the control group using the two-sided Dunn test.

If one or more group variance(s) equal 0, means were compared using non-parametric procedures.

C. METHODS

1. Clinical examination.

Animals were checked twice daily on weekdays and once daily on weekends and holidays for morbidity or moribundity. The nature, onset, severity, reversibility, and duration of clinical signs was recorded. Detailed physical examinations were conducted at least weekly during the treatment period.

2. Body weight

Each animal was weighed on the first day of test substance administration and at weekly intervals throughout the treatment period, and at necropsy.

3. Food intake

The weight of food supplied and that remaining at the end of the food consumption period were recorded weekly for all animals during the treatment period, and any food spillage was noted.

4. Ophthalmological examination

During the acclimation period, all animals were subjected to ophthalmological examination. After instillation of mydriaticum, each eye was examined by an indirect ophthalmoscope. During week 4, all animals were re-examined in the same manner.

5. Blood sampling

On study days 2, 7, 14, 21, the day prior to necropsy (before fasting), and the day of necropsy (after fasting), blood samples were taken from all animals in all groups by removing the extreme tip of the tail of each rat using a scalpel. Animals were anesthetized by inhalation of isoflurane. Two 40ul aliquots from each animal were collected onto 7% perchloric acid. After centrifugation at approximately 5000 rpm for 5 minutes, aliquots of the supernatant were frozen at -20C until determination by HPLC of tyrosine concentration.

6. Necropsy

On study days 30 or 31, all animals from all groups were sacrificed by exsanguination under deep anesthesia induced by inhalation of isoflurane. An approximately equal number of animals randomly distributed among all groups were sacrificed on each day. Animals were diet fasted overnight prior to sacrifice.

All animals were necropsied. The necropsy included the examination of the external surfaces, all orifices, and all major organs, tissues, and body cavities.

Selected organs were weighed, preserved for histopathology, and / or examined by light microscopy.

Table 5.8.2-15: Table of organs weighed, fixed, and / or examined by histopathology.

Organ / Tissue	Weighed	Fixed	Exam.	Organ / Tissue	Weighed	Fixed	Exam.
Adrenals		x		Oesophagus		x	
Aorta		x		Ovaries		x	
Articular surface (femoro-tibial joint)		x		Pancreas		x	x
Bone (sternum) and bone marrow		x		Pituitary		x	
Brain	x	x		Prostate		x	
Caecum		x		Rectum		x	
Colon		x		Salivary gland – submaxillary		x	
Duodenum		x		Sciatic nerves		x	
Epididymides		x		Seminal vesicles		x	
Eyes and optic nerves		x		Skeletal muscle, thigh		x	
Harderian glands		x		Skin		x	
Heart		x		Spinal cord		x	
Ileum		x		Spleen		x	
Jejunum		x		Stomach		x	
Kidneys		x	x	Testes		x	
Lachrymal glands		x		Thymus		x	
Larynx / pharynx		x		Thyroid with parathyroids	x1	x	x
Liver	x	x	x	Tongue		x	
Lungs		x		Trachea		x	
Lymph nodes – submaxillary		x		Urinary bladder		x	
Lymph nodes –		x		Uterus with cervix		x	

mesenteric							
Mammary gland		x		Vagina		x	
Nasal cavities		x					

II. RESULTS AND DISCUSSION

A. OBSERVATIONS

1. Mortality
There were no mortalities during the study.

2. Clinical signs

Treatment-related clinical signs were limited to the NTBC + TYR group. These signs were white area on the eye in all males and one female between study days 24 and 30, and half closed eyes in four of five males between study days 22 and 30, on one or more occasions.

3. Body Weight

In the NTBC + TYR group in females, mean body weight and mean body weight gain were slightly reduced. Although the decreases were not statistically significant, the effect is considered to be biologically relevant.

Table 5.8.2-16: Body weight and body weight change after administration of NTBC, tyrosine, or co-administration of NTBC + Tyrosine in rats

	Males				Females			
	Control	TYR	NTBC	NTBC + TYR	Control	TYR	NTBC	NTBC + TYR
Body weight, g, day 1	251	250	247	244	186	183	185	187
Body weight, g, day 29	396	400	393	382	250	241	251	244
Body weight gain, g	144	160	146	137	64	58	66	57

4. Food Consumption

In females of the NTBC + TYR group, food consumption was decreased throughout the study, with the greatest effect observed during week 2. There was no effect on food consumption in any other group.

B. OPHTHALMOLOGICAL EXAMINATION

Treatment-related ophthalmological findings were limited to the NTBC + TYR group, and consisted of corneal edema and snowflake corneal opacities in all 5 males, neovascularization of the cornea in one male, snowflake corneal opacity in one female, and anterior synechia of the iris in another female.

C. BLOOD TYROSINE CONCENTRATIONS

In the NTBC + TYR group, a time-dependent increase in blood tyrosine was observed from the first measurement at study day 2, with a plateau reached at measurement on study day 21. In the NTBC group, blood tyrosine was increased relative to controls only on study day 29 or 30, prior to fasting. In the TYR group, blood tyrosine was increased relative to controls at all time points.

Measurement of blood tyrosine concentration in samples taken on study day 30 or 31, after overnight fasting, showed plasma tyrosine concentrations similar to those observed in the previous study (data point 5.10, [redacted], 2006). Blood tyrosine concentration was similar to controls in the TYR group, and markedly elevated in both the NTBC and the NTBC + TYR groups.

Table5.8.2-17: Plasma tyrosine concentration, in nmol/ml, after administration of NTBC, tyrosine, or co-administration of NTBC + Tyrosine in rats

	Males				Females			
	Control	TYR	NTBC	NTBC + TYR	Control	TYR	NTBC	NTBC + TYR
Day 2	73.78	341.08	81.79	367.57	50.65	208.84	56.12	152.99
Day 7	73.29	310.83	84.66	389.98	46.28	148.13	55.18	104.75
Day 14	73.96	314.26	83.12	1166.95	44.40	141.84	66.37	235.88
Day 21	76.94	279.71	98.57	1852.20	50.15	152.11	66.37	921.57
Day 29 / 30	75.39	229.26	249.02	1981.35	52.92	138.64	306.31	892.32
Day 30 / 31	67.66	73.85	1231.86	1846.68	54.63	61.66	1451.51	1421.71

The exact reason for this decrease in the TYR groups, and increase in the NTBC group, is not clear. However, the results of this study suggest that if plasma tyrosine concentrations had been measured at earlier timepoints and / or prior to fasting in the previous study, the data would have resembled those shown for the present study.

D. ORGAN WEIGHTS

In females in the NTBC + TYR group, relative liver weight was increased compared to control animals. There were no other differences in organ weight.

Table 5.8.2-18: Terminal body weight and organ weight after administration of NTBC, tyrosine, or co-administration of NTBC + Tyrosine in rats

	Males				Females			
	Control	TYR	NTBC	NTBC + TYR	Control	TYR	NTBC	NTBC + TYR
Terminal body weight	362.0	374.1	363.6	357.1	233.3	225.5	232.3	224.8
Brain wt, g	2.01	1.99	2.03	2.00	1.96	1.90	1.88	1.94
Liver weight, g	9.68	10.14	9.59	10.16	6.15	5.97	5.97	6.48
Liver wt, % body wt	2.677	2.710	2.639	2.844	2.636	2.647	2.570	2.884**
Liver wt, % brain wt	481.5	512.2	473.4	507.2	314.9	314.8	319.0	334.4

Statistically significant at : * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

E. GROSS PATHOLOGY

At necropsy, in the NTBC + TYR group, all 5 males and one of 5 females were noted with eye opacity. There were no other treatment-related macroscopic findings.

F. HISTOPATHOLOGY

Findings which are considered to be treatment-related were observed in the pancreas, thyroid, and eye in the male and female rats in the NTBC + TYR group.

Findings in the pancreas were diffuse interstitial mixed inflammation, along with acinar degeneration / apoptosis of the exocrine pancreas. In the thyroid gland, the only treatment related findings was colloid alteration, and was only observed in the males. In the eye, the treatment-related change was bilateral keratitis.

Table 5.8.2-19: Treatment-related histopathological findings after administration of NTBC, tyrosine, or co-administration of NTBC + Tyrosine in rats

	Males				**Females**			
	Control	**TYR**	**NTBC**	**NTBC + TYR**	**Control**	**TYR**	**NTBC**	**NTBC + TYR**
N examined	5	5	5	5	5	5	5	5
Pancreas								
Acinar degeneration / apoptosis: focal / multifocal	1	1	1	3	0	0	1	5
Interstitial mixed cell inflammation: diffuse	0	0	0	2	0	0	1	1
Thyroid								
Follicular cell hypertrophy: diffuse	0	0	0	1	0	0	0	0
Colloid alteration	0	0	0	5	0	0	0	0
Eye								
Keratitis: diffuse bilateral	0	0	0	5	0	0	0	1

V. CONCLUSION

The results of this study clearly show that sustained systemic tyrosinemia provokes unilateral or bilateral keratitis of the eye, focal or multifocal acinar degeneration or apoptosis of the exocrine pancreas, and minimal to slight colloid alteration of the thyroid follicles. This data taken together supports the hypothesis that a threshold plasma tyrosine concentration exists below which tyrosine-related findings will not be observed.

Report:	[redacted]*;[redacted];2006;M-263626-01
Title:	Effect of tyrosinaemia on pregnancy and embryo-fetal development in the rat
Report No:	SA 05192
Document No:	M-263626-01-1
Guidelines:	**O.E.C.D. guideline 414 (January, 2001).** **E.E.C. Directive 2004/73/EC, Method B.31 (April 30, 2004)** **US E.P.A., OPPTS Series 870, Health Effects Testing Guidelines, N°870.3700 (August 1998).** **M.A.F.F. IN JAPAN notification 12 Nousan N°8147, (November 24, 2000);** **Deviations: not specified**
GLP/GEP:	**yes**

Executive Summary
To determine the effects of increased maternal plasma tyrosine in fetal development, pregnant rats were administered tyrosine-supplemented diet, NTBC by oral gavage, or tyrosine-supplemented diet plus NTBC by oral gavage. Diets containing 2% tyrosine were fed to animals from gestation day 6 through the end of the study, while NTBC was administered at a dose of 10 ug/kg bw/day on gestation days 6 through 20 inclusive.

Maternal body weight gain was slightly decreased at various time points in the groups receiving NTBC, either with or without dietary tyrosine. Four animals in the NTBC + Tyrosine group showed corneal opacities, a characteristic sign of elevated plasma tyrosine concentration. This was supported by the marked increase in plasma tyrosine in the NTBC + Tyrosine group, when compared to controls or to the tyrosine or NTBC groups.

Fetal body weight was statistically significantly decreased in the group receiving both NTBC and tyrosine, while no effects were observed with either compound alone. At examination of selected skeletal endpoints, there was a clear decrease in the ossification of specific structures, and a clear increase in the number of extra ossification points on the 14th thoracic vertebra.

The fetal findings observed in the NTBC + Tyrosine group are similar to those observed with HPPDase inhibitors such as isoxaflutole. When NTBC was administered alone at a low dose of 10 ug/kg bw/day, there was no clear effect on the skeletal endpoints. However, administration of both excess dietary tyrosine and NTBC resulted in a marked increase in maternal plasma tyrosine concentrations and in the incidence of typical fetal findings.

Thus, it can be concluded from this study that fetal findings such as decreased ossification of specific structures and increased incidence of 14th ribs or extra ossification points on the 14th thoracic vertebra are secondary to increased maternal plasma tyrosine concentrations. As rats and rabbits are less able to metabolize tyrosine via alternative pathways when HPPDase is inhibited, while mice and man are able to produce HPLA and other metabolites, rats and rabbits are uniquely sensitive to increased plasma tyrosine while mice and man are not sensitive. Thus, the fetal skeletal effects observed with HPPDase inhibitors such as isoxaflutole are not relevant to man.

I . MATERIALS AND METHODS

A. MATERIALS:

1. **Test Material:** 2-(2-nitro-4-trifluoromethyl-benzoyl)-1,3-cyclohexanedione (NTBC)

Description:	light beige powder
Lot/Batch:	MKH13222-3-2
Purity:	99.7%
CAS:	104206-65-7
Stability of test compound:	
Test Material:	L-tyrosine
Description:	white powder
Lot/Batch:	111K0888 and 078H06822
Purity:	> 99%
CAS:	60-18-4
Stability of test compound:	

2. Vehicle and/or positive control: demineralized water (for NTBC)

3. Test animals:

Species:	female rat
Strain:	Sprague-Dawley
Age:	not described in report
Weight at dosing:	234-294g on gestation day 0
Source:	[redacted], France
Acclimation period:	13 days
Diet:	A04C-10PI (Scientific Animal Food and Engineering, Augy, France)
Water:	tap water
Housing:	individual housing in suspended stainless steel wire mesh cages
Environmental conditions –	
Temperature:	20-24°C
Humidity:	40-70%
Air changes:	approximately 10-15 air changes per hour
Photoperiod:	12 hour light, 12 hour dark

B. STUDY DESIGN

1. In life dates: 2 August – 2 September 2005

2. Animal assignment and treatment
Females were mated on a one-to-one basis with stock males of the same strain and same supplier. Each morning following pairing, rats showing spermatozoa in a vaginal smear or sperm plug in situ were considered as pregnant animals.

The females were assigned to control and treated groups using a body weight procedure for each day of pairing. Body weight means were checked after the mating period to ensure similar means among all groups.

Four groups of 23 mated females were formed, and from gestation day 6 through gestation day 20 (oral gavage with vehicle or NTBC) or 21 (diet containing 2% tyrosine) animals received standard

diet and oral gavage of water, diet containing 2% tyrosine, oral gavage with 10 ug/kg bw/day NTBC, or both NTBC at 10 ug/kg bw/day and diet with 2% tyrosine.

Table 5.8.2-20: Design of a study to examine the developmental toxicity of elevated maternal plasma tyrosine after administration of NTBC, tyrosine, or co-administration of NTBC + Tyrosine in rats

Group	Diet	Gavage	Females
Control	Normal	Water	23
TYR	2% tyrosine	Water	23
NTBC	Normal	10 ug/kg bw/day NTBC	23
NTBC + TYR	2% tyrosine	10 ug/kg bw/day NTBC	23

3. Test substance formulations
The appropriate amount of NTBC was suspended in demineralized water and stored at approximately 5°C. The solutions were mixed continuously before and during dosing with an electromagnetic stirrer.

L-Tyrosine was ground to a fine powder before being incorporated into the diet by dry mixing to provide the required dietary concentration of 20,000 ppm, or 2%. When not in use, the diet supplemented with 2% L-tyrosine was stored at room temperature.

4. Statistics
Maternal body weight gain and corrected body weight gain, food consumption, liver weight, and number of corpora lutea, implantation sites, and early or late resorptions, as well as pre- and post-implantation loss percentages, were tested for statistical significance by the Bartlett test followed by ANOVA, two-sided Dunnett test, two-sided Dunn test, and / or non-parametric Kruskal-Wallis test.

C. METHODS

1. Clinical examination
All animals were examined daily from gestation day 0 through gestation day 21. A detailed physical examination including careful examination of the eyes was conducted on gestation days 1, 7, 14, and 21.

2. Body weight
Body weights were recorded on gestation days 0, 6, 8, 10, 12, 14, 16, 18, and 21.

3. Food intake
Feeders were weighed on gestation days 1, 6, 8, 10, 12, 14, 16, 18, and 21, and food consumption was calculated for each interval.

4. Blood collection
Two blood samples of approximately 20ul each were collected from all surviving animals on gestation day 21. The extreme tip of the tail was removed using a scalpel, and blood samples were collected into eppendorf tubes containing 7% perchloric acid. The samples were centrifuged at 5000 rpm for 5 minutes, then aliquots of the supernatant were used for HPLC measurement of tyrosine concentration.

5. Maternal necropsy
Animals killed in extremis were sacrificed by inhalation of carbon dioxide, a macroscopic examination of the viscera was conducted, and where possible the number of implantations and corpora lutea were noted. On gestation day 21, all females surviving were sacrificed by inhalation of carbon dioxide. Macroscopic examination of the viscera was conducted, the liver was weighed, liver, thyroid gland, and pancreas were preserved in 10% neutral buffered formalin, eyes were preserved in Davidson's fixative, and the reproductive tract was dissected out.

The reproductive tract was weighed, and the number of corpora lutea, implantations, early or late resorptions, number of live and dead fetuses, sex of live fetuses, and individual weights of live fetuses were recorded. Runt fetuses were defined as live fetuses weighing less than 4.0g at cesarean section.

6. Fetal examinations
All the live fetuses were killed by subcutaneous injection of 0.02ml Dolethal and subjected to an external examination. Fetuses were then skinned, eviscerated, and placed in absolute ethanol before staining with alizarin red S and alcian blue for targeted skeletal examination of specific bones and cartilage on approximately half the fetuses from each litter.

Structural deviations are classified as malformations (very rare or obviously lethal changes), minor anomalies (slight, relatively rare structural changes not obviously detrimental), or common variants (structural changes occurring in more than approximately 5% of the control population.

II. RESULTS AND DISCUSSION

A. OBSERVATIONS

1. Mortality

On dam in the NTBC group was sacrificed for humane reasons on gestation day 13, after showing clinical signs of reduced motor activity and tilting head on gestation days 12 and 13, as well as body weight loss of 21g between gestation days 10 and 12. There was no clear relationship to treatment.

2. Clinical signs

No treatment-related clinical signs were observed in any treatment group.

3. Body Weight

In the NTBC group, there was an initial, statistically non-significant decrease in body weight gain between gestation days 6 and 8, but for the remainder of gestation body weight gains were comparable to controls in this group. In the NTBC + TYR group, maternal body weight gain was also statistically non-significantly decreased between gestation days 6 and 8. Mean maternal body weight gains were similar to the control values thereafter, however overall body weight gain was still slightly reduced in this group when compared to controls, from gestation days 6 to 21.

Maternal corrected body weight gain was similar to control in all treated groups.

Table 5.8.2-21: Maternal body weight change after administration of NTBC, tyrosine, or co-administration of NTBC + Tyrosine in rats

		Control	TYR	NTBC	NTBC + TYR
Maternal body weight gain, g	0-6	29.0	30.7	29.5	30.9
	6-8	6.6	4.7	2.8	5.0
	6-10	15.9	12.6	11.4	12.4
	6-14	33.9	32.4	30.9	30.7
	6-18	75.2	74.3	70.8	73.0
	6-21	123.7	124.1	120.4	116.1
Maternal corrected body weight change, g		45.7	50.7	48.7	45.9

4 Food Consumption

There was no treatment-related effect on food consumption in any group.

5. Pregnancy rate

There was no effect of treatment on the pregnancy rate.

B. MATERNAL NECROPSY FINDINGS

Mottled kidney of minimal to slight severity was observed in all treatment groups, but not in the control group. Four animals showed minimal unilateral or bilateral corneal opacity in the NTBC + TYR group; no corneal opacities were observed in other groups. There was no effect of treatment on liver weight in any group.

C. MATERNAL PLASMA TYROSINE CONCENTRATION

Maternal plasma tyrosine concentrations were increased in all groups, with the greatest increase observed in the NTBC + TYR group.

Table 5.8.2-22: Maternal plasma tyrosine concentration, in nmol/ml, after administration of NTBC, tyrosine, or co-administration of NTBC + Tyrosine in rats

	Control	TYR	NTBC	NTBC + TYR
Maternal plasma tyrosine, nmol/ml	46.04	216.4	388.6	2888

D. LITTER DATA

There were no effects of treatment on the number of live or dead fetuses, the number of corpora lutea, early or late resorptions, or pre- or post-implantation losses.

Fetal body weight was statistically significantly decreased in the NTBC + TYR group. There was also a slight, statistically non-significant decrease in fetal body weight in the NTBC group.

Table 5.8.2-23: Fetal body weight after administration to dams of NTBC, tyrosine, or co-administration of NTBC + Tyrosine in rats

	Control	TYR	NTBC	NTBC + TYR
Fetal body weight, both sexes, g	5.43	5.39	5.30	5.04**
Fetal body weight, males, g	5.56	5.54	5.43	5.17**
Fetal body weight, females, g	5.31	5.25	5.16	4.93**

Statistically significant at : * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

E. FETAL NECROPSY FINDINGS

There were no external findings observed which were considered to be treatment-related. There was no effect of treatment on the number of runt fetuses observed.

A limited number of skeletal structures was examined, namely those which were observed to differ from controls in other studies conducted with HPPDase inhibitors. In the NTBC + TYR group, there was a decrease in ossification of specific bones, and an increase in the incidence of short 14th rib and extra ossification points on the 14th thoracic vertebrae. All of these findings are classified as variants.

Table 5.8.2-24: Fetal skeletal observations after administration to dams of NTBC, tyrosine, or co-administration of NTBC + Tyrosine in rats

	Fetuses				Litters			
	Control	TYR	NTBC	NTBC + TYR	Control	TYR	NTBC	NTBC + TYR
N examined	175	162	146	170	23	22	20	23
7th cervical centrum, unossified / normal cartilage	1	6	9	72	1	5	6	19
5th sternebra, incomplete ossification / normal cartilage	49	36	57	71	20	12	17	21
6th sternebra, incomplete ossification / normal cartilage	0	1	0	10	0	1	0	2
5th sternebra, unossified / normal cartilage	1	2	1	12	1	2	1	6
14th thoracic rib (uni- / bilateral), short	2	2	0	4	1	2	0	4
Extra ossification point (uni- / bilateral) on 14th thoracic vertebra	2	8	5	20	2	5	4	11
Forepaw, 3rd and / or 4th phalanges, unossified / normal cartilage	0	1	0	8	0	1	0	3
5th metacarpal, incomplete ossification / normal cartilage or unossified / normal cartilage	7	7	6	21	5	4	5	8
1st metatarsal, unossified / normal cartilage	1	4	2	6	1	3	2	4
Less than 9 sacrocaudal vertebrae ossified / normal cartilage	1	0	0	7	1	0	0	4

VI. CONCLUSION

The results obtained in this study confirm the hypothesis that marked increases in maternal plasma tyrosine concentration result in decreased ossification of specific skeletal structures in the fetuses. Furthermore, the results observed with animals treated with only tyrosine or only NTBC, where maternal plasma tyrosine was not markedly increased and where there was little or no effect on fetal ossification, show that the effects of increased maternal plasma tyrosine on fetal ossification are threshold-based. Marked increases in maternal plasma tyrosine concentration are required for fetal effects to be observed.

Report:	[redacted]q; [redacted];2006;M-264099-01
Title:	NTBC - In vitro inhibition of HPPDase using LiverbeadsTM from different species
Report No:	SA04276
Document No:	M-264099-01-1
Guidelines:	**not specified;** **Deviations: not specified**
GLP/GEP:	**yes**

Executive Summary
To compare the metabolism of tyrosine in untreated and HPPDase-inhibited conditions across species, hepatocyte preparations (LiverbeadsTM) from rat, mouse, rabbit, dog, and human were incubated with tyrosine, in the absence or the presence of NTBC, a potent inhibitor of the HPPDase enzyme. Incubation times ranged from 0 to 4 hours, and at the end of the incubation the concentration of tyrosine and hydroxyphenyl lactic acid (HPLA) were measured.

There were no species differences in tyrosine concentrations in either the presence or the absence of NTBC. There was a slight production of HPLA in baseline incubations using LiverbeadsTM from mouse cells. Production of HPLA in the presence of NTBC was observed in cultures using LiverbeadsTM from human, mouse, rabbit, and rat, although there was a faster and greater response in the mouse and human than in either the rabbit or the rat.

These results demonstrate that the species of interest can be divided into two groups, based on their ability to produce an alternate metabolite of tyrosine when the HPPDase enzyme is inhibited. Furthermore, humans are shown in vitro to be more like mice, which in vivo show a lower maximum tyrosine concentration after administration of an HPPDase inhibitor and show few if any responses to HPPDase inhibitors in the key target organs (eye, liver, pancreas, thyroid, kidney, skeletal development).

I. MATERIALS AND METHODS

A. MATERIALS:

1. Test Material: 2-(2-nitro-4-trifluoromethyl-benzoyl)-1,3-cyclohexanedione (NTBC)
Description: powder
Lot/Batch: MKH13222-3-2
Purity: 99.7%
CAS: 104206-65-7
Stability of test compound:

2. Vehicle and/or positive control: DMSO

3. Test system: LiverbeadsTM
Species: Wistar male rat, CD1 male mice, male beagle dog, male New Zealand white rabbit, female human
Source: [redacted], France

B. STUDY DESIGN

1. In life dates: 23 November 2004 - 15 April 2005

2. Incubation of LiverbeadsTM

Vials containing LiverbeadsTM (immobilized hepatocytes entrapped within an alginate matrix) were thawed, and cells were pooled within each species in either basal medium (HBSS plus glucose (25 mM)), or in basal + tyrosine (HBSS plus glucose (25mM) plug 100 mg/L tyrosine to increase enzymatic turn-over and mimic stressed conditions). The LiverbeadsTM were then seeded into 12-well incubation plates, with one plate for each incubation time and species.

Table 5.8.2-25: Design of an in vitro assay to compare metabolism of tyrosine in hepatocytes from various species, with or without inhibition of HPPDase

Basal	Basal + NTBC	Basal + Tyrosine	Basal + Tyrosine + NTBC
Basal	Basal + NTBC	Basal + Tyrosine	Basal + Tyrosine + NTBC
Basal	Basal + NTBC	Basal + Tyrosine	Basal + Tyrosine + NTBC

Each assay (test and control) was run in triplicate, and each experiment (one species, one incubation time on one plate) was run in duplicate.

The inhibition of the HPPDase enzyme was initiated by the addition of NTBC in DMSO at a final concentration of 30 uM. The vehicle alone was added to the basal or basal + TYR wells. Plates were then placed in an incubator at 37C with gentle shaking, and incubated for 0, 2, or 4 hours.

At the end of the incubation time, the LiverbeadsTM were first dissolved using EDTA-sodium, and then the hepatocytes were sonicated for 20 seconds. The hepatocytes plus incubation medium plus dissolved LiverbeadsTM from each well were then transferred into separated vials and immediately stored at -80C until either HPLC analysis or protein concentration measurement.

3. Analyses conducted

After thawing, hepatocyte incubations were centrifuged and the supernatants were directly injected into an HPLC system for determination of the concentrations of tyrosine and hydroxyphenyl lactic acid (HPLA), one of the metabolites of tyrosine.

The protein concentration of each hepatocyte incubation was measured using the Biorad colorimetric assay.

4. Statistics

For each incubation condition on each plate, the mean concentrations of tyrosine and HPLA were determined. Some values of HPLA were observed as lower than the limit of quantification; in these cases, the HPLA concentration was re-calculated by dividing the limit of quantification by the protein concentration in the well. This led to artificial overestimation of HPLA concentrations in some cases.

For comparisons of mean tyrosine or HPLA concentrations, the F test was performed to compare homogeneity of sample variances. If the F test was not significant, sample means were compared using the one-sided t-test. If the F test was significant, sample means were compared using the modified one-sided t-test with degrees of freedom calculated according to the Satterthwaite's approximation.

II. RESULTS AND DISCUSSION

A. OBSERVATIONS

1. Tyrosine concentrations

Basal tyrosine levels were similar across the species, and did not change during the incubation period with the exception of the rabbit; tyrosine levels in rabbit incubations were slightly lower than those of other species. After the addition of NTBC to the basal medium, tyrosine levels were similar for each species to those observed in the basal incubations.

Incubations of LiverbeadsTM in basal + tyrosine medium did not lead to appreciable differences in tyrosine concentration across the species. Similar results were observed in the incubations in basal + tyrosine medium to which NTBC was added.

Table .8.2-26: Tyrosine concentration in incubation medium after incubation of hepatocytes with or without excess tyrosine and with or without NTBC

Condition	Time, h	Rat	Dog	Rabbit	Mouse	Human
Basal	0	23.69	26.22	15.25	29.12	24.33
	2	25.93	26.47	17.95	20.75	23.03
	4	25.18	26.22	16.27	21.13	24.40
Basal + NTBC	0	23.82	26.25	15.35	28.48	24.42
	2	26.87	27.60	17.30	24.63	24.48
	4	27.60	27.48	16.85	29.25	27.33
Basal + TYR	0	82.17	77.18	74.45	69.45	76.07
	2	74.42	81.18	74.55	60.63	74.03
	4	74.78	82.60	78.13	54.02	74.47
Basal + TYR + NTBC	0	84.92	78.77	75.60	70.12	74.62
	2	79.07	82.77	76.05	68.52	76.73
	4	79.30	81.38	79.77	73.17	77.70

2. HPLA concentrations

HPLA was not detected in incubations with only basal medium, except in the mouse incubations. After addition of NTBC to basal medium, HPLA was not observed in the rat, dog, or rabbit LiverbeadsTM incubations at any time point. In mouse and human incubations, however, HPLA concentration increased with time.

In basal medium supplemented with tyrosine, HPLA was similarly only observed in mice. After the addition of NTBC to basal + TYR medium, HPLA was observed in the rat, rabbit, mouse, and human incubations. The concentration of HPLA increased with time in the mouse and human incubations, while in the rat there was little or no increase in HPLA with time. In the rabbit, HPLA was only observed at the 4-hour time point. HPLA was not detected in dog LiverbeadsTM incubations under any conditions.

Table 5.8.2-27: HPLA concentration in incubation medium after incubation of hepatocytes with or without excess tyrosine and with or without NTBC

Condition	Time, h	Rat	Dog	Rabbit	Mouse	Human
Basal	0	< LOQ	< LOQ	< LOQ	0.15	< LOQ
	2	< LOQ	< LOQ	< LOQ	0.24	< LOQ
	4	< LOQ	< LOQ	< LOQ	0.25	< LOQ
Basal + NTBC	0	< LOQ	< LOQ	< LOQ	0.18	< LOQ
	2	< LOQ	< LOQ	< LOQ	0.42	0.33
	4	< LOQ	< LOQ	< LOQ	0.69	0.54
Basal + TYR	0	< LOQ	< LOQ	< LOQ	0.17	< LOQ
	2	< LOQ	< LOQ	< LOQ	0.20	< LOQ
	4	< LOQ	< LOQ	< LOQ	0.26	< LOQ
Basal + TYR + NTBC	0	< LOQ	< LOQ	< LOQ	0.12	< LOQ
	2	0.19	< LOQ	< LOQ	0.73	0.54
	4	0.23	< LOQ	0.36	1.31	1.08

VII. CONCLUSION

Based on findings observed with HPPDase inhibitors including isoxaflutole in various test animal species, it was hypothesized that animal species including humans could be divided into two groups, one which is able to use an "alternative" pathway via HPLA for degrading tyrosine under the conditions of HPPDase inhibition, and one which is much less able to channel excess tyrosine through HPLA when the HPPDase enzyme is inhibited.

The results of this study clearly show that mice and humans are more efficient at producing HPLA from excess tyrosine under the conditions of excess tyrosine and inhibition of HPPDase by NTBC than are rats, dogs, or rabbits.

Report:	[redacted]; [redacted];2010;M-390522-01
Title:	28-day immunotoxicity study in the male Sprague-Dawley rat by dietary administration
Report No:	SA 09405
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Guidelines:	**U.S.E.P.A., OPPTS Series 870, Health Effects Testing Guidelines, No 870.7800 (August 1998); Deviations: not specified**
GLP/GEP:	**yes**

Executive Summary

Groups of 10 male Sprague Dawley rats were fed diets containing isoxaflutole (Batch 6464/5/8/9, 97.8% pure) at concentrations of 0, 160, 800, and 4000 ppm for at least 28 days, for mean compound intake of 0, 6, 57, and 279 mg/kg bw/day respectively. An additional group of 10 male rats were administered cyclophosphamide at 3.5 mg/kg bw/day by oral gavage for 28 days, and acted as a positive control group. Four days prior to necropsy, all animals were immunized with sheep red blood cell antigen (SRBC) by intravenous injection of 2.5 x 10^9 SRBC per animal via the tail vein. Blood samples were collected on study day 30 just prior to necropsy for analysis of specific anti-SRBC IgM. All animals were then necropsied, gross pathology observations were performed, and spleen and thymus were weighed.

In the group receiving isoxaflutole via the diet at 4000 ppm, body weight and body weight gain were reduced compared to control animals. There were no effects of body weight or body weight gain at either 800 or 160 ppm. There was no decrease in IgM production after SRBC injection.

In the animals receiving cyclophosphamide by oral gavage, there was a slight reduction in both body weight and body weight gain. Absolute and relative spleen and thymus weight were reduced, compared to the negative control group, and at necropsy several animals were noted to have atrophic / small spleen and / or thymus. The IgM response to SRBC injection was also markedly reduced in these animals.

The NOAEL for immunotoxicological parameters in this study was 4000 ppm (approximately 279 mg/kg bw/day), the highest dose tested.

I. MATERIALS AND METHODS

A. MATERIALS:

1. **Test Material:** Isoxaflutole
   - **Description:** white powder
   - **Lot/Batch:** 6464/5/8/9
   - **Purity:** 98.7%
   - **CAS:** 141112-29-0
   - **Stability of test compound:**
2. **Vehicle and/or positive control:** cyclophosphamide
3. **Test animals:**
   - **Species**: male rat
   - **Strain:** Sprague-Dawley

Age:	approx. 7 weeks at start of treatment
Weight at dosing:	262-321g
Source:	[redacted] France
Acclimation period:	12 days
Diet:	A04CP1-10 (Scientific Animal Food and Engineering, Augy, France)
Water:	tap water
Housing:	individual housing in suspended stainless steel wire mesh cages
Environmental conditions –	
Temperature:	20-24°C
Humidity:	40-70%
Air changes:	approximately 10-15 air changes per hour
Photoperiod:	12 hour light, 12 hour dark

B. STUDY DESIGN:

1. In life dates: 26 April 2010 – 25 May 2010

2. Animal assignment and treatment
On the day of randomization, all animals were weighed, and a computerized randomization procedure was used to select aniamls for the study from the middle of the weight range of available animals. This ensured a similar body weight distribution among groups (within ± 20% of the mean body weight on the day of randomization.

Dose levels for isoxaflutole were set based on the results of previous studies, in which administration at 10 or 100 mg/kg bw/day by dietary route decreased lymphocyte counts without any effect on body weight, and on the reduction of body weight and body weight gain in a separate study at 250 and 750 mg/kg bw/day. The dose level of 3.5 mg/kg bw/day for the positive control cyclophosphamide was selected after evaluating a separate 28-day study with cyclophosphamide alone.

3. Diet preparation and analysis
The test substance isoxaflutole was ground to a fine powder before being incorporated into the diet by dry mixing to provide the required concentrations. When not in use, the diet formulations were stored at approximately -18C.

The homogeneity of the test substance in the diet was verified before the study for the lowest and highest concentrations, to demonstrate adequate formulation procedures. Dietary concetnrations of the test substance were verified for each concentration.

The dosing formulation of cyclophosphamide was prepared by dissolving the compound in sterilized water to produce the required dosing concentration. The formulation was prepared and stored in air-tight light resistant containers at approximately +5C when not in use.

The homogeneity of cyclophosphamide in vehicle was verified to demonstrate adequate formulation procedures, and concentration of cyclophosphamide in water was verified.

C. METHODS:

1. Observations
All animals were checked for moribundity and mortality twice daily, once daily on weekends or public holidays, and were observed for clinical signs at least once daily. Detailed physical examinations were performed at least weekly during the treatment period.

2. Body weight
Each animal was weighed at least weekly during the acclimatization period, on the start of treatment, at weekly intervals throughout the treatment period, and before necropsy.

3. Food consumption and compound intake
Food consumption was determined weekly through subtracting the weight of food remaining from the weight of food originally provided.

4. Sheep Red Blood Cell challenge
Sheep red blood cells (SRBC; BioMerieux, reference number 72 141) were selected as an appropriate antigen as it is recommended by the guideline.

On the day of injection, SRBC were washed in phosphate buffered saline, counted, and diluted in phosphate buffered saline to obtain a preparation containing 5 x 10^9 cells per ml. SRBC preparation was then kept on ice until use. On study day 26, all animals in all groups were immunized by intravenous injection of 0.5 ml SRBC in the tail vein.

5. Blood sampling and SRBC-specific IgM assay
At terminal sacrifice 4 days after SRBC immunization, animals (not diet-fasted) were anesthetized by inhalation of isoflurane and blood samples were taken from all animals in all groups by puncture of the retro-orbital venous plexus. Blood was placed into tubes with clot activator and centrifuged, and serum aliquots were then stored at approximately -74C until analysis.

ELISA techniques were used to determine SRBC-specific IgM in serum from all animals.

6. Sacrifice and pathology
On study day 30, all animals were sacrificed by exsanguination while under deep anesthesia through isoflurane inhalation. All animals were necropsied, including the examination of all major organs, tissues, and body cavities. Spleen and thymus were weighed.

II. RESULTS AND DISCUSSION

A. OBSERVATIONS:

1. Clinical signs of toxicity
No clinical signs were noted in any animals in any group.

2. Mortality
There were no mortalities during the study.

B. BODY WEIGHT AND BODY WEIGHT GAIN:
Body weight was marginally decreased in the groups receiving cyclophosphamide at 3.5 mg/kg bw/day, or isoxaflutole at 4000 ppm. Overall body weight gain was reduced in both groups as well.

Table 5.8.2-28. Body weight and overall body weight gain in male rats administered either isoxaflutole in the diet, or cyclophosphamide by oral gavage.

	Isoxaflutole, dietary concentration in ppm				Cyclophosphamide, mg/kg bw/day
Day	**0**	**160**	**800**	**4000**	**3.5**
1	297	293	294	296	295
8	342	336	335	332	331
22	400	398	390	386	386
29	429	427	418	410	409
Body weight gain, g	133	134	124	114	114

C. FOOD CONSUMPTION:
There was no effect on food consumption in any group.

D. SRBC-SPECIFIC IGM RESPONSE
There was a great degree of inter-individual response in all groups, including both positive and negative controls. However, there was no effect of isoxaflutole administration on anti-SRBC IgM production, while cyclophosphamide decreased the response to SRBC as expected.

Table 5.8.2-29. SRBC-specific IgM (u/ml) in male rats administered either isoxaflutole in the diet, or cyclophosphamide by oral gavage.

	Isoxaflutole, dietary concentration in ppm				Cyclophosphamide, mg/kg bw/day
	0	**160**	**800**	**4000**	**3.5**
Mean	9844	8941	9665	14099	1386**
Standard deviation	10133	6409	6268	14249	541

** statistically significant at $p < 0.01$

E. SACRIFICE AND PATHOLOGY:

1. Body and organ weight

Terminal body weight was decreased in animals receiving 4000 ppm isoxaflutole, and in animals receiving cyclophosphamide at 3.5 mg/kg bw/day, but this decrease was not statistically significant in either group. Spleen and thymus weight were significantly decreased only in the cyclophosphamide group.

Table 5.8.2-30. Terminal body weight, and spleen and thymus weight, in male rats administered either isoxaflutole in the diet, or cyclophosphamide by oral gavage.

	Isoxaflutole, dietary concentration in ppm				Cyclophosphamide, mg/kg bw/day
	0	**160**	**800**	**4000**	**3.5**
Terminal body wt, g	434.9	430.3	423.0	414.1	413.3**
Spleen weight, g	0.774	0.765	0.745	0.738	0.561**
Relative spleen wt, %	0.178	0.178	0.177	0.179	0.135**
Thymus weight, g	0.587	0.558	0.530	0.494	0.440**
Relative thymus wt, %	0.135	0.130	0.126	0.119	0.107*

* statistically significant at $p < 0.05$ ** statistically significant at $p < 0.01$

2. Gross pathology

No macroscopic changes were observed in either spleen or thymus of animals receiving isoxaflutole at any concentration in the diet. In animals administered cyclophosphamide at 3.5 mg/kg bw/day atropyhic / small spleen (4/10) or thymus (3/10) were noted at necropsy.

III. CONCLUSION

The immunosuppressive properties of cyclophosphamide were clearly shown in this study, by markedly decreased IgM response to SRBC injection and by decreased spleen and thymus weight and reduction in spleen and thymus size at gross necropsy.

No immunosuppressive properties were observed after dietary administration of isoxaflutole at up to 4000 ppm in the diet. A dose level of 4000 ppm (approximately 279 mg/kg bw/day) was a No Observed Adverse Effect Level in male rats for the immunotoxicological parameters observed.

CA 5.8.3 Endocrine disrupting properties

Based on a complete toxicological data set, there is no evidence of any endocrine disrupting potential of Isoxaflutole in mammals. Furthermore isoxaflutole does not fall under the interim criteria for endocrine disruption.

Studies submitted for evaluation during the initial evaluation of Isoxaflutole demonstrated that Isoxaflutole is an inducer of hepatic phase I and phase II xenobiotic metabolizing enzymes. Secondary to this induction, alterations of thyroid homeostasis through a known mechanism may be observed in some sensitive species. Isoxaflutole itself does not possess endocrine disrupting properties.

Further details of the relevant studies can be found in sections CA 5.3 and CA 5.8.2.

CA 5.9 Medical data

CA 5.9.1 Medical surveillance on manufacturing plant personnel and monitoring studies

Isoxaflutole has been produced externally at an annual average amount of 41 tons since 1997. A workforce of 58 to 63 employees has been involved in this activity. No incidents have occurred or complaints been voiced. Regular medical examinations are done every 1 to 5 years, depending on age and job tasks. The examinations include blood count, fasting blood sugar, liver enzymes, blood fats, blood pressure, audiometry and lung function testing, and also address life-style factors like nutrition. Biomonitoring is also included.
Since 1997 these examinations have revealed no abnormal results related to isoxaflutole.

CA 5.9.2 Data collected on humans

No clinical cases nor poisoning incidents have been published in the literature. Additionally, no data on general population exposure or epidemiological studies are available.

CA 5.9.3 Direct observations

No clinical cases nor poisoning incidents have been published in the literature. Additionally, no data on general population exposure or epidemiological studies are available.

CA 5.9.4 Epidemiological studies

No data on general population exposure or epidemiological studies are available.

CA 5.9.5 Diagnosis of poisoning (determination of active substance, metabolites), specific signs of poisoning, clinical tests

As there have been no poisoning cases and in regard of the low toxicity of the compound no such data are available.

CA 5.9.6 Proposed treatment: first aid measures, antidotes, medical treatment

First Aid:

- Remove patient from exposure/terminate exposure
- Thorough skin decontamination with copious amounts water and soap, if available with polyethylene glycol 300 followed by water
    - **Note:** Most formulations with this active ingredient can be decontaminated with water (and soap), so for formulations polyethylene glycol 300 is not required.
- Flushing of the eyes with lukewarm water for 15 minutes
- Induction of vomiting does not seem to be required in regard of the low toxicity. It should only be considered if a large amount has been swallowed, if the ingestion was less than one hour ago, and if the patient is fully conscious.
    - Induced vomiting can remove maximum 50% of the ingested substance.
    - **Note:** Induction of vomiting is forbidden, if a formulation containing organic solvents has been ingested!

CA 5.9.7 Expected effects of poisoning

No data is available, as no poisonings have occurred.